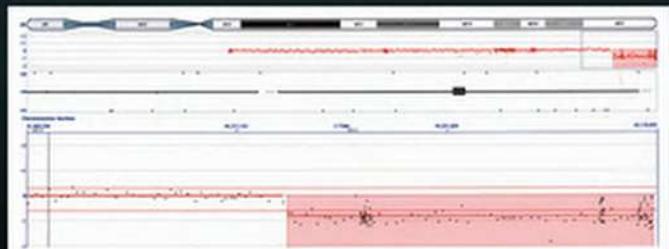


FOURTH EDITION

THE AGT CYTOGENETICS LABORATORY MANUAL

EDITORS: MARILYN S. ARSHAM, MARGARET J. BARCH,
AND HELEN J. LAWCE



The AGT Cytogenetics Laboratory Manual



The AGT Cytogenetics Laboratory Manual

Fourth Edition

Edited by

Marilyn S. Arsham

Cytogenetic Technologist II (retired)

Western Connecticut Health Network, Danbury Hospital Campus
Danbury, Connecticut, USA

Margaret J. Barch, M.S., CG(ASCP)^{CM}

(formerly) Associate Director, Frank F Yen Cytogenetics Laboratory
Weisskopf Child Evaluation Center
University of Louisville
Louisville, Kentucky, USA

Helen J. Lawce, B.S., CG(ASCP)^{CM}

Cytogenetic Technologist

Oregon Health & Science University Knight Diagnostic Laboratory
Portland, Oregon, USA



WILEY Blackwell

Copyright © 2017 by The Association of Genetic Technologists. All rights reserved

Published by John Wiley & Sons, Inc., Hoboken, New Jersey
Published simultaneously in Canada

No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, recording, scanning, or otherwise, except as permitted under Section 107 or 108 of the 1976 United States Copyright Act, without either the prior written permission of the Publisher, or authorization through payment of the appropriate per-copy fee to the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, (978) 750-8400, fax (978) 750-4470, or on the web at www.copyright.com. Requests to the Publisher for permission should be addressed to the Permissions Department, John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, (201) 748-6011, fax (201) 748-6008, or online at <http://www.wiley.com/go/permissions>.

Limit of Liability/Disclaimer of Warranty: While the publisher and author have used their best efforts in preparing this book, they make no representations or warranties with respect to the accuracy or completeness of the contents of this book and specifically disclaim any implied warranties of merchantability or fitness for a particular purpose. No warranty may be created or extended by sales representatives or written sales materials. The advice and strategies contained herein may not be suitable for your situation. You should consult with a professional where appropriate. Neither the publisher nor author shall be liable for any loss of profit or any other commercial damages, including but not limited to special, incidental, consequential, or other damages.

For general information on our other products and services or for technical support, please contact our Customer Care Department within the United States at (800) 762-2974, outside the United States at (317) 572-3993 or fax (317) 572-4002.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic formats. For more information about Wiley products, visit our web site at www.wiley.com.

Library of Congress Cataloging-in-Publication Data

Names: Arsham, Marilyn S., editor. | Barch, Margaret J., editor. | Lawce, Helen J., editor. |
Association of Genetic Technologists, issuing body.

Title: The AGT cytogenetics laboratory manual / edited by Marilyn S. Arsham,
Margaret J. Barch, Helen J. Lawce.

Description: Fourth edition. | Hoboken, New Jersey : John Wiley & Sons Inc., [2016] |
Includes bibliographical references and index.

Identifiers: LCCN 2016029052 | ISBN 9781119061229 (cloth) | ISBN 9781119061281 (epub) |
ISBN 9781119061175 (Adobe PDF)

Subjects: | MESH: Cytogenetic Analysis—methods | Laboratory Manuals

Classification: LCC RB44 | NLM QY 95 | DDC 616/.042—dc23

LC record available at <https://lccn.loc.gov/2016029052>

Cover Design: Wiley

Cover Image: Courtesy of the editors

Printed in the United States of America

We dedicate this work to the memories of three extraordinary cytogeneticists who have recently departed, and will be truly missed.

Janet Rowley
April 5, 1925, to December 17, 2013

Dr. Rowley went against the prevailing theories of the causes of cancer to argue that they were chromosomal or genetic in nature. She discovered the first cancer causing translocation, the t(15;17) of APL, and also found that the Philadelphia chromosome of CML was caused by a translocation of chromosomes 9 and 22.

R. Ellen Magenis
September 24, 1925, to February 4, 2014

Dr. Magenis was also unafraid to go against prevailing ideas in science, and was on the cutting edge of research revealing that the seemingly same deletion on the long arm of chromosome 15 was responsible for two different syndromes, Prader–Willi and Angelman. This led to the discovery that imprinting was important in humans, as well as other animals. She contributed the imprinting chapter for this book. There is a genetic disease that even bears her name because of the critical role she played in discovering the genetic cause—Smith-Magenis syndrome, which is caused by a small deletion within the short arm of chromosome 17.

Margaret Barch
July 28, 1945, to February 8, 2015

Margaret was editor of the 2nd edition of the *Association of Cytogenetic Technologists Laboratory Manual*, and co-editor with Turid Knutsen and Jack Spurbeck of the *Association of Genetics Technologists Laboratory Manual*, 3rd edition, as well as an author of several chapters. She was in the process of finishing this 4th edition, along with co-editors Helen Lawce and Marilyn Arsham, and had re-written both the Cell and Cell Division chapter and the Microscopy chapter, when she died unexpectedly. Her spirit is evident throughout this book.

Contents

Contributing authors	xxvii
Preface	xxix
Acknowledgments	xxxi
1 The cell and cell division	1
<i>Margaret J. Barch and Helen J. Lawce</i>	
1.1 The cell	1
1.1.1 Cell membrane	1
1.1.2 Cytoplasm	3
1.1.3 Nucleus	5
1.1.4 Chromosomes and their proteins	10
1.1.5 X inactivation	12
1.1.6 Satellite DNA	13
1.2 The cell cycle	14
1.2.1 Interphase	14
1.2.2 Cell division	14
1.2.3 Mitosis	15
1.2.4 Meiosis	16
1.3 Recombinant DNA techniques	19
1.3.1 Bacterial-plasmid cloning	19
1.3.2 Electrophoresis	19
1.3.3 Southern blotting	19
1.3.4 Synthetic oligonucleotides	21
1.3.5 Polymerase chain reaction	21
1.4 The human genome	21
1.4.1 Genomic DNA variations	21
References	22
2 Cytogenetics: an overview	25
<i>Helen J. Lawce and Michael G. Brown</i>	
2.1 Introduction	25
2.2 History of human cytogenetics	25
2.3 Cytogenetics methods	29
2.3.1 Work flow	29
2.3.2 Culture methods	33
2.3.3 Harvesting	35
2.3.4 Removal of attached cells and centrifugation steps	35
2.3.5 Mitotic arrest: Colcemid®	37
2.3.6 Hypotonic treatment	37
2.3.7 Fixation	40
2.3.8 In situ harvesting	41
2.3.9 Chromosome anticontraction methods	43
2.3.10 Mechanism of action of synchrony chemicals	46
2.3.11 Additives to prevent chromosome contraction	48
2.3.12 Combination of synchrony and additives for longer chromosomes	49
2.3.13 Automatic harvesting devices and slide-making chambers/drying chambers	49

2.4	Slide-making	49	
2.4.1	History of slide-making	49	
2.4.2	Theory of slide-making	50	
2.4.3	Slide-making variables	53	
2.4.4	Wet versus dry slides	53	
2.4.5	Angle of the slide	55	
2.4.6	Ambient humidity and temperature	55	
2.4.7	Fixative ratio	56	
2.4.8	Quality and freshness of fixative	56	
2.4.9	Height from which cells are dropped	56	
2.4.10	Wicking effects	56	
2.4.11	Air flow	57	
2.4.12	Dilution factor	57	
2.4.13	Slide cleaning and labeling	57	
2.4.14	Slide type	57	
2.4.15	Cell and culture type	57	
2.4.16	Culturing and harvesting techniques	57	
2.4.17	In situ cell drying	58	
2.4.18	Slide-making for FISH studies	58	
2.4.19	Slide aging	58	
2.5	Chromosome staining	58	
2.6	Chromosome microscopy/analysis	59	
2.6.1	Chromosome abnormalities	60	
2.6.2	Mosaicism	62	
2.6.3	Chromosome breakage	65	
2.6.4	Karyotyping a cell	66	
2.6.5	Banded karyograms	67	
2.6.6	Haploid band number and band levels	67	
2.6.7	The complete cytogenetic study	68	
2.6.8	Karyograms, karyotypes, and the final report	68	
2.6.9	Sources of error in analysis and reporting	69	
2.7	Laboratory procedure manual	69	
	References	70	
	Contributed protocols	75	
	Protocol 2.1	Slide-making	75
	Protocol 2.2	Slide-making	76
	Protocol 2.3	Making wet slides for chromosome analysis	78
	Protocol 2.4	Slide-making	82
	Protocol 2.5	Slide preparation	82
	Protocol 2.6	Slide preparation procedure	84
3	Peripheral blood cytogenetic methods	87	
	<i>Helen J. Lawce and Michael G. Brown</i>		
3.1	Using peripheral blood for cytogenetic analysis	87	
3.2	Special uses of peripheral blood cultures	88	
3.2.1	Chromosome instability syndromes	88	
3.2.2	Fragile sites	89	
3.3	Peripheral blood constituents	89	
3.4	Specimen handling	91	
3.4.1	Stimulants	91	
3.4.2	Anticoagulants	92	
3.4.3	Culturing	92	
3.5	Cell culture equipment and supplies	93	
3.5.1	Materials for cell culture	93	
3.5.2	Solutions for tissue culture	93	

3.6 Harvesting peripheral blood cultures	95
3.7 Chromosome analysis of peripheral blood	95
3.8 Storage of fixed specimens	95
Acknowledgments	95
References	95
Contributed protocols	98
Protocol 3.1 Blood culture and harvest procedure	98
Protocol 3.2 High-resolution peripheral blood method	100
Protocol 3.3 Constitutional cytogenetic studies on peripheral blood	108
Protocol 3.4 Blood culture and harvest procedure for microarray confirmation studies	115
4 General cell culture principles and fibroblast culture	119
<i>Debra F. Saxe, Kristin M. May and Jean H. Priest</i>	
4.1 Definitions of a culture	119
4.1.1 Time in culture	119
4.1.2 Growth characteristics	120
4.1.3 Morphology	120
4.1.4 Tissue source	121
4.2 Basic considerations in cell culture	121
4.2.1 Culture containers	122
4.2.2 Sterilization and washing principles of cell culture	123
4.2.3 Water for cell culture	124
4.2.4 Temperature, pH, CO ₂ , and humidity control	124
4.2.5 Media	125
4.2.6 Balanced salt solution	126
4.2.7 Dispersion of monolayer cells for subculture	127
4.2.8 Antimicrobial agents and monitoring for contamination	127
4.3 Fibroblast culture	128
4.3.1 Tissue sampling and transport	128
4.3.2 Tissue sampling to exclude mosaicism	128
4.3.3 Specimen setup	128
4.3.4 Routine handling and maintenance of monolayer cultures	129
4.3.5 Preparation of cultured cells for analysis	130
4.3.6 Tracking culture age	131
4.3.7 Freezing and storage of cell lines	131
4.3.8 Transport and shipping cultured cells	132
4.4 Lymphoblastoid cell lines	132
Glossary	132
Reference	133
Additional readings	133
Contributed protocols section	134
Protocol 4.1 Solid tissue collection for establishing cultures	134
Protocol 4.2 Solid tissue transport and sendout media	135
Protocol 4.3 Tissue culture reagents	138
Protocol 4.4 Phosphate buffer solution deficient in Ca ²⁺ and Mg ²⁺	141
Protocol 4.5 Solid tissue and fibroblast culture setup	141
Protocol 4.6 Solid tissue setup and processing	142
Protocol 4.7 Flask and coverslip setup for POC/fibroblast cultures	145
Protocol 4.8 Coverslip setup for solid tissue biopsy specimens	147
Protocol 4.9 Solid tissue (fibroblast) culturing and harvesting	150
Protocol 4.10 Fibroblast culture maintenance: media feeding and changing	154
Protocol 4.11 Routine subculture of fibroblast cultures	155
Protocol 4.12 Manual harvest for flasks	157
Protocol 4.13 Treated media for contamination	158
Protocol 4.14 Fungizone–mycostatin solution for treatment of fungus/yeast contaminated cultures	158

Protocol 4.15 Mycoplasma testing	159
Protocol 4.16 Plating efficiency of serum	160
Protocol 4.17 Routine replication plating for human diploid cells	160
Protocol 4.18 Cell counting chamber method	161
Protocol 4.19 Cell viability by dye exclusion	161
Protocol 4.20 Mitotic index	161
Protocol 4.21 Growth rate-estimation of mean population doubling time during logarithmic growth	162
Protocol 4.22 Maintenance of fibroblast cultures as non-mitotic population	163
Protocol 4.23 Synchronization at S-phase with BrdU	163
Protocol 4.24 Making direct FISH preparations from abortus tissue	164
Protocol 4.25 Cryopreservation	165
Protocol 4.26 Cryopreservation with Nalgene cryogenic container	166
Protocol 4.27 Lymphoblastoid lines	167
Protocol 4.28 Freezing tissue cultures (cryopreservation)	171

5 Prenatal chromosome diagnosis 173

Kristin M. May, Debra F. Saxe and Jean H. Priest

5.1 Introduction	173
5.2 Amniotic fluid	173
5.2.1 Amniocentesis	173
5.2.2 Amniotic fluid characteristics	173
5.2.3 Cells in amniotic fluid	174
5.2.4 Specimen size	175
5.2.5 Collection container	175
5.2.6 Specimen transport	175
5.3 Culture of amniotic fluid	175
5.3.1 Open versus closed system for culture	175
5.3.2 In situ versus flask growth methods	176
5.3.3 Media and additives	176
5.3.4 Number of cultures per specimen	176
5.3.5 Number of days to first culture check	177
5.3.6 Medium addition or changes	177
5.3.7 Treatment of precipitation in cultures	177
5.3.8 Treatment of microbial contamination	177
5.3.9 Treatment of bloody specimens	177
5.3.10 Treatment of poor growth	178
5.3.11 Notifying the physician about poor growth or no growth	178
5.4 Analysis of amniotic fluid	178
5.4.1 Determining harvest times	178
5.4.2 Steps involved in harvest and slide-making	178
5.4.3 Number of slides per patient	179
5.4.4 Stains performed	179
5.4.5 Analysis of amniotic fluid	179
5.4.6 Average time to report cases	179
5.4.7 Maternal cell contamination	180
5.4.8 Mosaicism	180
5.5 Chorionic villus sampling	180
5.5.1 Amniocentesis versus chorionic villus sampling	180
5.5.2 Chorionic villus sampling procedures	181
5.5.3 Structure and cell types	181
5.5.4 Specimen transport	182
5.5.5 Specimen cleaning	182
5.5.6 Direct harvest	184
5.5.7 Villus culture	184

5.6 Analysis of chorionic villi	184
5.6.1 Routine chromosome analysis	184
5.6.2 Confined placental mosaicism and uniparental disomy	185
5.6.3 Maternal cell contamination	186
5.6.4 Fetal blood sampling	186
References	186
Contributed protocols section	188
Protocol 5.1 Amniotic fluid culture setup and routine maintenance	188
Protocol 5.2 Coverslip (in situ) harvest procedure for chromosome preparations from amniotic fluid, CVS, or tissues (manual method)	191
Protocol 5.3 Harvest of flask amniocyte cultures	193
Protocol 5.4 Amniotic fluid culturing, subculturing, and harvesting (flask method)	195
Protocol 5.5 Criteria for interpreting mosaic amniotic fluid cultures	198
Protocol 5.6 Chorionic villi sampling – setup, direct harvest, and culture	199
Protocol 5.7 Chorionic villus sampling	204
Protocol 5.8 G-Banding with Leishman's stain (GTL)	208
Protocol 5.9 Cystic hygroma fluid protocol	209
6 Chromosome stains	213
<i>Helen J. Lawce</i>	
6.1 Introduction	213
6.1.1 Definitions of banding patterns and reference tables	215
6.1.2 Slide mounting	215
6.1.3 Mounting media	216
6.1.4 Slide aging for chromosome staining methods	218
6.1.5 Conventional (solid) staining methods	219
6.2 Chromosome banding methods	220
6.2.1 Quinacrine banding (QFQ)	220
6.2.2 Giemsa banding (GTG, GTW, GAG, GTL)	222
6.2.3 C-banding (CBG)	230
6.2.4 G-11 staining	233
6.2.5 Centromere/kinetochore staining	235
6.2.6 Reverse banding (R-bands)	238
6.2.7 DAPI/distamycin A staining (DA-DAPI)	241
6.2.8 Silver staining (AgNOR) for nucleolus organizing regions	243
6.3 5-bromo-2'-deoxyuridine methodologies	246
6.3.1 Historical and theoretical perspectives	249
6.3.2 Replication banding	250
6.3.3 Sister chromatid exchanges	251
6.3.4 Technical considerations for replication banding and SCEs	251
6.4 T-banding/CT-banding	252
6.4.1 Historical and theoretical perspectives	252
6.4.2 Technical considerations	252
6.5 Antibody banding and restriction endonuclease banding	252
6.6 Destaining slides	252
6.7 FISH DAPI bands	252
6.8 Sequential staining	253
Acknowledgments	253
References	253
Contributed protocols section	266
Protocol 6.1 Conventional Giemsa staining (unbanded)	266
Protocol 6.2 Leishman's stain	266
Protocol 6.3 Quinacrine mustard chromosome staining (Q-bands)	266
Protocol 6.4 C-banding	268

Protocol 6.5 C-banding	270
Protocol 6.6 C-banding	271
Protocol 6.7 C-banding of blood slides	272
Protocol 6.8 Giemsa-11 staining technique	274
Protocol 6.9 Distamycin A/DAPI staining	275
Protocol 6.10 Chromomycin/methyl green and chromomycin/distamycin fluorescent R-banding method	277
Protocol 6.11 Bone marrow and cancer blood G-banding	278
Protocol 6.12 Trypsin G-banding	280
Protocol 6.13 Giemsa-trypsin banding with Wright stain (GTW) for suspension culture slides and in situ culture coverslips	281
Protocol 6.14 G-banding blood lymphocyte slides	284
Protocol 6.15 Cd staining	285
Protocol 6.16 CREST/CENP antibody staining	286
Protocol 6.17 AgNOR (silver staining)	287
Protocol 6.18 Sister chromatid exchange blood culture and staining	289
Protocol 6.19 Sister chromatid exchange fibroblast culture and staining	291
Protocol 6.20 T-banding by thermal denaturation	294
Protocol 6.21 CT-banding	295
Protocol 6.22 Lymphocyte culture and staining procedures for late replication analysis	295
Protocol 6.23 Destaining and sequential staining of slides	298
Protocol 6.24 Restaining permanently mounted slides	299
7 Human chromosomes: identification and variations	301
<i>Helen J. Lawce and Luke Boyd</i>	
7.1 Understanding the basics	301
7.1.1 Chromosome heteromorphisms	301
7.2 Description of human chromosome shapes	302
7.2.1 Chromosome shapes	302
7.2.2 Karyogram arrangement	302
7.2.3 A group	302
7.2.4 B group	302
7.2.5 C group	303
7.2.6 D group	303
7.2.7 E group	303
7.2.8 F group	303
7.2.9 G Group	303
7.2.10 Sex chromosomes	303
7.3 Determination of G-banded chromosome resolution	355
7.3.1 Vancouver method	355
7.3.2 Johnson and Stallard method	355
7.3.3 Welborn method	355
7.3.4 Band determination quality control	355
Acknowledgments	356
Glossary	356
References	357
8 ISCN: the universal language of cytogenetics	359
<i>Marilyn S. Arsham and Lisa G. Shaffer</i>	
8.1 Introduction	359
8.2 Language	359
8.2.1 Centromere	360
8.2.2 Homologue	361
8.2.3 Idiogram	362
8.2.4 Karyogram	362

8.3 Karyotype	364
8.3.1 Chromosome count	365
8.3.2 Event symbols	367
8.3.3 Structural breakpoint	367
8.3.4 Spaces in a karyotype	372
8.3.5 Short form karyotype	372
8.3.6 Detailed karyotype	374
8.3.7 Karyotype order priority	374
8.3.8 Repeated description	376
8.3.9 Amending a cytogenetic karyotype	377
8.4 Numerical events	378
8.4.1 Polyploidy and endoreduplication	378
8.4.2 Near ploidy	379
8.4.3 Autosome aneuploidy	379
8.4.4 Sex chromosome aneuploidy	379
8.4.5 Pseudodiploid	380
8.5 Structural events	380
8.5.1 Deletion (del)	380
8.5.2 Dicentric (dic)	381
8.5.3 Duplication (dup)	383
8.5.4 Insertion (ins)	385
8.5.5 Inversion (inv)	385
8.5.6 Isochromosome (i)	386
8.5.7 Isodicentric (idic)	387
8.5.8 Constitutional origin (mat, pat, dn, inh, and c)	388
8.5.9 Recombinant (rec)	389
8.5.10 Ring of known centric origin (r)	390
8.5.11 Telomeric association (tas)	391
8.5.12 Translocation (t)	391
8.5.13 Uniparental disomy (upd) and loss of heterozygosity (LOH)	394
8.6 Derivative chromosomes (der)	394
8.6.1 One centric derivative	394
8.6.2 Homologue derivative	394
8.6.3 Isoderivative	395
8.6.4 Multiple events derivative	395
8.6.5 Neocentromere derivative	396
8.6.6 Ring derivative involving more than one chromosome	396
8.6.7 Whole arm derivative	396
8.7 Symbols of uncertainty	397
8.7.1 Uncertainty (?)	397
8.7.2 Additional material (add)	399
8.7.3 Homogeneously staining region (hsr)	399
8.7.4 Or	399
8.7.5 Approximation (~) range	399
8.7.6 Rings of unknown origin (r)	400
8.7.7 Marker (mar)	401
8.7.8 Double minute (dmin)	402
8.7.9 Incomplete (inc)	402
8.7.10 Composite karyotype [cp20]	402
8.8 Random versus reportable	403
8.8.1 Guidelines for reporting an abnormality	403
8.8.2 Heteromorphic variations	403
8.8.3 Common fragile sites	404
8.8.4 Rare fragile sites	404

8.9	Multiple cell lines and clones	404
8.9.1	Constitutional mosaicism	405
8.9.2	Neoplastic clonal evolvement	405
8.9.3	Mainline	405
8.9.4	Stemline (sl), sideline (sdl) and idem	406
8.9.5	Neoplastic polyploidy	407
8.9.6	Multiple stemlines	408
8.9.7	Jumping translocations	408
8.10	Fluorescence in situ hybridization	408
8.10.1	Probe validation and normal cut-off values	408
8.10.2	Signal patterns	409
8.10.3	Probe name	410
8.10.4	Metaphase FISH karyotype	410
8.10.5	Metaphase fusion FISH strategy	412
8.10.6	Chromosome paints	413
8.10.7	Interphase FISH karyotype	413
8.10.8	Interphase fusion karyotype	414
8.10.9	Break-apart probe strategy	415
8.10.10	Building a string of interphase FISH results	416
8.10.11	Paraffin-embedded malignant tissue	418
8.10.12	Bone marrow transplant chimerism	420
8.11	Microarray (arr) and region-specific assay (rsa)	420
8.12	Conclusion	422
	Acknowledgments	422
	Addendum for <i>ISCN 2016</i> updates	426
	References	426
9	Constitutional chromosome abnormalities	429
	<i>Kathleen Kaiser-Rogers</i>	
9.1	Numerical abnormalities	429
9.1.1	Clinical consequences of numerical aneuploidy	430
9.1.2	Mechanisms of aneuploidy	430
9.1.3	Mosaicism	438
9.1.4	Trisomy	439
9.1.5	Monosomy	441
9.1.6	Euploidy	441
9.1.7	Triploidy	442
9.1.8	Mosaic triploidy	443
9.1.9	Tetraploidy	443
9.2	Structural rearrangements	444
9.2.1	Mechanism for structural rearrangements	446
9.2.2	Deletions	449
9.2.3	Duplications	450
9.2.4	Inversions	451
9.2.5	Neocentromeric or analphoid chromosomes	455
9.2.6	Dicentric chromosomes	455
9.2.7	Isochromosomes	456
9.2.8	Rings	457
9.2.9	Marker chromosomes	459
9.2.10	Reciprocal translocations	460
9.2.11	Robertsonian translocations	462
9.2.12	Insertions	464
9.2.13	Complex chromosome rearrangements	466
	References	472

10 Genomic imprinting	481
<i>R. Ellen Magenis</i>	
10.1 Introduction	481
10.2 Human genomic disease and imprinting	488
10.2.1 Chromosomal syndromes	488
10.2.2 Whole chromosome uniparental disomies	490
10.2.3 Partial (segmental) uniparental disomies	492
10.3 Germ cell tumors – UPD and imprinting	493
10.3.1 Common chromosome abnormalities	493
10.3.2 Imprinting status	493
Glossary	494
References	496
11 Cytogenetic analysis of hematologic malignant diseases	499
<i>Nyla A. Heerema</i>	
11.1 Introduction	499
11.2 Myeloid leukemias	508
11.2.1 Acute myeloid leukemia (AML)	508
11.2.2 Common recurring cytogenetic abnormalities in AML	510
11.2.3 Other abnormalities recognized by the WHO	514
11.2.4 Therapy-related MDS and AML (t-MDS and t-AML)	514
11.3 Myelodysplastic syndromes	514
11.4 Myeloproliferative neoplasms	515
11.4.1 Chronic myelogenous leukemia	515
11.4.2 Chronic myelomonocytic leukemia	515
11.4.3 Polycythemia vera	516
11.4.4 Essential thrombocythemia	516
11.4.5 Primary myelofibrosis	516
11.4.6 Chronic neutrophilic leukemia	516
11.4.7 Chronic eosinophilic leukemia	516
11.4.8 Other myeloid and lymphoid disorders	516
11.5 B- and T-cell lymphoid neoplasms	517
11.5.1 Acute lymphoblastic leukemia	517
11.5.2 Chronic lymphocytic neoplasias	521
11.6 Lymphomas	522
11.6.1 B-cell lymphomas	522
11.6.2 T-cell lymphomas	525
11.7 Laboratory practices	525
11.7.1 Common cytogenetic aberrations	525
11.7.2 Cytogenetic methodology	525
Acknowledgments	533
Glossary of hematopoietic malignancies	533
References	535
Contributed protocols section	553
Protocol 11.1 Cancer cytogenetics procedure	553
Protocol 11.2 Bone marrow/leukemic peripheral blood setup and harvest procedure	558
Protocol 11.3 Bone marrow and leukemic blood culture and harvest procedure using DSP30 CPG oligonucleotide/interleukin-2 for B-cell mitogenic stimulation	560
Protocol 11.4 Culture of CpG-stimulated peripheral blood and bone marrow in chronic lymphocytic leukemia	562
Protocol 11.5 Plasma cell separation and harvest procedure for FISH analysis	567
Protocol 11.6 Plasma cell separation and harvest procedure for FISH	569

Protocol 11.7 Bone marrow GTG-banding	571
Protocol 11.8 GTW banding procedure (G-bands by trypsin using Wright stain)	573
12 Cytogenetic methods and findings in human solid tumors	577
<i>Marilyn Nelson</i>	
12.1 Introduction	577
12.1.1 Historical review	577
12.1.2 Tumor classification	578
12.2 Processing tumor specimens	579
12.2.1 Culture of tumor cells	579
12.2.2 Specimen requirements	579
12.2.3 Specimen transport	580
12.2.4 Specimen receipt	580
12.2.5 Culture media and supplementation	581
12.2.6 Culture vessels	581
12.2.7 Tissue dissociation	581
12.2.8 Cell dilution and culture initiation	583
12.2.9 Culture maintenance and duration	583
12.2.10 Mitotic arrest	584
12.2.11 Cell synchrony	585
12.2.12 Determination of harvest time	585
12.2.13 Hypotonic treatment	585
12.2.14 Fixation	586
12.2.15 Slide preparation and staining	586
12.2.16 Chromosome analysis	587
12.3 Recurrent cytogenetic abnormalities	592
12.3.1 Benign adipose tumors	592
12.3.2 Malignant adipocytic tumors	594
12.3.3 Skeletal muscle tumors	596
12.3.4 Tumors of uncertain differentiation	597
12.3.5 Bone tumors	599
12.3.6 Tumors of the nervous system	599
12.3.7 Tumors of the lung	603
12.3.8 Tumors of the liver	604
12.3.9 Tumors of the thymus	604
12.3.10 Tumors of the salivary glands	604
12.3.11 Tumors of the prostate	605
12.3.12 Tumors of the kidney	605
12.3.13 Tumors involving germ cells of testicular or ovarian origin	607
12.3.14 Tumors of the pleura	607
12.4 Molecular genetic and cytogenetic techniques	608
12.4.1 FISH applications	608
12.4.2 RT-PCR	611
12.4.3 Chromosome microarray analysis and multicolor karyotyping	611
12.5 Conclusion	612
Glossary	612
References	613
Contributed protocol section	631
Protocol 12.1 Solid tumor cell culture and harvest	631
Protocol 12.2 Solid tumor cell culture and harvest	637
Protocol 12.3 Solid tumor culture	643
Protocol 12.4 Solid tumor harvest: monolayer and flask methods	644
Protocol 12.5 Solid tumor culturing and harvesting	646

13 Chromosome instability syndromes	653
<i>Yasmine Akkari</i>	
13.1 Introduction	653
13.1.1 Cytogenetics versus molecular diagnosis	654
13.2 Fanconi anemia	656
13.2.1 Cytogenetic diagnosis of Fanconi anemia	656
13.2.2 Somatic mosaicism in Fanconi anemia	658
13.3 Bloom syndrome	658
13.4 Ataxia–telangiectasia	658
13.5 Nijmegen breakage syndrome	659
13.6 Immunodeficiency, centromeric instability, and facial anomalies syndrome	660
13.7 Roberts syndrome	661
13.8 Werner syndrome	661
13.9 Rothmund–Thomson syndrome	662
13.10 Proficiency testing	662
Glossary	662
References	667
Contributed protocol section	671
Protocol 13.1 Fanconi anemia chromosome breakage procedure for whole blood	671
Protocol 13.2 Supplemental procedure; Ficoll separation of whole blood	675
Protocol 13.3 Fanconi anemia fibroblast set up, culture, subculture, and harvest procedure	676
Protocol 13.4 Fanconi anemia chromosome breakage analysis policy	681
Protocol 13.5 Table for breakage studies result interpretation	682
Protocol 13.6 Fanconi anemia	684
14 Microscopy and imaging	687
<i>Margaret J. Barch and Helen J. Lawce</i>	
14.1 The standard microscope	687
14.1.1 The light path	688
14.1.2 Magnification, numerical aperture, and resolution	689
14.1.3 Lenses	690
14.1.4 Condensers	692
14.1.5 Eyepieces (or oculars)	693
14.1.6 Homogenous system	694
14.1.7 Mechanical stages	694
14.1.8 Practical microscopy	694
14.1.9 Cleaning the microscope	694
14.2 Brightfield microscopy	695
14.2.1 Köhler illumination	695
14.2.2 Filters	695
14.2.3 Immersion oil	696
14.2.4 Coverglass	696
14.2.5 Slides	696
14.2.6 Eyepiece adjustment	697
14.3 Fluorescence microscopy	697
14.3.1 Light sources for fluorescence	697
14.3.2 Filters for fluorescence	699
14.3.3 Practical advice for fluorescence	699
14.4 Specialized microscopy	699
14.4.1 Phase contrast microscopy	699
14.4.2 Inverted microscope	701
14.5 Capturing the microscopic image	701
14.5.1 Brightfield photography	701
14.5.2 Digital imaging	702

14.5.3	Printer	703
14.5.4	Modern advances in microscopy tools	703
References		703
15 Computer imaging		705
<i>Christine E. Haessig</i>		
15.1	Introduction	705
15.2	Techniques to improve karyogram image quality	705
15.3	Metaphase preparation	706
15.3.1	Use of phase contrast	706
15.3.2	Chromosome length and staining	706
15.3.3	Band resolution	706
15.4	Microscopy	706
15.4.1	Köhler illumination	706
15.4.2	Magnification	707
15.4.3	Focus	707
15.5	Image capture	707
15.5.1	Computer gray levels	707
15.5.2	Gray scale	708
15.5.3	Indicator chromosomes	710
15.6	Enhancement	710
15.6.1	Sharpening	710
15.6.2	Contrast	710
15.7	Advanced contrast	710
15.7.1	Pink/blue sliders	711
15.7.2	Brighten/darken contrast slider	711
15.7.3	Cutoff % sliders	711
15.8	Macro programming	712
15.9	FISH imaging	713
15.9.1	Microscope setup	713
15.9.2	Thresholding	714
15.9.3	Probe enhancement tips	714
15.10	Printing	715
15.11	Quality control	715
15.12	Archiving	715
Acknowledgments		715
References		715
16 Fluorescence in situ hybridization (FISH)		717
<i>Helen J. Lawce and Jeffrey S. Sanford</i>		
16.1	Introduction	717
16.1.1	Molecular mechanisms of FISH	717
16.1.2	Historical development of FISH	717
16.1.3	FISH of today	719
16.2	Clinical applications of FISH probes	722
16.2.1	In vitro diagnostics versus analyte-specific reagents	722
16.2.2	Probe designs	722
16.3	Deletion/duplication probes for constitutional abnormalities	730
16.3.1	Subtelomeric-specific probes	732
16.3.2	All-human telomere probes	733
16.4	Hematology/oncology and solid tumor probes	734
16.4.1	Cancer-related deletion probes	734
16.4.2	Cancer-related enumeration probes	735

16.4.3	Cancer-related locus-specific probes	735
16.4.4	Cancer-related paint probes	735
16.4.5	Cancer probe panels	736
16.5	Sources and characteristics of probes available to the clinical cytogenetics laboratory	736
16.5.1	Probe size	737
16.5.2	Probe concentration	738
16.5.3	Probe terminology/nomenclature	738
16.5.4	Probe storage	738
16.6	Special uses of probes	738
16.6.1	Mixing probes of different brands or kinds	738
16.6.2	Off-label use of probes	739
16.7	Important FISH probe adjuvants	739
16.7.1	Cot-1® blocking DNA	739
16.7.2	Probe buffers	740
16.8	Principles of FISH	741
16.8.1	Principles of fluorescence	741
16.8.2	Stringency in FISH assays	741
16.8.3	Fluorescent background in the FISH assay	743
16.9	FISH methods – an overview	744
16.9.1	Specimen	745
16.9.2	Harvest/cell preparation	747
16.9.3	Slide preparation	748
16.9.4	Pretreatments for FISH slides	749
16.9.5	Denaturation of probes and target	751
16.9.6	Coverslips, sealants, and alternatives	752
16.9.7	Renaturation (reannealing) times and temperatures	753
16.9.8	Post-hybridization wash	753
16.9.9	Detection	753
16.9.10	Slide mounting and counterstains	754
16.9.11	Storage of hybridized slides	754
16.9.12	Safety precautions	757
16.10	FISH analysis and reporting	757
16.10.1	Microscopy	757
16.10.2	Scoring cells	758
16.10.3	Capturing and working with FISH signal images	764
16.10.4	Reporting results	765
16.10.5	FISH and mosaicism	765
16.11	FISH probe testing and validation	765
16.11.1	Sensitivity, specificity, and efficiency	765
16.11.2	Establishing cutoffs	766
16.11.3	FISH controls	768
16.12	FISH for special investigation	768
16.12.1	Sequential staining methods combined with FISH	768
16.12.2	Rx-FISH	769
16.12.3	Fiber FISH	770
16.13	Preimplantation genetic FISH	771
16.13.1	Obtaining samples for PGD FISH	771
16.13.2	PGD technical issues	773
16.13.3	Designing translocation probe sets for balanced carrier patients	773
16.13.4	Preimplantation FISH abnormalities	773
16.13.5	Technical issues for preimplantation FISH	774
16.13.6	PGD FISH methods	774
16.13.7	Scoring PGD samples	775
16.13.8	Sperm FISH	775
16.13.9	PRINS	775

16.14 Other applications	776
16.14.1 Array CGH confirmation FISH	776
16.14.2 FISH for bladder cancer	776
16.15 Variants in FISH signal patterns	777
16.15.1 Alpha satellite DNA probes	777
16.15.2 Subtelomere-specific probes	777
16.15.3 Locus-specific probes	777
16.16 Conclusion	777
Acknowledgments	778
Glossary	778
References	780
Contributed protocols	790
Protocol 16.1 FISH (fluorescence in situ hybridization) methods	790
Protocol 16.2 LSI, CEP, and paint probe protocol	796
Protocol 16.3 FISH protocol for multiprobe® FISH panels	799
Protocol 16.4 Slide pretreatment with pepsin for FISH	800
Protocol 16.5 Interphase FISH for amniotic fluid specimen aneuploidy	801
Protocol 16.6 FISH on direct preparations from abortus tissue	803
Protocol 16.7 FISH on cultured non-mitotic abortus tissue	804
Protocol 16.8 FISH on smears	806
Protocol 16.9 FISH on very small samples	808
Protocol 16.10 Paraffin-embedded tissue FISH method	810
Protocol 16.11 VP2000 automated slide processor method for FFPE FISH	811
Protocol 16.12 Plasma cell targeted FISH	814
Protocol 16.13 Plasma cell separation for interphase FISH using easy SEP magnet method	815
Protocol 16.14 Preimplantation genetic testing (PGD) for aneuploidy	818
Protocol 16.15 Preimplantation genetic testing (PGD) FISH for translocations	821
Protocol 16.16 Post-FISH BrdU antibody detection	823
Protocol 16.17 Same-day HER2 IQ-FISH pharmDx™ for breast tissue	824

17 Multicolor FISH (SKY and M-FISH) and CGH 833

Turid Knutsen

17.1 Introduction	833
17.1.1 Online databases	833
17.2 Multicolor FISH (SKY/M-FISH)	834
17.2.1 Introduction	834
17.2.2 Other multicolor FISH techniques	834
17.2.3 Theory	835
17.2.4 Applications of multicolor FISH	837
17.2.5 Methodology	840
17.3 Comparative genomic hybridization	849
17.3.1 Introduction	849
17.3.2 Theory	851
17.3.3 Advantages and limitations	851
17.3.4 Applications of CGH	851
17.3.5 Methodology	853
17.4 Conclusion	859
Acknowledgments	859
References	859
Contributed protocols section	864
Protocol 17.1 Spectral karyotyping (SKY)	864
Protocol 17.2 Spectral karyotyping (SKY)	877
Protocol 17.3 DNA spectral karyotyping	878

Protocol 17.4 Multicolor-FISH method (M-FISH) I	881
Protocol 17.5 Multicolor FISH (M-FISH) or 24-color FISH II	884
Protocol 17.6 Multicolor FISH (M-FISH) III	888
Protocol 17.7 Comparative genomic hybridization I	891
Protocol 17.8 Comparative genomic hybridization II	898
18 Genomic microarray technologies for the cytogenetics laboratory	903
<i>Bhavana J. Davé and Warren G. Sanger</i>	
18.1 Introduction	903
18.1.1 Principle and advances	903
18.1.2 Advantages	905
18.1.3 Methods	906
18.2 Applications	907
18.2.1 CMA for identification of congenital genetic defects (constitutional abnormalities)	907
18.2.2 CMA for characterization of acquired genetic changes	909
18.2.3 Use of CMA in prenatal diagnostics	911
18.2.4 CMA in determination of genomic variations and polymorphisms	911
18.2.5 Evolutionary characterization with CMA	912
18.2.6 Limitations	912
18.3 Genomic microarray in a cytogenetics laboratory	913
18.3.1 General considerations	913
18.3.2 Specimens	913
18.3.3 Types of microarray used in the clinical laboratory	914
18.3.4 Microarray data analysis	914
18.3.5 Aspects specific to the validation of home-brew microarrays	914
18.3.6 Validation of FDA-approved commercial, IVD commercial, or home-brew microarrays	914
18.3.7 Confirmation of abnormal CMA results	915
18.3.8 Genomic polymorphisms and variations	915
18.3.9 Reporting CMA results	915
18.3.10 Examples	915
18.4 Conclusion	922
Acknowledgment	922
Authors' note	923
References	923
19 Mathematics for the cytogenetic technologist	937
<i>Patricia K. Dowling</i>	
19.1 General concepts	937
19.1.1 Scientific notation	937
19.1.2 Manipulating numbers written in scientific notation	939
19.1.3 Unit conversion	940
19.2 Solutions	942
19.2.1 Definitions	942
19.2.2 Moles and molarity	943
19.2.3 Preparing a molar solution	943
19.2.4 Making molar solutions using hydrated compounds	945
19.2.5 Making percent solutions	946
19.2.6 Stock solutions	947
19.2.7 Diluting molar solutions	947
19.2.8 Diluting a stock molar solution to a specific concentration	947
19.2.9 Using dilution ratios	949
19.2.10 Converting molarity to percent	951
19.2.11 Converting percent to molarity	951
19.2.12 Serial dilutions (application for dose-response experiments)	951

19.3 Statistical tools	956
19.3.1 Determining the normal cut-off for FISH validation	956
19.3.2 Other statistics of use in the cytogenetics laboratory	961
19.3.3 Choosing the right method to fit the situation	965
19.4 Using a hemacytometer	968
19.4.1 Determining cell count by using a hemacytometer	968
19.4.2 Determining cell viability using a hemacytometer	972
19.5 Quantification and purity determination of DNA using spectroscopy	973
Reference	974
Additional readings	974
20 Selected topics on safety, equipment maintenance, and compliance for the cytogenetics laboratory	975
<i>Helen Jenks and Janet Krueger</i>	
20.1 Introduction	975
20.2 Biological hazard safety	975
20.2.1 Safety program	975
20.2.2 Work practice controls	977
20.2.3 Personal protective equipment	977
20.2.4 Universal (or standard) precautions	977
20.2.5 Engineering controls	979
20.2.6 Housekeeping procedures	979
20.2.7 Biological hazard spills	979
20.3 Chemical safety	980
20.3.1 Know your chemicals	980
20.3.2 Proper chemical storage	983
20.3.3 Fume hoods	984
20.3.4 Working with chemicals	985
20.3.5 Chemical emergencies	985
20.4 Fire safety	986
20.4.1 Fire prevention unit	986
20.4.2 Fire drills	986
20.4.3 Fire extinguishers	986
20.5 Electrical safety	987
20.6 Disaster plan	988
20.7 Equipment operation, maintenance, and safety	988
20.7.1 Autoclaves	990
20.7.2 Compressed gas cylinders	990
20.7.3 Centrifuges	990
20.7.4 Thermometers	991
20.7.5 Refrigerators/freezers	991
20.7.6 Ovens	992
20.7.7 Water baths	992
20.7.8 Microscopes	992
20.7.9 Environmental control chamber	992
20.7.10 Biological safety cabinets	993
20.7.11 Pipettes	993
20.7.12 pH meter	994
20.7.13 Scales	994
20.7.14 Timers	994
20.7.15 Incubators	994
20.7.16 Fume hoods	995
20.7.17 Automated hybridization system	995
20.7.18 Robotic harvester	995
20.8 Ergonomics	996

20.9 Regulatory considerations	998
20.9.1 Incident reporting	998
20.9.2 Privacy act	998
20.9.3 Safety inspections/drills/training	999
20.9.4 Compliance	999
20.9.5 US regulatory and accreditation agencies	1000
Acknowledgments	1001
References	1001
Contributed protocols section	1003
Protocol 20.1 Autoclave sterilization, liquid nitrogen, pro-par	1003
Protocol 20.2 Dishwashing procedure	1003
Protocol 20.3 Eppendorf pipette calibration	1004
Protocol 20.4 NIST thermometer calibration	1006
Protocol 20.5 Thermometer calibration	1008
Protocol 20.6 Timer calibration	1008
21 A system approach to quality	1011
<i>Peggy J. Stupca and Sheryl A. Tran</i>	
21.1 Quality system	1011
21.1.1 What is the quality system?	1011
21.1.2 Organization	1012
21.1.3 Facilities and safety	1012
21.1.4 Personnel	1012
21.1.5 Purchasing and inventory	1012
21.1.6 Equipment	1012
21.1.7 Process management	1012
21.1.8 Documents and records	1012
21.1.9 Nonconforming event management	1012
21.1.10 Assessments	1012
21.1.11 Customer focus	1012
21.1.12 Information management	1013
21.1.13 Continual improvement	1013
21.2 Process management	1013
21.2.1 Validating new tests, changed tests, and new or moved equipment	1013
21.2.2 Validation plan	1013
21.2.3 Specimen number for validation	1014
21.2.4 Data analytical evaluation	1015
21.2.5 Reference range maintenance	1015
21.3 Documents and records	1015
21.3.1 Document creation and control	1016
21.3.2 Records	1018
21.4 Assessments	1018
21.4.1 Internal assessments	1019
21.4.2 External assessments	1021
21.5 Continual improvement	1022
21.6 Summary	1023
References	1023
Contributed protocols section	1025
Protocol 21.1 Quality control overview document	1025
Protocol 21.2 Monitoring specimen quality from off-hill sites	1030
22 Laboratory management	1031
<i>Mervat S. Ayad and Adam Sbeiti</i>	
22.1 Introduction	1031
22.2 Management concepts and functions	1032
22.2.1 Planning (goal setting)	1032
22.2.2 Organizing/staffing needs	1032

22.2.3	Directing (leading)	1032
22.2.4	Monitoring (controlling)	1032
22.3	Personnel management	1033
22.3.1	Staff level assessment	1033
22.3.2	Job descriptions	1033
22.3.3	Selecting your team	1033
22.3.4	Training	1034
22.3.5	Competency	1034
22.3.6	Performance evaluation and appraisals	1035
22.3.7	Staff motivation and retention	1035
22.3.8	Policy manual	1036
22.4	Quality management and control	1036
22.4.1	Technology assessment and implementation	1036
22.4.2	Financial management	1037
22.4.3	Cost per test	1038
22.4.4	Productivity and workload	1038
22.5	Budget development and monitoring	1039
22.5.1	Forecast	1039
22.5.2	Budgeting	1040
22.5.3	Capital expenditure	1041
22.5.4	Trends and measures	1042
22.6	Conclusion	1043
References		1043
Suggested reading		1043

23 Laboratory information system 1045

Peining Li and Richard Van Rheeden

23.1	Historical perspective	1045
23.2	General description of LIS	1045
23.2.1	LIS concept	1045
23.2.2	Software architecture and hardware considerations	1046
23.2.3	Validation and implementation	1047
23.2.4	Compliance and security	1048
23.3	LIS in cytogenetics laboratories	1048
23.3.1	The CytoGen system from Washington University in St. Louis	1048
23.3.2	Examples of other cytogenetic LIS systems	1051
23.4	Trends for the future LIS	1051
Acknowledgments		1052
References		1052

24 Animal cytogenetics 1055

Marlys L. Houck, Teri L. Lear and Suellen J. Charter

24.1	Introduction	1055
24.2	Domestic animal fertility	1056
24.2.1	Cattle	1056
24.2.2	Horses	1056
24.3	Captive management	1057
24.3.1	Species integrity	1057
24.3.2	Chromosome abnormalities	1057
24.3.3	Studbooks	1058
24.4	Wildlife conservation	1059
24.5	General sample collection considerations	1060
24.5.1	Regulations	1060
24.5.2	Record keeping	1062

24.6 Fibroblast cell culture	1062
24.6.1 Bioresource banking	1062
24.6.2 Fibroblast sample sources	1062
24.6.3 Fibroblast culture conditions	1063
24.7 Peripheral blood culture	1063
24.8 Chromosome analysis	1064
24.8.1 Conventional staining	1064
24.8.2 Banding	1065
24.8.3 Digital imaging	1066
24.8.4 Karyotyping	1066
24.8.5 Karyotype standards and precedents	1068
24.9 Molecular and comparative cytogenetics	1070
24.9.1 Zoo-FISH	1070
24.9.2 Reciprocal chromosome painting	1070
24.9.3 BAC maps	1071
24.9.4 Future directions	1071
Acknowledgments	1071
Glossary	1072
References	1072
Contributed protocol section	1078
Protocol 24.1 Blood feather collection	1078
Protocol 24.2 Avian lymphocyte culture (for large birds)	1078
Protocol 24.3 Lymphocyte culture using whole blood	1084
Protocol 24.4 Lymphocyte culture using autologous plasma/buffy coat (AP/BC)	1085
Protocol 24.5 Horse lymphocyte culture method	1087
Protocol 24.6 Rhino blood culture	1089
Protocol 24.7 Organ tissue collection protocol from carcass	1090
Protocol 24.8 Skin biopsy procedure	1090
Protocol 24.9 Placenta biopsy procedure	1091
Protocol 24.10 Freezing of fibroblast cell cultures	1092
Protocol 24.11 Freezing tissue biopsy samples for later initiation of cell culture (tissue piecing)	1094
Protocol 24.12 Preparation of primary cultures from feather pulp	1095
Protocol 24.13 Preparation of primary cultures from solid tissue (explants)	1096
Protocol 24.14 Preparation of primary cultures using enzyme digestion	1097
Protocol 24.15 Harvesting of fibroblast cell cultures	1098
Protocol 24.16 Preparation of competitor DNA for FISH hybridization	1099
Protocol 24.17 In situ hybridization of BAC clones labeled with spectrum fluorochromes: probe and slide preparation	1100
Protocol 24.18 Labeling DNA with spectrum fluorochromes	1102
25 Online genetic resources and references	1103
<i>Wahab A. Khan</i>	
25.1 Introduction	1103
25.2 Resource information	1103
25.2.1 Databases, laboratory tools and educational tutorials	1103
25.2.2 Bioinformatic resources	1105
25.2.3 Links to cytogenetics and genomics support groups	1106
25.2.4 Prominent peer-reviewed journals pertaining to genetics	1107
25.2.5 Cytogenetics and medical genetics textbooks	1107
25.2.6 Vendor products/equipment and lab support	1108
25.2.7 Credentialing and guidelines	1110
25.2.8 Genetics training programs and courses	1111
25.2.9 Professional organizations	1112
25.2.10 Job search	1112
Index	1113

Contributing authors

Yassmine Akkari, PhD, FACMG *Scientific Director, Cytogenetics; Technical Director, Molecular Pathology, FISH; Legacy Health, Portland, OR, USA*

Marilyn S. Arsham (retired) *Laboratory Technologist II, Western Connecticut Health Network, Danbury Hospital campus, Danbury, CT, USA*

Mervat S. Ayad, BS, EMBA, CG(ASCP)^{CM}, DLM^{CM}, CCS *Director of Laboratory Operations, Quest Diagnostics Nichols Institute, San Juan Capistrano, CA, USA*

Margaret J. Barch, MS, CG(ASCP)^{CM} (deceased) *formerly, Associate Director, Frank F Yen Cytogenetics Laboratory, Weisskopf Child Evaluation Center, University of Louisville, Louisville, KY, USA*

Luke Boyd, BS, CG(ASCP) *Clinical Cytogenetics, Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA*

Michael G. Brown, MS, CG(ASCP)^{CM}, MB(ASCP)^{CM} (retired) *Clinical Cytogenetics, Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA*

Suellen J. Charter *Research Coordinator, San Diego Zoo Institute for Conservation Research, San Diego, CA, USA*

Bhavana J. Davé, PhD, FACMG *Professor, Department of Pediatrics, Pathology and Microbiology, Munroe Meyer Institute; Associate Director, Human Genetics Laboratory, Munroe Meyer Institute for Genetics and Rehabilitation, University of Nebraska Medical Center, Omaha, NE, USA*

Patricia K. Dowling, PhD, FACMG *Director/Cytogenetics, Pathline Emerge Pathology Services, Ramsey, NJ, USA*

Christine E. Haessig, Hons BSc, RT(CG), CG(ASCP)^{CM} (retired) *Cytogenetics Supervisor, Cytogenetics Laboratory, Vancouver General Hospital, Vancouver, BC, Canada*

Nyla A. Heerema, PhD, ABMG, FACMG *Professor, Director of Cytogenetics, Department of Pathology, The Ohio State University, Columbus, OH, USA*

Marlys L. Houck, BA, CG(ASCP) *Senior Researcher, San Diego Zoo Institute for Conservation Research, San Diego, CA, USA*

Helen Jenks, CLS, CG(ASCP)^{CM} (retired) *UC Davis Health System, Molecular/Cytogenetic Laboratory, Specialty Testing Center, Sacramento, CA, USA*

Kathleen Kaiser-Rogers, PhD, FACMG *Clinical Professor, Pathology & Laboratory Medicine, Pediatrics, Genetics; Director, UNC Hospitals Cytogenetics Laboratory, University of North Carolina at Chapel Hill, NC, USA*

Wahab A. Khan, PhD, CG(ASCP)^{CM} *Icahn School of Medicine at Mount Sinai, New York, NY, USA*

Turid Knutsen, MT(ASCP), CG(ASCP)^{CM} (retired) *Section of Cancer Genomics, Genetics Branch, Center for Cancer Research National Cancer Institute, NIH, Bethesda, MD, USA*

Janet Krueger, CLS, CG(ASCP)^{CM} (retired) *UC Davis Health System, Molecular/Cytogenetic Laboratory, Specialty Testing Center, Sacramento, CA, USA*

Helen J. Lawce, BS, CG(ASCP)^{CM} *Clinical Cytogenetics, Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA*

Teri L. Lear, MS, PhD (deceased) *formerly, Research Associate Professor, M.H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY, USA*

Peining Li, PhD *Associate Professor, Director, Clinical Cytogenetics Laboratory, Department of Genetics, Yale University School of Medicine, New Haven, CT, USA*

R. Ellen Magenis, MD, ABMG (deceased) *formerly, Professor emeritus, Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA*

Kristin M. May, PhD, FACMG *Director, Genetic Diagnostic Laboratory, Children's Hospital at Erlanger, Chattanooga, TN, USA*

Marilu Nelson, MS, CG(ASCP)^{CM}, MB(ASCP)^{CM} *Human Genetics Laboratories, Munroe-Meyer Institute, University of Nebraska Medical Center, Omaha, NE, USA*

Jean H. Priest, MD, FACMG *Professor Emeritus, Department of Pediatrics, Division of Medical Genetics, Emory University School of Medicine, Atlanta, GA, USA*

Jeffrey S. Sanford *Probe Sales and Support Manager, MetaSystems Group, Inc., Newtown, MA, USA*

Warren G. Sanger, PhD, FACMG (deceased) *formerly, Professor, Department of Pediatrics, Pathology and Microbiology, Munroe Meyer Institute; Director, Human Genetics Laboratory, Munroe Meyer Institute for Genetics and Rehabilitation, University of Nebraska Medical Center, Omaha, NE, USA*

Debra F. Saxe, PhD, FACMG *Laboratory Director, Oncology Cytogenetics, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA, USA*

Adam Sbeiti, CG(ASCP)^{CM} *Supervisor, Quest Diagnostics Nichols Institute, San Juan Capistrano, CA, USA*

Lisa G. Shaffer, PhD, FACMG *CEO, Paw Print Genetics, Genetic Veterinary Sciences, Inc., Spokane, WA, USA*

Peggy J. Stupca, MS, CG(ASCP), DLM(ASCP) (retired) *Mayo Clinic, Rochester, MN, USA*

Sheryl A. Tran, BS, *Compliance Office Operations Manager, Mayo Clinic, Rochester, MN, USA*

Richard Van Rheeden, MS, CG(ASCP) *Supervisor, Clinical Cytogenetics Laboratory, Department of Pediatrics, University of Iowa Healthcare, Iowa City, IA, USA*

Preface

The *ACT Cytogenetics Laboratory Manual*, also known as the ACT Technical Manual, was first published by the Association of Cytogenetic Technologists in 1980. Its two editors, technologists Marilyn S. Arsham and Helen J. Lawce, were the driving force behind this work. They did much of the writing themselves and acquired the input of many practicing technologists who shared protocols, advice and troubleshooting strategies. Two additional chapters were published in 1984 and 1985 under the editorship of Gitta Wahrenburg.

In 1991 a second comprehensive edition, edited by Margaret J. Barch, was published with the goal of maintaining the philosophy and quality of content of the original while updating material, including an index, adding chapters on solid tumors, fragile sites and molecular cytogenetics, and producing a professionally published volume. The third edition, edited by Margaret J. Barch, Turid Knutsen, and Jack Spurbeck, was entitled the *AGT Cytogenetics Laboratory Manual* reflecting the change of ACT to AGT (the Association of Genetic Technologists) in 1996. The name change was officiated in order to incorporate the genetic technologies that complement the cytogenetic ones. The third edition also included an expanded treatment of molecular cytogenetics, including comparative genomic hybridization (CGH).

This fourth edition is completely updated and includes chapters on peripheral blood culture, continuous cell lines, prenatal diagnosis and culture, hematological and solid tumor malignancies, fragile sites, an expanded fluorescence in situ hybridization (FISH) chapter, molecular cytogenetics, a treatment of ISCN (International System for Human Cytogenomic Nomenclature), and a new approach to human chromosome identification. Other new topics to the fourth edition include safety, equipment maintenance, compliance, quality control, managerial tools, laboratory information software, laboratory mathematics, and even animal cytogenetics. Together, this constellation of chapters provides a comprehensive resource of both knowledge and skills that are essential to the daily operation of a cytogenetics laboratory.

Our goal has been to collect together in one volume a book which provides an in-depth treatment of the theoretical basis of the field, combined with a wealth of complementary protocols. This manual satisfies that goal by including an exhaustive survey of over 100 techniques for visualization and analysis of chromosome patterns in diagnosis and research. In most cases, protocols, along with their tips, hints, and processing detail, have been submitted by individuals who perform the test. Some procedures may have been edited in order to conform to the manual's general style. Important: Any new procedure, or changes to an existing one, must be properly validated by the performing laboratory before it can be used for clinical purposes.

We have designed this volume to be useful to students as well as to novice and experienced technologists. It is our hope that this new edition captures the vitality and fascination that the field of cytogenetics still holds as it continues to change.

Marilyn S. Arsham
Margaret J. Barch
Helen J. Lawce

Acknowledgments

An African proverb says, “It takes a whole village to raise a child.” This manual is the collective efforts of a village of exceptional people - professionals at all levels who were willing to donate their time, expertise, and insight to create chapters that are rich with information; reviewers who were willing to honestly and carefully critique each manuscript; and editors who helped structure the flow of information. As you read each chapter, you too will appreciate these incredible authors who voluntarily shared their expertise with us. We thank them for their generous contributions and time.

What you won’t see listed in the chapters are the names of the many experts on the review board who worked behind the scenes. We sincerely thank everyone on our review board for their suggestions and evaluations; we couldn’t have done this without you. Please forgive any omissions: Charles Dana Bangs, Jane Bayani, Sue Ann Berend, Adam Coovadia, Pat Dowling, Betty Dunn, Viola Freeman, Atieh Hajianpour, Bing Huang, Betsy Jackle, Wahab Khan, Turid Knutsen, Peining Li, James L. Marks, Susan Olson, Katy Phelan, Richard Van Rheeden, Thomas Wan, Su Yang.

Three individuals deserve a special mention for their extra help. Turid Knutsen, you were the first to complete your chapter, which gave us an example to query publishers, and you stepped in to help edit chapters and format protocols without once asking for accolades. Charles Dana Bangs, your critical eye, encouraging words, and countless donations of time and material brought this manual to a higher level, again without expecting accolades. And Helen Jenks, your willingness and eagerness to step in, with your special way with words, helped us get through a very stressful period. We can never thank you three enough for your support and expertise.

On a personal note, we want to thank all those around us, both personal and professional, family and friends, who helped us along the way. There are too many to individually name, but we want you to know how much we appreciate your willingness to share your time, tips, protocols, pictures, advice, generators, and patience, while we plugged away at this manual, even in the face of nor’easters and hurricanes. Thank you, Bryan Arsham, for your assistance and patience in setting up this new computer so that uploading to AGT and Wiley’s clouds did not take hours per file. We give special thanks to AGT Past President Mervat Ayad for your support in helping build the interface with the publisher and to the editors at Wiley for making the process painless. In particular, we thank Purvi Patel, Metilda Shummy, and the invisible team behind them, for making this project come alive. Most of all, we thank our spouses, Ron, Gary, and John, for your unwavering support. These past 10 years have been an especially challenging time for us, and you have never once demanded anything of us but to finish this manual. You are our true heroes in each of our personal villages.

Finally, and most difficult of all, we thank you, Margaret. You are no longer here as we write these final words for you. You left us too early, and we miss you terribly. Thank you for keeping this manual alive, for giving so much love to each of us, and for being the gracious Southern Belle that you will always be in our eyes. Gary, we know her loss can never be mended, but we want you to know how much we love you for all the love you gave and still give “our” Margaret.

Our “village” is AGT, which reaches across all continents, a vision that far exceeds the expectations of the original 40 California technologists who started it all. This manual is the “child” of AGT’s dedicated technologists. It was conceived four decades ago, when Marilyn nervously stood up at the first annual AGT business meeting in Boston and proposed the concept. Helen joined her a year later, and Margaret kept it going for three more decades. Its need was evident in 1975 and is still evident today. It is therefore our turn to thank you all collectively for sharing your discoveries with us over these last 40 years.

CHAPTER 1

The cell and cell division

Margaret J. Barch¹ and Helen J. Lawce²

¹*(deceased) formerly, Frank F Yen Cytogenetics Laboratory, Weisskopf Child Evaluation Center, University of Louisville, Louisville, KY, USA

²Oregon Health & Science University Knight Diagnostic Laboratory, Portland, OR, USA

1.1 The cell [1,2]

The cell is the basic unit of life – the simplest structure capable of independent existence. The simplest organisms consist of only one cell. Higher organisms are composed of complex colonies of interdependent cells, each colony with a specialized function necessary for the survival of the organism. Cells that have the same general function are often grouped together to form tissues, such as muscle, bone, and connective tissue. Tissues may be combined in larger functional units called organs, such as kidneys, skin, and heart. Organs can in turn be grouped by function into organ systems, such as the respiratory and circulatory systems.

Cells vary greatly in size, but they all must be able to survive and reproduce to be successful organisms. The cell membrane that envelops its contents must be able to control the movement of nutrients into the cell and of ions, molecules, and proteins out of the cell. Energy is converted from food and/or light and is used to synthesize internal components. The information for reproducing cell structures is encoded within its genetic makeup, thus providing the cell with its own self-sufficient capability to reproduce life-supporting needs and to repair generic damage as needed. When functioning properly, the cell contains all the necessary tools to survive.

1.1.1 Cell membrane

Composition

The cell generally consists of cytoplasm, bounded by a cell membrane, and a nucleus, also enclosed in a membrane. There are exceptions to this model, such as red blood cells that have lost their nuclei during differentiation. The plasma membrane, or cell membrane, defines the boundary of the cell (Figure 1.1) and consists primarily of phospholipids and proteins. The phospholipids form a bimolecular layer, with their hydrophilic ends at the outer surfaces of the membrane and their hydrophobic chains extending into the middle of the membrane. The protein components of the membrane are globular particles distributed through the lipid bilayer; their polar amino acids may be exposed on an outer surface, but nonpolar portions remain in the interior.

Physical barrier

The cell membrane serves as a physical barrier for the cell contents, but it is rather fragile. If one were to tear a hole in this membrane by micromanipulation, the contents would spill out into the surrounding medium. An intact cell can rapidly repair minor membrane damage, but more extensive damage leads to cell death.

* Editors' note: We lost Margaret in the final stages of producing this book. May her spirit shine through and the reader be touched by her love of science, and her passion for passing it on.

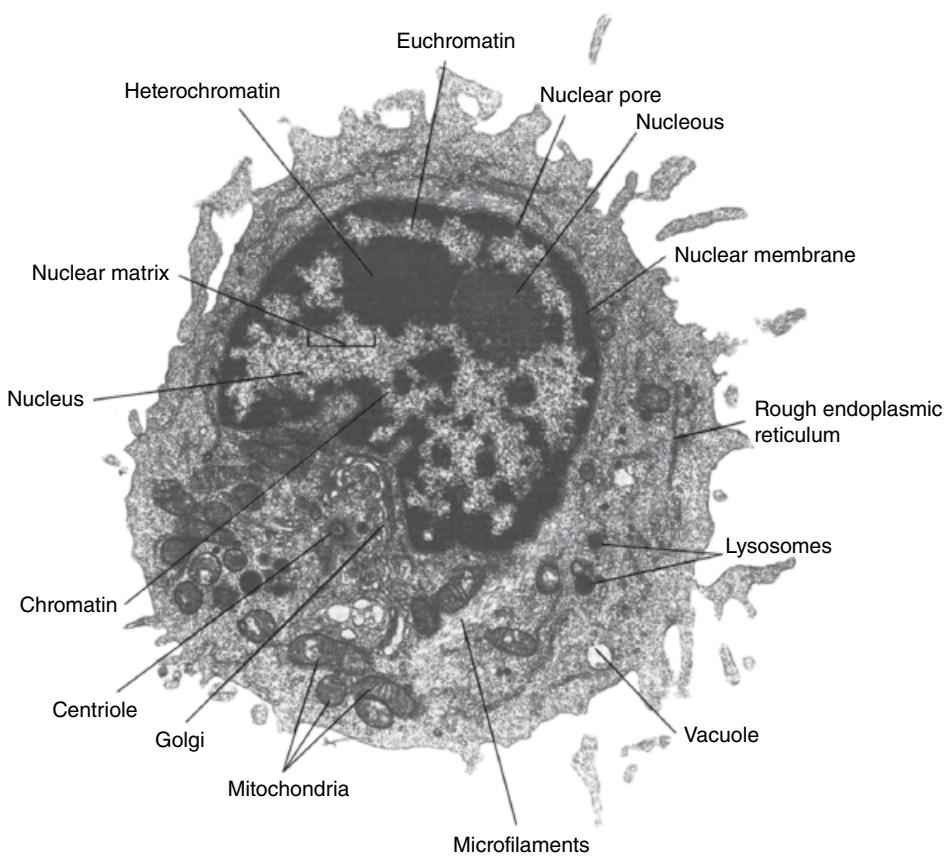


Figure 1.1 An electron micrograph showing the various components of a eukaryotic (human) cell.

Regulatory barrier

The membrane also acts as a regulatory barrier for the entry and exit of molecules and particles. This ability to regulate the passing of substances is called *selective permeability*. Substances can cross the cell membrane by three mechanisms: by free diffusion along a gradient, meaning that substances travel from regions of high concentration to regions of lower concentration; by active transport, which requires energy and moves substances against a concentration gradient; and by enclosure in vesicles that move substances into the cell (endocytosis) or out of the cell (exocytosis). Water can move freely across cell membranes in both directions; it is this property that allows hypotonic solutions (those less concentrated than the inside of the cell) to swell the mitotic cell, thus facilitating chromosome spreading for cytogenetic study.

Glycoprotein functionality

Molecules of glycoprotein (proteins with sugar molecules attached at points along the amino acid chain) exist on the surface of the protein–lipid membrane and sometimes project through it, into the cell. These glycoproteins function in cell adhesion, both to other cells and to culture flask surfaces. Trypsin, a protease (an enzyme that digests proteins), removes these molecules, thereby freeing cells for subculture or harvest. Glycoproteins can be antigenic (e.g., in red cells they determine blood type), and can serve as receptors for viruses, plant agglutinins (e.g., phytohemagglutinin), and hormones. They are further implicated in contact inhibition, a process in which normal cells stop dividing as cultures become confluent. Tumor cells often lose this property and tend to keep growing unchecked in a disorganized fashion when the growth surface is limited. Glycoproteins on the cell surface are also important in cell–cell recognition. If lymphocytes are stripped of their glycoproteins, they no longer accumulate in the lymph nodes.

1.1.2 Cytoplasm

Cytoplasm is the part of the cell within the cell membrane, excluding the nucleus, that consists of water, inorganic ions or molecules, and a variety of organic compounds. In many ways it resembles a colloid, with particles suspended in a continuous gel-like substance called the cytosol. The cytosol, in turn, contains a cytoskeleton of tubules and filaments, dissolved molecules, and water. Among the inorganic molecules are potassium, sodium, magnesium, and calcium. Trace amounts of many heavy metals are also present, as are bicarbonate and phosphate. Tiny granules can also be seen with a light microscope. These granules have been shown to be a series of vacuolar structures, bound by lipoprotein membranes similar to the cell membrane, with some even further differentiated into a complex system of internal membranes.

The large organic molecules (called macromolecules), which give the cytoplasm its colloidal properties, can be grouped into three main classes: proteins, nucleic acids, and polysaccharides. Each class is a polymer built from different subunits (monomers): proteins are made up of amino acid subunits; nucleic acids are polymers of nucleotides; and polysaccharides are built from sugar monomers. Together, the organelles described below and the cytosol make up the cytoplasm.

Proteins

Proteins carry out several important functions within the cell, including structural support, catalysis of metabolic reactions, and regulation of complex cellular processes. Examples of structural proteins are actin and myosin in muscle, and keratin in hair, nails, and hooves. Regulatory proteins include hormones, growth factors, and receptors.

Polysaccharides

Polysaccharides function as food storage molecules and as structural molecules. The two most important polysaccharide food reserves in higher organisms are starch and glycogen, both of which are polymers of glucose sugar. Structural polysaccharides include cellulose and chitin: cellulose is the major constituent of cell walls in plants, and chitin is found in the exoskeletons of insects and crustaceans.

Lipids

Another important organic molecule, although it is not classified as a macromolecule, is the lipid. Lipids encompass a diverse group of compounds that are all soluble in nonpolar, organic solvents. Included in this class are fats, which are used primarily for energy storage; phospholipids, which are found in cell membranes; sphingolipids, which are especially prominent in the cell membranes of brain and nervous tissue; glycolipids, which are important in the myelin sheath of nervous tissue; steroids, which include male and female sex hormones, bile acids, adrenocortical hormones, and cholesterol; and fatty acids, which are components of energy storage molecules.

Endoplasmic reticulum

Endoplasmic reticulum (ER) is contiguous with the outer membrane of the nucleus. It is the site for folding proteins and assembling large molecules in an oxidizing environment. The ER consists of membranous channels lacking ribosomes (smooth ER) or containing ribosomes (rough ER). In the rough ER, ribosomes actively synthesize protein that accumulates in the lumen of the ER. These proteins include secretory proteins that make their way to the cell surface via a complex route, e.g., the rough ER, Golgi complex (see later), and secretory vesicles. Smooth ER is the site of synthesis of lipids and steroids and also for the inactivation and detoxification of drugs and other compounds harmful to cells.

Golgi complex

The Golgi complex (or Golgi apparatus) is a region of flattened vesicles closely related to the smooth ER both in proximity and function. It processes and packages secretory proteins and synthesizes complex polysaccharides. The Golgi also accepts vesicles that “bud off” the ER. These vesicles and their protein contents are processed further and then passed on, via vesicle budding of the Golgi complex, to other components of the cell. Therefore, the Golgi complex is a processing station for both receiving vesicles that fuse with it and also producing vesicles from it in a repackaged form, usually ready for export from the cell.

Lysosome and peroxisome

Two structurally similar organelles are the lysosome and peroxisome, each contained by a single membrane. Lysosomes are storage structures for hydrolases, i.e., enzymes that digest food and cell components that are no longer needed. Peroxisomes generate and degrade hydrogen peroxide. Animal peroxisomes also detoxify other harmful compounds, such as, ethanol, methanol, formate, and formaldehyde, and generate some unusual substances, such as D-amino acids.

Mitochondria

The mitochondrion (plural: mitochondria) is quite large, relative to other organelles, i.e., several micrometers (microns) in length and 1 micrometer in width, about the size of a bacterial cell. All mitochondria in the cells of an individual are maternally derived from those that were present in the egg at the time of fertilization. Therefore, unlike nuclear DNA in which the paternal contribution of genes is 50%, all mitochondrial DNA comes from the mother.

Depending on the organism and cell type, a cell may have only one mitochondrion or it may have several thousand. A typical human cell contains hundreds of mitochondria, each with 2–10 copies of mitochondrial DNA (mtDNA), resulting in thousands of copies of mtDNA per cell. Again in contrast with nuclear DNA inheritance, where each cell receives exactly half the genetic material at cell division, mitochondria are not always evenly partitioned into daughter cells – one cell may receive more (or fewer) copies of mitochondria. Therefore, the number of mitochondria and constituent mtDNAs can be heterogeneous between tissues and even within a given tissue; this is termed *heteroplasmy*.

Within the mitochondria, oxidation of nutrients (oxidative phosphorylation) takes place, providing energy to synthesize adenosine triphosphate (ATP). ATP conserves the energy from the oxidative reaction that would otherwise have been lost as heat and makes it available to the cell for work. Thus, the mitochondria have been called the powerhouses of the cell. They have a double membrane, an outer membrane plus an inner membrane, which are infolded into numerous projections called *cristae*, where oxidation of nutrients takes place.

Mitochondria also command special interest because they contain their own DNA (mtDNA) and ribosomes, although the ribosomes in mitochondria are more similar to those in prokaryotes in size and nucleotide sequence than to ribosomes elsewhere in the eukaryotic cell. mtDNA is usually circular, like a bacterial genome, with no histones attached. Human mtDNA contains 37 genes, including those that specify transfer RNAs, ribosomal RNA, and polypeptides important in ATP synthesis. The mitochondrion even encodes some of its own RNA and polypeptides, about 5% of those it needs. Mutations of mitochondrial genes, even when only a fraction contain mutant mtDNAs, can cause disease if they are located in tissues where mitochondrial function is important; for example, mitochondrial mutations have been implicated in several metabolic diseases, heart disease, and aging.

Ribosomes

In addition to the membranous organelles mentioned above, cells contain other important structures. The ribosome, made up of 50–80 different proteins and three or four different kinds of RNA molecules, is a tiny spherical body on which the synthesis of proteins takes place. They are found either free in the cytoplasm or attached to mitochondria, ER, or the outer surface of the nuclear membrane. Proteins needed for use in the cytosol are usually synthesized on single ribosomes.

Polypeptide chains are made on groups of ribosomes called polyribosomes, or polysomes. The polysome contains a variable number of ribosomes held together by a messenger RNA (mRNA) strand. This mRNA strand determines the sequence of amino acids in the synthesized protein. Signals residing in the mRNA also determine initiation, elongation, and termination of the polypeptide. Antibiotics, such as streptomycin, chloramphenicol, and puromycin, block protein synthesis at one of these three stages.

Centrioles

Tubules and filaments are other versatile cell components. Electron microscopy has shown that centrioles (or basal bodies), which are important in cell division, are found near the nucleus. The centriole contains nine microtubule triplets around its periphery. These bodies occur in pairs, called a diplosome or centrosome, which are perpendicular to each other and are attached to the outside of the nucleus. During the G1/S cell cycle transition the centrioles self-duplicate and migrate to opposite ends of the cell, where they form spindle fibers (also made of microtubules). Spindle fibers help separate chromosomes to their respective daughter cells in cell division.

Many proteins interact with and regulate the centrosomes. Alterations in these centrosome-associated proteins can have pathological consequences. For example, mutations in the TP53 gene can lead to extra copies of the centrosome, predisposing the cell to misshapen spindle apparatus formation, aneuploidy, and tumor formation [3].

In the laboratory, colchicine inhibits cells from completing mitosis by binding to the monomer tubulin, thereby blocking its assembly into polymeric spindle fibers. Colchicine also indirectly disassembles already-formed spindle fibers. Without spindle fibers, chromosomes are unable to move away from the metaphase plate and complete cell division.

Cilia and flagella

Cilia and flagella, the external hair-like projections that function in cell motility, are also made of microtubules. Nine doublet tubules are arranged around the periphery and a tenth doublet forms the core. Like their close relatives, the microfilaments, microtubules are involved in cell movement, cytoplasmic streaming, cell cleavage, and membrane invaginations. Microtubule-initiated motion almost always requires ATP as an energy source.

1.1.3 Nucleus

The nucleus is the information headquarters for the cell. Unlike prokaryotes, such as bacteria and blue green algae that carry their genetic material in the cytoplasm, other more complex organisms confine their genetic information, DNA, within a nucleus. These more evolved plant and animal organisms, including humans, are termed eukaryotes (eu = true; karyon = nucleus). Every eukaryotic cell has a nucleus at some stage of its existence. Some cells have more than one nucleus, and some, such as red cells and platelets, lose their nuclei when they mature. Cells lacking nuclei, however, are severely limited in their metabolic activities.

The nucleus contains a nuclear membrane, chromatin, and nucleoli (see Figure 1.1). It is also the site of ribosome precursor assembly. The term *nuclear matrix* refers to the fibrous material that remains if the chromatin and nucleoli are extracted. DNA within the nucleus determines the cell's morphological, biochemical, and metabolic characteristics.

The appearance of the nucleus is markedly different in interphase (nondividing) and mitotic (dividing) cells. First noted by Brown in plant cells in 1831 [4], the interphase nucleus is a conspicuous spherical body in the cell interior. By light microscopy, it appears as an amorphous network of variably condensed fibers, called chromatin, which are not distinguishable as individual entities. Highly condensed chromatin stains darkly with nuclear stains and is known as *heterochromatin*; the more dispersed chromatin, which stains lightly or not at all, is called *euchromatin*. In cell division, the chromatin condenses into deeply staining, threadlike or rod-like structures called chromosomes (chromo = color; soma = body), which are present in specific numbers in each cell of a given species. This process of chromatin condensation to form chromosomes during division is necessary for the equal parceling of genetic information to daughter cells.

The nucleus is spatially organized with each chromosome in a specific region. This serves to prevent one chromosome from getting tangled with another. Telomeres are attached to the nuclear membrane. Between chromosomal subcompartments are chromatin-free interchromosomal domains. Here reside RNA molecules being processed for export to the cytoplasm. Highly transcribed portions of the chromosomes are positioned next to the interchromosomal domains and since different genes are transcribed in different cell types; the arrangement can vary from cell to cell.

Under the electron microscope, chromatin and chromosomes appear as fibrous structures. This is understandable since they comprise DNA molecules that are themselves filamentous. Fibers of DNA with associated proteins are about 30 nanometers (nm) in diameter, but protein-depleted strands are only about 10 nm in diameter. Chromatin fibers with diameters greater than 30 nm are occasionally observed and are believed to represent coiling or folding of these main fibers.

Nuclear envelope

The nuclear envelope, as the membrane surrounding the nucleus is called, is a porous double membrane with ribosomes attached to the outside. Numerous pores serve as channels for water-soluble molecules to travel between the nucleus and the cytoplasm. Ribosomes, mRNA, chromosomal proteins, and enzymes needed for nuclear activities are also thought to travel through these nuclear pores. The outer membrane is contiguous with the ER at many sites. Inside the nucleus are two obvious structural elements – the nucleolus and the chromatin. During cell division, this nuclear envelope disappears.

Nucleolus

One to four nucleoli appear as darkly staining bodies eccentrically placed within the normal nucleus. They comprise primarily RNA and protein but contain some DNA. Their size will vary, based on the cell type and the metabolic state, i.e., larger nucleoli are seen in rapidly dividing cells and in cells active in protein synthesis. Each nucleolus is formed along the nucleolar-organizing region (NOR) of one or more specific chromosomes; these regions are recognizable during cell division. The nucleolus is the site of ribosome precursor assembly; therefore, all ribosomes in the cytoplasm originate in the nucleolus.

Nucleic acids

The nucleus contains the nucleic acids DNA and RNA along with structural and regulatory proteins. Nucleic acids are involved with protein synthesis and the storage of genetic information. There are two kinds of nucleic acids: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), each a polymer of *nucleotides*. Nucleotides consist of one purine or pyrimidine, a five-carbon sugar, and a phosphate group (see DNA). The sugar in DNA is deoxyribose; in RNA (see RNA) it is ribose.

DNA is the genetic material, and RNA is responsible for carrying out the instructions coded by the DNA. The primary functions of nucleic acids are *gene replication*, i.e., the process of copying sequences of DNA (genes) for distribution to daughter cells, and *gene transcription*, i.e., the process of copying sequences of DNA into complementary strands of RNA. These transcript RNAs may then be translated into corresponding sequences of amino acids during the synthesis of polypeptides (proteins). As previously discussed (see Ribosomes), protein synthesis occurs on cytoplasmic ribosomes.

DNA

The story of how scientists searched for the hereditary material and eventually established that DNA is the genetic material in almost all organisms is a fascinating one. Gregor Mendel's "hereditary factors," Walther Flemming's chromosomal threads, and Walter Sutton's chromosome theory of heredity led the way. Johan Miescher discovered DNA in 1869, calling it nuclein. The beauty of its structure and the logic of the coding process still inspire those who study them today.

Studies with sister chromatid exchange, electron microscopy, and other techniques demonstrate that a chromatid, one of a pair of metaphase chromosome strands, contains a single, uninterrupted, highly folded molecule of DNA. DNA itself is a double helix made up of two strands. Each strand is comprised of nucleotides, each consisting of a sugar molecule, a phosphate group, and one of four bases: adenine (A), guanine (G), thymine (T), or cytosine (C). The nucleotides are arranged side by side, with two bases forming one rung of a twisted ladder, and the phosphate and sugar form the outer structure (Figure 1.2). The sugar in

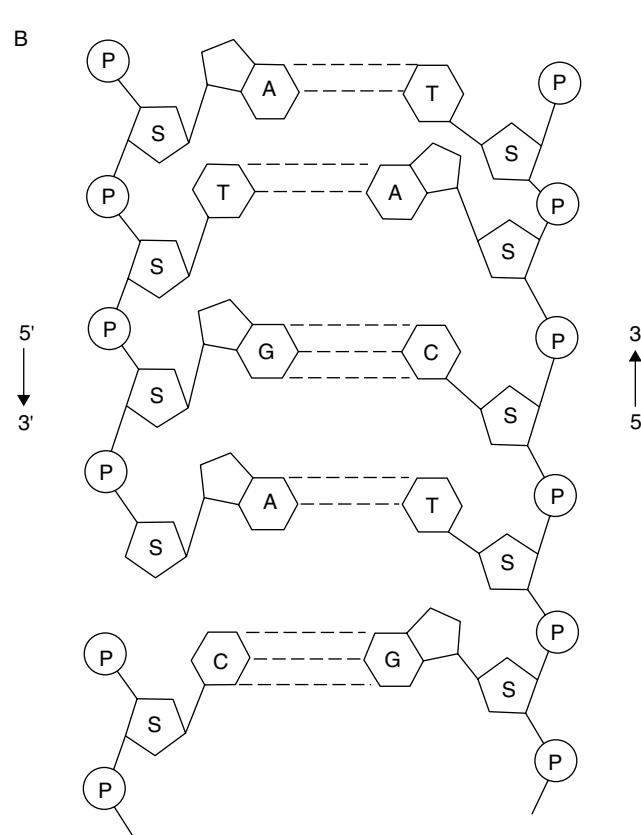


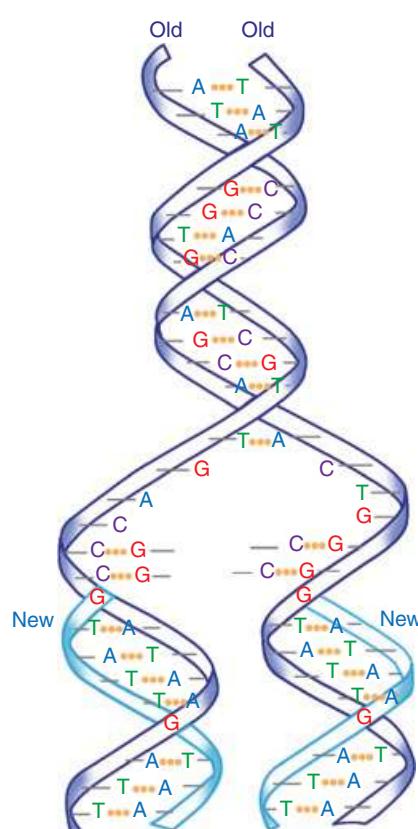
Figure 1.2 Chemical structure where A is adenine, T is thymidine, G is guanine, C is cytosine, S is sugar, and P is phosphate. The left strand polarity is from the 5' base to the 3' base, and the right strand has a 3' to 5' opposite polarity.

DNA, deoxyribose, has five carbon atoms, the third and fifth of which are bonded together by a phosphate (phosphodiester) linkage. Thus, a single strand of DNA is a polymer of deoxyribonucleotides held together by a 3'-5' phosphate linkage between their sugars. This is called the sugar-phosphate backbone of the DNA molecule, and it lies on the outside of the DNA fiber; the bases extend inward from the backbone. The free 3' and 5' ends give the molecule a polarity, or direction.

Watson and Crick [5,6] determined the double helical structure of DNA in the 1960s using models and X-ray diffraction images. The two strands of polynucleotides have opposite polarity. The bases hold the two strands together by hydrogen bonds (see Figure 1.2). Both strands are coiled in the same direction, so they cannot be separated without unwinding. Minor bases present in mammalian DNA include 6-methyl adenine and 5-methyl cytosine; the latter is found throughout the human genome but is often concentrated in areas of heterochromatin, such as in chromosomes 1, 9, 15, 16, and Y.

The bases in DNA are flat molecules that can stack on top of one another. The double-helical nature of DNA is maintained by these stacking forces and by the hydrogen bonds between the bases. The regularity of the double helix along its axis is possible because an AT pair is the same size and shape as a GC pair.

Prior to cell division, new DNA must be synthesized with great fidelity. This is accomplished by separation of the two strands so that each acts as a template for the assembly of a complementary strand (see Figure 1.3). Thus, two identical copies of the original DNA are produced, each composed of one original strand and one newly synthesized strand (semiconservative replication). This mechanism for producing a faithful copy of the genetic information for each daughter cell is fundamental to understanding techniques such as sister chromatid exchange (see Chapter 6, Chromosome stains). Of the four bases, two are purines (A and G), and two are pyrimidines (T and C). The precise replication of DNA is possible because the pairing of bases is specific: A pairs with T, and G pairs with C. Thus, the sequence of bases in one strand specifies the bases and their order in the complementary strand.



DNA replication illustrating two new helices being replicated semiconservatively.

Figure 1.3 This diagram illustrates two new helices being replicated semiconservatively (Adapted from Lince-Faria et al. 2009 [26]. See insert for color representation of this figure.).

Table 1.1 Genetic code

DNA triplet	RNA triplet	Amino acid
AAA	UUU	Phenylalanine
AAT	UUA	Leucine
TAA	AUU	Isoleucine
TAC	AUG	Methionine (start)
AGA	UCU	Serine
GGA	CCU	Proline
TGA	ACU	Threonine
CGA	GCU	Alanine
ATA	UAU	Tyrosine
ATT	UAA	(stop)
GTA	CAU	Histidine
GTT	CAA	Glutamine
TTA	AAU	Asparagine
TTT	AAA	Lysine
CTA	GAU	Aspartic acid
CTT	GAA	Glutamic acid
ACA	UGU	Cysteine
ACT	UGA	(stop)
ACC	UGG	Tryptophan
GCA	CGU	Arginine
CCA	GGU	Glycine

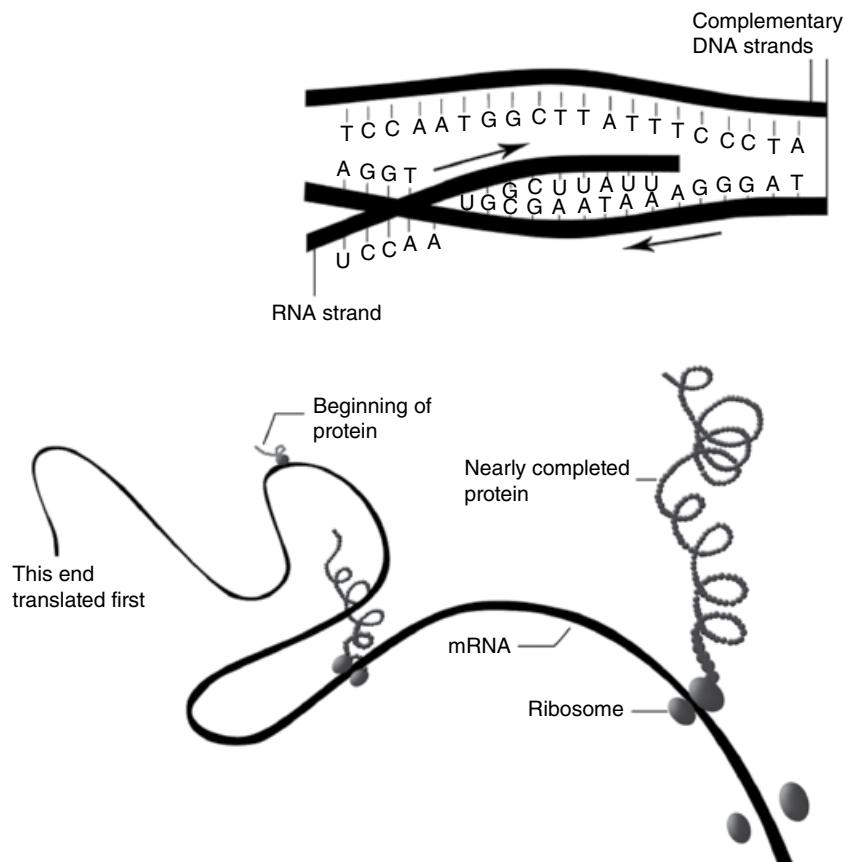
The nucleotide triplet in DNA specifies a triplet in RNA, which specifies an amino acid (or a start or stop signal). The code is “degenerate” in that each codon is not unique; for instance, UUA, UUG, CUU, CUC, CUA, and CUG all specify the amino acid leucine. A, adenine; C, cytosine; G, guanine; T, thymine; U, uracil.

The way DNA stores information was elucidated in the 1960s [5,6]. It was learned that the genetic code consists of three bases per code word; one triplet, or codon, codes for one amino acid (Table 1.1). A gene, then, can be understood as a linear arrangement of codons giving the instructions for the building of a protein with specific amino acids in a particular order.

It was later discovered that in higher eukaryotes, the coding instructions in a gene are often interrupted by DNA sequences that are not present in the mRNA and are not translated into amino acids in that gene’s protein. These interrupting sequences are called introns (for intervening sequence), and the DNA sequences translated into the mRNA that usually code for protein are called exons [7]. The introns are spliced out of the mRNA before it codes for a protein. It is now known that a single gene can make more than one protein. Alternative splicing of introns can lead to multiple transcripts.

High temperatures or high pH conditions break the hydrogen bonds, and the double-stranded helix unwinds, or denatures, into two single-stranded helices. Because G–C pairs have three hydrogen bonds and A–T pairs have only two, the A–T pairs tend to be more unstable, denaturing before the G–C pairs. Therefore, the temperature at which a given DNA will be half denatured, or melted, is used as an index of the amount of G and C in that DNA. The curve of the rate at which this denatured DNA renatures (becomes double-helical once more) is called its Cot curve (Co = concentration of single stranded DNA, t = time). This curve yields other information about the DNA, such as how many sequences are present in multiple copies (repetitive DNA) versus how many are unique. Denaturation of DNA is an important step in fluorescence in situ hybridization (FISH) procedures discussed in a subsequent chapter.

Another measure of the G–C content is the buoyant density of the DNA. This is measured by forming gradients of concentration (and therefore of density) in cesium chloride during centrifugation. The DNA will collect at the



DNA transcription: synthesis of an RNA molecule complementary to one strand of the DNA (top). RNA translation: synthesis of protein molecules specified by the RNA sequence (bottom).

Figure 1.4 DNA transcription involves synthesis of a RNA molecule complementary to one strand of the DNA (top). RNA translation refers to the synthesis of protein molecules specified by the RNA sequence (bottom). Watson 1983 [28]. Reproduced with permission of John Wiley and Sons.

band where the gradient density is equal to the DNA density. This buoyant density depends upon DNA strandedness (single or double) and base composition. (See also 1.1.6, Satellite DNA.)

RNA

Like DNA, RNA is a polymer of ribonucleotides linked by 3'-5' phosphodiester bonds. RNA differs from DNA in three respects: its ribose sugar has a 2'-H group instead of 2'-OH group; it is single-stranded, rather than double-stranded; and it substitutes the base uracil for thymine to pair with adenine.

DNA does not specify a protein directly; rather, the gene for forming the protein is expressed through an intermediary molecule, the mRNA. Transcription, or mRNA synthesis, uses one strand of the DNA as a template for a complementary strand of RNA (Figure 1.4). After transcription, introns are spliced out and the mRNA molecule moves out of the nucleus to the cytoplasm, where it directs the synthesis of protein in the presence of ribosomes. Transfer RNA (tRNA) binds the appropriate amino acid to its anticodon, a base triplet complementary to a codon in mRNA. Ribosomal RNA assists in actual protein synthesis, by binding the anticodons of the tRNA molecules with the matching codons of the mRNA molecule so that the attached amino acids are covalently linked in the proper linear order.

Approximately 1.2% of the genome encodes for protein via mRNA; yet about 93% produces RNA transcripts. For example, micro RNA (miRNA) performs its regulatory function by binding to a matching region on a strand of mRNA; this will block

the ribosome from reading the strand and will thus disrupt protein synthesis. Likewise, small interfering RNA (siRNA) binds to mRNA and cuts it, thereby preventing translation. The functions of these and other RNAs are under study.

1.1.4 Chromosomes and their proteins

The two main categories of chromosome proteins are histones and nonhistones. Interphase chromatin contains mostly histone proteins, characterized by their basic pH, which is due to large numbers of the amino acids arginine and lysine. Their isoelectric points (pH at which the average charge of the molecule is zero) are always more than 10. Proteins with an isoelectric point less than 10 are classified as nonhistone proteins (NHPs). NHPs tend to be acidic, although their isoelectric points vary from 4 to 9, and are a mixture of proteins with different structural, enzymatic, and regulatory functions.

Histones

Histones, which can be found in a 1:1 ratio by weight to DNA, are classified into five major classes: lysine-rich H1, slightly lysine-rich H2A and H2B, and arginine-rich H3 and H4. More specialized forms can also be found in specific structures, such as, H5, the histone that replaces H1 in nucleated erythrocytes, and protamines, a group of highly basic proteins with a low molecular weight, which replace the histones in mature sperm. H3 and H4 have been highly conserved in evolution, and may actually express the same functionality in all eukaryotes [8]. Histones are also highly conserved in organisms from one tissue to another and between species; therefore, cows and peas have virtually the same histones. In the laboratory, histones can be extracted from chromatin by dilute acids or by high-molarity salt solutions. Acetic acid and methanol, commonly used to “fix” chromosomes, dissolve out some, if not most, of the histones.

Nucleosome

Histones H2A, H2B, H3, and H4 form octomers containing two molecules of each histone, giving rise to a 10-nm sphere or disk (visible with the electron microscope) called a nu-body, or nucleosome. The nucleosome appears to be the basic unit of eukaryotic chromatin. The fifth histone, H1, is implicated in the linking and compaction of these nucleosomes.

The nucleosome is present in dispersed or condensed chromatin, in repetitive areas or unique sequences, and in interphase and metaphase nuclei. It is associated with roughly 140 base pairs (bp) of DNA, which is wound twice around the spherical nucleosome. For reference, an average structural gene is approximately 1200 bp, which would span about six nucleosomes. When chromatin is extended by the removal of H1 histones, a linker region of about 60 base pairs of DNA can be seen between nucleosomes. When this region is uncoiled, the nucleosomes are seen located along the naked DNA like beads on a string. The H1 protein is responsible for condensing these beads into a 10-nm fiber. This is coiled again into the 25-nm strands, which look lumpy or kinky under the electron microscope (see Figure 1.5).

Nonhistone proteins

There are several hundred nonhistone proteins [9], which include all proteins of chromatin other than histones. Even though these proteins are thought to be more numerous and more variable than the histones, they actually make up much less of the chromatin mass [10]. Nonhistone proteins are involved in chromosomal metabolism [1,2], gene expression, and higher order structure.

Euchromatin and heterochromatin

Positive and negative heteropyknosis is used when referring to staining intensity that reflects the degree of coiling or condensation of the chromatin filaments. During the cell cycle, chromosomes condense and decondense, with maximum condensation at metaphase. Chromosomes and segments of chromosomes that are more heavily stained than the rest exhibit positive heteropyknosis. Others that are more lightly stained exhibit negative heteropyknosis. The chromatin in these variable regions, showing condensation unlike the remainder of the chromatin, is termed heterochromatin, while other regions are known as euchromatin. Under the electron microscope there is no difference in the basic structure of euchromatic and heterochromatic chromatin; therefore, differential staining has been attributed to fiber packaging within the heterochromatic regions. In the literature, the term heterochromatin usually refers to positive-heteropyknotic areas, but a given area may be negatively heteropyknotic in one banding technique and positive in another; therefore, use of the term should specify the stain with which it is being evaluated.

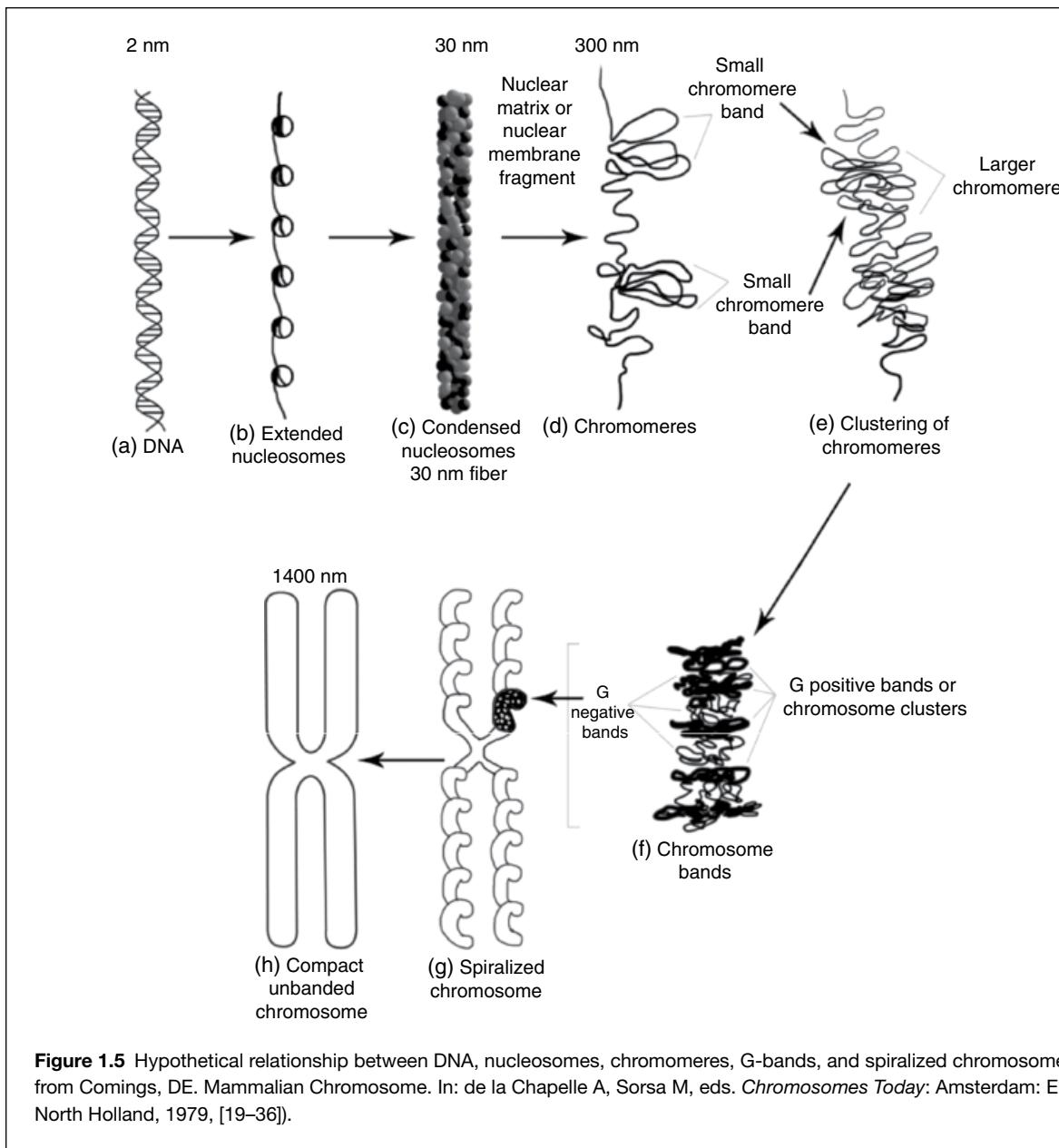


Figure 1.5 Hypothetical relationship between DNA, nucleosomes, chromomeres, G-bands, and spiralized chromosomes (Adapted from Comings, DE. Mammalian Chromosome. In: de la Chapelle A, Sorsa M, eds. *Chromosomes Today*: Amsterdam: Elsevier/North Holland, 1979, [19–36]).

There are two distinct types of heterochromatin: facultative and constitutive. In humans, facultative heterochromatin is the name given to the condensed, inactive chromatin of X chromosomes in excess of one. It may represent one X in a given cell and its homolog in another, owing to the randomness of X inactivation (see 1.1.5, X inactivation). Constitutive heterochromatin is the name for the differentially staining areas of chromatin and chromosomes, which are evident with different stains and banding techniques and are constant from cell to cell.

Facultative heterochromatin and constitutive heterochromatin resemble each other in several ways: neither codes for protein (in most cases being genetically inactive), and both replicate late in the synthesis phase of the cell cycle. They differ in that constitutive heterochromatin is often rich in repetitive DNA, stains differently from euchromatin with banding techniques, and never elongates or decondenses. Facultative heterochromatin has sequences similar to active DNA, does not stain differently with standard banding techniques, and in some cases can become decondensed and active, as the X chromosome does during meiosis and early embryogenesis.

1.1.5 X inactivation

The manifestation of facultative heterochromatin in most female mammals, including humans, is a visible sex chromatin body that is derived from the second X chromosome. It is commonly referred to as a Barr body. Various staining techniques, as well as phase contrast, can be used to visualize the Barr body. It can be found in almost all tissue cells.

Barr body

Although earlier investigators noticed a “basophilic nucleolus” or “paranucleus,” this triangular staining body on the periphery of the nuclear membrane was not related to sex until 1949, when Murray L. Barr and Ewart G. Bertram published their observation of a “paranucleus” in cells from the hypoglossal nerve of female cats, but not of the males, called the Barr body [11]. Expanding on the work by Liane Russell, Mary Lyon in 1961 put forth what we now know as the *Russell–Lyon hypothesis* [12], which stated the following:

1. One of the two X chromosomes is inactivated in human females.
2. The inactivated X may have either maternal or paternal origin in a given cell of an individual, and the choice is random.
3. The inactivation occurs in early embryogenesis.
4. Inactivation is stable, and descendants of a cell with an inactive X inherit that same X in an inactive state except in the germ cells (see later).

X inactivation is often called dosage compensation, that is, a mechanism for producing equal amounts of gene products in females having two X chromosomes and males having only one. We know that this compensation is effected by some mechanism, because females homozygous for hemophilia A have clotting times similar to those of affected hemizygous males. Also, glucose-6-phosphate dehydrogenase (G6PD) levels are similar in normal females (most of whom are homozygous) and in normal hemizygous males [13].

This mechanism, however, is by no means universal. In Drosophila, females are the homogametic sex (XX) and males are the heterogametic sex (XY), just as in mammals. The difference is that in Drosophila both Xs are active (transcribed), but the single X in males produces comparable amounts of gene products. Birds, reptiles, and butterflies have no dosage compensation of Z-linked genes, and the homogametic sex (ZZ), which is male, produces twice the level of gene products as the heterogametic sex (ZW).

Because of the random inactivation of X chromosomes, female mammals are mosaics for the genes on the X chromosome [14]. This is demonstrated by coat color in tortoise-shell and calico cats, which are heterozygous for black and orange X-linked alleles. Another example is the expression of G6PD in women who are heterozygous for the trait. Clones from a single cell produce either the mutant or the wild-type enzyme, never both, but in a random sampling of cells, about 50% produce the wild type and 50% the mutant clones.

Inactivation appears irreversible, even in human/rodent hybrid cells in which the inactive human X is present alone against a rodent background. It also remains stable in cells maintained in culture for many generations [15,16]. Reactivation of the entire X chromosome takes place only in the oocytes at some time before meiosis, and both X chromosomes are transcribed. This has been supported by experiments in which the presence of the X-linked enzyme HPRT (hypoxanthine phosphoribosyl transferase) was found to be at levels twice as great in females as in males in the morula stage of mouse embryogenesis. X inactivation occurs in waves from day 3.5 to day 13 of development. Germ cell progenitor cells in females are inactivated by day 12 [15].

In mammalian cells, an inactivation center is believed to reside on the long arm of the X chromosome at band Xq13 (XIC). There is no known case in which this proximal Xq area, called the *c region*, is deleted in the X chromosome, leading to the presumption that two active X chromosomes would not be viable [15]. In metaphase cells, the inactive X often appears shorter than its active homologue, and is frequently bent in the proximal long arm [17]. In X chromosomes with two c regions, bipartite Barr bodies are seen in some cells.

DNA methylation is believed to play a role in maintaining this repression of the inactive X. The gene *XIST* (inactive X specific transcript) has been found to be active only on the inactive X chromosome [18,19]. This gene, located in the region of the X inactivation center (XIC), does not code for a protein but rather produces an RNA product that “coats” the X chromosome on which it is expressed (i.e., acts in *cis*), and recruits other factors, including those that result in histone deacetylation and methylation, with the overall result of transcriptional repression of genes on the same chromosome. Experimental treatment of cells with 5-azacytidine produces hypomethylation of DNA and partial reactivation of selected loci, but not of the entire X [15,16].

X inactivation and conversion to the heterochromatic state involves interaction between noncoding transcripts such as *Xist*, chromatin modifiers, and factors involved in nuclear organization. These produce changes in chromatin structure and in the spatial reorganization of the X chromosome [20].

In individuals having one normal and one abnormal X, the abnormal X is usually the inactive one. There are indications, however, that inactivation is initially random even in these individuals but that the cells with an active normal X survive [15,16]. In X;autosome translocations, the normal X is usually inactive, but minority cell lines have been demonstrated. In unbalanced X;autosome translocations (usually offspring of balanced translocation carriers), the translocation chromosome is also usually inactive, but what is interesting is that the inactivation may or may not extend into the autosome.

Some genes on the X chromosome escape inactivation [21]. It is known that DNA synthesis is not synchronous in the late-replicating X but that it starts around the centromere and is followed by the short arm and the proximal part of the long arm. Several of the genes escaping inactivation are found in the early-replicating regions of the otherwise late-replicating X and are thought to cause the abnormalities associated with extra X chromosomes [16].

In the laboratory, the inactive X can be identified by growing cells in bromodeoxyuridine (BrdU) for 40–44 hours, and adding thymidine 6–7 hours before fixation. After staining with Hoechst 33258, the late-labeling X will be bright when examined by fluorescent microscopy. Alternatively, cultures can be grown in the presence of thymidine and pulsed with BrdU, resulting in a pale-staining, late-labeling X.

1.1.6 Satellite DNA

Repetitive DNA found in constitutive heterochromatin is often called satellite DNA because much of it separates from main band DNA by density gradient centrifugation. Satellite DNA has come to mean any highly repeated sequence, whether separable by ultracentrifugation or not. The satellite bands originally described are called classical satellites I, II, III, and so forth (Table 1.2). A substantial portion of each fraction is made up of a single family of simple repeats designated by the Arabic numeral corresponding to the Roman numeral [14]. Other pure sequences may be designated by lower case Greek letters, which also relate to the fraction from which they were derived. Polymorphisms that have arisen from mutations can be detected by restriction endonuclease digestion and electrophoretic separation. *Consensus sequences* are ones that are substantially the same, differing by only a few bases.

Alpha (α) and beta (β) satellite DNA, as well as classical satellite DNA, are found at the centromeres of all human chromosomes. Alpha satellite DNA has a consensus sequence so that probes made from a mix of the α satellite probes can be visualized at the centromeres of all the human chromosomes [16] (Table 1.2). More specific α satellite probes can also identify the centromeric regions of specific chromosomes (see Chapter 16, Fluorescence *in situ* hybridization).

In contrast to the undispersed repetitive sequences found in heterochromatin, dispersed repetitive sequences are found throughout the genome. They are the short (<500 bp) interspersed elements (SINES) and long interspersed sequences (INES) [16]. SINES contain cleavage sites recognized by the restriction endonuclease Alu1 and are located in the quinacrine pale bands. INES have cleavage sites for L1 and are located in the quinacrine bright bands. Together the Alu1 and L1 families make up about a third of the total repetitive DNA [14].

Other satellites of interest are microsatellites, SSR (simple sequence repeats), and minisatellites, all of which are interspersed throughout the genome. Microsatellites are di- or trinucleotide tandem repeats and are highly polymorphic. SSR are 3- to 6-bp repeats found in coding and noncoding DNAs and are also highly polymorphic. Minisatellites have longer repeats, usually more than 10 bp, and are usually located at the distal ends of chromosomes. These satellite DNAs are useful for DNA fingerprinting because of their highly polymorphic nature and are also useful as *in situ* probes [22].

Table 1.2 Characteristics of satellite DNA

Type of satellite DNA	Location	Length	Repeat sequences
Alpha satellite DNA	All human chromosome centromeres; Yq	0.17/0.34 kb	Varies between chromosomes
Beta satellite DNA	Chromosome 1, 9, 13, 14, 15, 21, 22, Yq	50–300 kb	68-bp monomers
Satellite I Unit A	Most human chromosomes	0.04 kb	17 bp
Satellite I Unit B	Most human chromosomes	25–48 bp in arrays	(A-B-A-B-A) ⁿ
Satellite II	Most chromosomes	0.05 kb	(GGAAT) ⁿ
Satellite III	Chromosomes 9 and 15, also most others	0.05 kb	CAACCCGA ^A / _G T(GGAAT) ⁿ

1.2 The cell cycle

1.2.1 Interphase

The transition from interphase to cell division (mitosis) and back to interphase is called the cell cycle; therefore, when the cell is not dividing, it is said to be in interphase. This is the time when the nucleus is metabolically active. The nucleus is spatially organized with each chromosome in a specific region. This separation serves to prevent one chromosome from getting tangled with another. Telomeres are attached to the nuclear membrane. Between chromosomal subcompartments are chromatin-free interchromosomal domains, where RNA molecules are being processed for export to the cytoplasm. Highly transcribed portions of the chromosomes are positioned next to the interchromosomal domains and since different genes are transcribed in different cell types, the arrangement can vary from cell to cell.

Originally, it was assumed that the cell synthesized new DNA just previous to cell division. With the use of autoradiography and Feulgen staining techniques in the early 1950s; however, DNA synthesis was found to take place hours before any sign of mitosis, during interphase [23]. Furthermore, it is now known that synthesis of new DNA does not take place all at once. During the synthesis (S) period of 6–8 hours, some DNA will replicate early, and other DNA will replicate late. A given part of the chromosome, however, will almost always replicate at a certain time in the S period; for example, the inactivated X will always replicate late, as well as most of the dark-staining G-bands, and the light-staining G-bands will replicate early. The late-replicating portions of the chromosomes are usually considered genetically less active than the early-replicating areas. The discrepancy in replication timing between regions (and even whole chromosomes in the case of the X chromosome homologues) provides the basis for the banding technique called replication banding (see Chapter 6, section 6.3.2, Replication banding), which is most useful in discriminating the active from the inactive X chromosome.

There are four distinct stages in the cell cycle. Each phase is driven by cyclin-dependent kinases (CDKs) in conjunction with regulatory subunits called the cyclins. Cyclins are negatively regulated by cyclin-dependent kinase inhibitors (CKIs) such as p16, p21, and p27. CDKs phosphorylate target proteins at different stages of the cell cycle. For example, the retinoblastoma protein, pRb, associates with a transcription factor called E2F when pRb is hypophosphorylated. This association physically prevents E2F from dimerizing with its partner protein DP and thus inhibits the cell's passage into the synthesis phase. When pRb is phosphorylated by CDKs, it no longer binds E2F, and the cell cycle is allowed to progress through the synthesis phase. Mutations in the *RB1* gene are responsible for the eye cancer called retinoblastoma. Another cell cycle regulatory protein, TP53, can likewise stall the cell cycle at the G1/S transition to allow time for DNA repair or to initiate programmed cell death (apoptosis) when the damage is severe. Indeed, cell cycle regulation is intimately tied to DNA damage response: it is crucial that the cell have a series of checkpoints to prevent damaged DNA from being replicated, but checkpoints also exist at other points in the cell cycle. Alterations in any of these elements (e.g., cyclins, CDKs, pRb, *ATM*, *TP53*, and many others) have been observed in many human cancers, and several have their own cancer syndrome associated with alterations (e.g., TP53 mutations and Li–Fraumeni syndrome, and *RB1* and retinoblastoma) [3].

The time intervals for the four stages are consistent within a given cell type but vary between cell types. The four stages of the cell cycle are G1 (gap one), S (synthesis of DNA), G2 (gap two), and M (mitosis). The histone proteins of the chromosomes are, like DNA, synthesized during S. When the cell is in a resting stage and is not cycling, as when nutrients are scarce, it is said to be in G0.

The average mammalian cell cycle spans 18 hours, with a range of 12–24 hours, and a typical schedule for the portions of the cycle is nine hours for G1, 5 hours for S, 3 hours for G2, and 1 hour for M (see Figure 1.6). However, early embryonic cells can complete the cycle in 30 minutes, and an adult liver cell may take 1 year. These differences are due to shorter or longer G1 and G2 stages. If the cell is arrested at some point in S or G2, it cannot undergo cell division. During G2, the final preparations for mitosis are completed, and unless protein synthesis is inhibited, the cell will divide.

Different drugs inhibit the cell cycle at various stages. S inhibitors include amethopterin (methotrexate), hydroxyurea, and cytosine arabinoside. Naturally occurring mitotic arrestants include Vinca alkaloids, colchicine (also an alkaloid), and podophyllin.

1.2.2 Cell division

It has been firmly established since the time of Louis Pasteur that cells arise from pre-existing cells [24,25]. In order to donate genes to progeny, the parent, whether it is a single cell or multicellular organism, duplicates its genetic material (e.g., chromosomes) and transfers the copies to the offspring. Diploid organisms possess chromosomes in pairs called homologues, one inherited maternally and one inherited paternally. These pairs of chromosomes are normally similar or identical in shape, with a given gene found in a specific position (locus) on each chromosome of a pair. Even though these homologous genes determine similar functions or characteristics, they often are not identical. There are commonly alternate forms (alleles) of a gene on each homologous chromosome that code for two different expressions of the same gene, such as body color in fruit

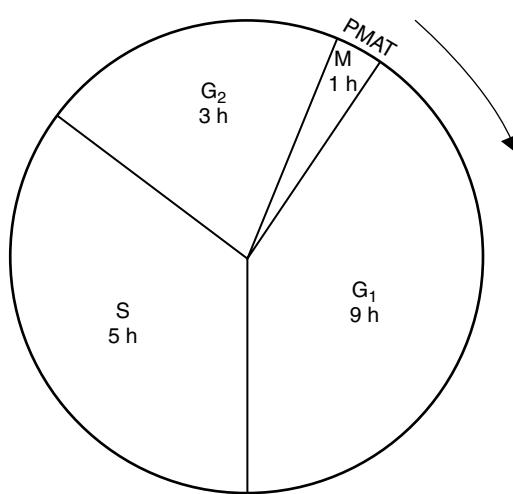


Figure 1.6 This pie graph demonstrates the four major stages of the human cell cycle and each stage's relative timeframe, e.g., Gap 1 (9 hours), Synthesis (5 hours), Gap 2 (3 hours), and Mitosis (Prophase, Metaphase, Anaphase, Telophase) (1 hour).

flies. If a fly inherits the allele for yellow from both parents, it is said to be homozygous for that trait. If, instead, it receives one allele for yellow and one for gray, it is said to be heterozygous for the trait. The color it expresses (gray or yellow) is called its phenotype, and the description of the actual genes it is carrying is its genotype.

In the case of the sex chromosomes in humans, males have only one X chromosome and the genes on that X are present unpaired, in a single dose. This condition is neither homozygous nor heterozygous, but hemizygous. Dosage compensation (see 1.1.5, X inactivation) provides a mechanism for the production of comparable gene products between homozygous and hemizygous individuals.

The genetic information present in each cell is transmitted during cell division, both in mitosis and meiosis. Mitosis is the division of somatic (nonreproductive) cells, and meiosis is the specialized division that occurs only in the formation of gametes (ova and sperm). One reason for the existence of two types of cell division is the fact that somatic cells need a full (diploid) complement of chromosomes; whereas, gametes need only one-half a complement (haploid), because they will ultimately fuse with another gamete during fertilization to become diploid in the resulting zygote.

1.2.3 Mitosis

In humans, the diploid ($2n$) number of chromosomes is 46. During the S stage of the cell cycle, each chromatid replicates itself. The cell at the end of S phase is still diploid, but the DNA content doubles as the chromatids are replicated to produce a chromosome comprised of two chromatids. Thus, at the end of the S phase, the cell is $2n$, but $4c$, where c = DNA content. At mitosis, the chromosomes line up at the metaphase plate and one chromatid from each chromosome goes to each daughter cell. The cycle continues and the chromatids are then replicated at S phase. Mitosis can be divided into four stages: prophase, metaphase, anaphase, and telophase (see Figure 1.7).

Prophase

Prophase is the stage of progressive coiling of the already doubled chromosomes, which appear long and threadlike. In middle to late prophase the chromosomes can be seen as discrete units, each containing two chromatids and a centromere. During prophase, the nucleolus becomes undetectable under the light microscope. With the electron microscope it is apparent that the nucleolus becomes dispersed throughout the nucleus. In the cytoplasm, the centrioles (one pair of which has budded off of the other) start to migrate to opposite poles of the cell and to form the microtubules that make up the mitotic spindle.

Between prophase and metaphase (in a stage often called prometaphase), the nuclear envelope breaks down in most organisms, releasing the chromosomes into the cytoplasm, which is contained by the cytoplasmic membrane. The chromosomes move rather erratically toward the equatorial plane (metaphase plate) of the cell. At this time the spindle fibers are not yet attached to the spot in the centromere (called the kinetochore) to which they later anchor for chromatid separation.

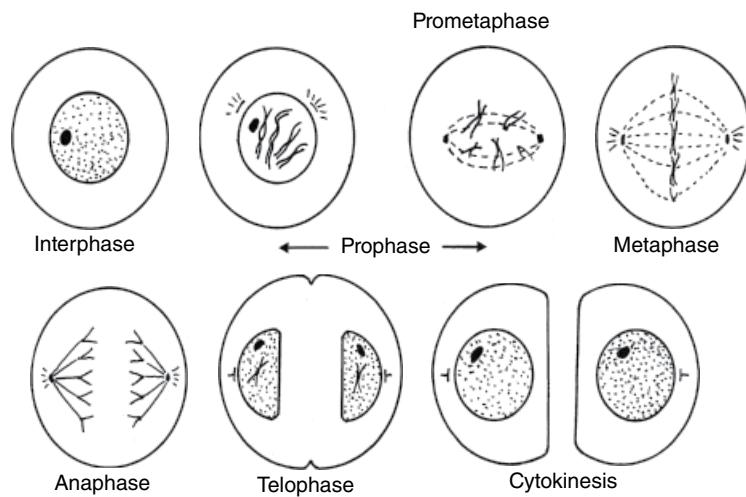


Figure 1.7 During the 1 hour that mitosis typically takes, replicated chromatin condenses in prophase to form identifiable chromosomes, and the nuclear envelope breaks down. Chromosomes that line up at the metaphase plate are attached at their kinetochores with spindle fibers that are connected at the other end to the centrioles at each of the poles. At anaphase, the spindle fibers pull the duplicated chromosome arms to opposite poles to form the two daughter cells in telophase, when the nuclear envelope is re-formed. Cytokinesis is the formation of new cell membranes between the daughter cells, each with a complete, identical set of genetic information.

Metaphase

At metaphase, the mitotic spindle is complete: the centrioles are in place at opposite poles, the chromosomes are lined up at the metaphase plate with the spindle fiber attached to each kinetochore, and the kinetochores are facing opposite poles of the cell. The mitotic spindle consists not only of these chromosome-to-pole fibers but also of continuous pole-to-pole fibers, which bypass the chromosomes altogether. At metaphase, chromosomes are at their most contracted state. This is also their least metabolically active state.

Anaphase

Anaphase begins with the division of the centromeres and, therefore, the separation of the chromatids. Once separate, each chromatid is called a daughter chromosome. The chromosomes move to opposite ends of the cell along the spindle fibers. Increased understanding of the mechanism of chromosome movement is being gained and some think there is potential to exploit this knowledge in the development of new cancer therapies [26,27].

Telophase

The final stage of mitosis is telophase. In telophase, the chromosomes uncoil, the nucleolus reappears, the nuclear envelope reappears, the spindle fibers disappear, and the nucleus takes on the morphology of the interphase cell. During or directly after telophase, the cytoplasm is divided by the formation of new cell membranes in a process called cytokinesis, and cell division is complete. The result of mitosis is two daughter cells, each with a complete, identical set of genetic information. For a video of mitosis in a live cell, search on “mitosis” or “cell division” on YouTube (www.youtube.com/watch?v=aDAw2Zg4IgE).

1.2.4 Meiosis

Meiosis is often called the reduction division, because it reduces the number of chromosomes in each daughter cell to the haploid (n) number, which is 23 in humans. Meiosis takes place in the reproductive organs – the ovaries in females and the testes in males. The process of meiosis transforms cells called primary spermatocytes in the male testis and primary oocytes in the female ovary into haploid spermatids and ova, respectively. When fertilization occurs, the ovum and sperm fuse to form a

diploid zygote. Meiosis differs from mitosis in its reduction to four separate n (haploid) nuclei and in the creation of new gene combinations by crossing over so that the daughter chromosomes are composites of the parent chromosomes. The parent cell, for example, contains a pair of chromosomes number 1, one from each of its parents. During meiosis, these homologs exchange genetic material between them (crossing over – see later) so that the spermatid or ovum receives a single chromosome 1 that is derived from, but is not identical to, either of the two chromosomes 1 that the parent possessed. The same sequence of events has simultaneously occurred for chromosomes 2, 3, and so on. The consequence of this feature of meiosis is an increase in the phenotypic variation of sexually reproducing organisms, which provides selective advantage of great importance. The mechanism of this genetic exchange will become apparent in the description of meiosis.

Meiosis has two nuclear divisions, meiosis I and meiosis II. In meiosis I the homologous chromosomes (homologues) separate, and in meiosis II the chromatids separate (as in mitosis). This results in four cells, each with one haploid set of chromosomes. In the female, only one of these becomes a viable ovum, and the rest become polar bodies. In the male, all four spermatids can mature into spermatozoa.

Compared with mitosis, meiosis is a complicated and lengthy process. Like mitosis, each stage of meiosis has a prophase, metaphase, anaphase, and telophase. Prophase I is especially complex and is divided into five consecutive substages: leptotene (also called leptonema), zygotene (zygonema), pachytene (pachynema), diplotene (diplonema), and diakinesis (see Figure 1.8).

Prophase I

Leptoneme

At this substage the nuclear chromatin begins to condense for division, but the chromosomes are not yet evident and the double nature of the strands cannot be discerned. The electron microscope shows that both ends (telomeres) of the chromosomes are attached to the nuclear envelope. By light microscopy, the leptotene cell has an enlarged nucleus and finely dispersed chromatin. Once the cell has entered leptonema, it is committed to meiosis.

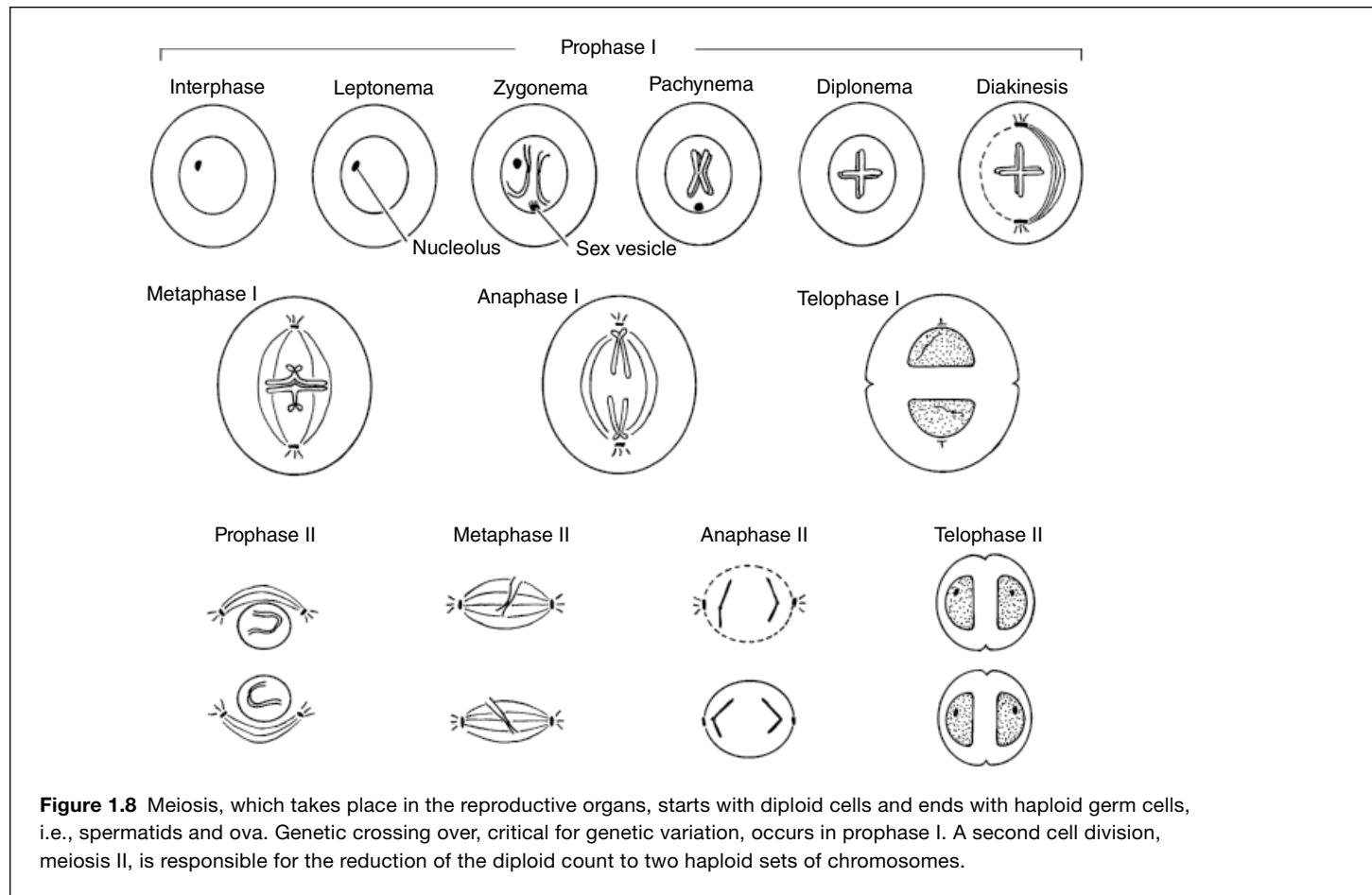


Figure 1.8 Meiosis, which takes place in the reproductive organs, starts with diploid cells and ends with haploid germ cells, i.e., spermatids and ova. Genetic crossing over, critical for genetic variation, occurs in prophase I. A second cell division, meiosis II, is responsible for the reduction of the diploid count to two haploid sets of chromosomes.

Zygotene

Homologous chromosomes, which still resemble long threads, align themselves side by side and attach to each other, allowing homologous loci to lie next to each other. This alignment is called synapsis (the Greek word for joining together). Electron microscopy reveals a structure holding the chromosomes close to each other; this structure is called the synaptonemal complex. The exchange of genetic material probably takes place in these complexes. This exchange is called crossing over, or recombination. In the female, the two X chromosomes are homologous and behave exactly like the other chromosomes. In males, however, the X and Y chromosomes are not homologous and condense to form a small, dark-staining body called the sex vesicle. Evidence shows that the X and Y are aligned end to end in this vesicle. Each set of homologues in synapse is called a bivalent, because the two chromosomes do not yet appear differentiated into chromatids. During zygotene the nucleolus is still visible and is associated closely with some of the bivalents. Zygonema ends when all homologues have been paired.

Pachytene

During this stage of prophase I, the bivalents shorten and become very thick, and crossing over occurs. Two nonsister chromatids cross over, but the other two remain unchanged. In good preparations, the bivalents can be seen as two parallel strands (chromosomes) on which there are a number of dark-staining, beadlike structures called chromomeres. These chromomeres are constant from preparation to preparation, so their number and position can identify some of the bivalents. Chromomeres are thought by some workers to be analogous to the G-band patterns of mitotic prometaphase chromosomes (see Figure 1.5). In this stage, the bivalent, owing to its four closely opposed chromatids, is known as a tetrad.

Diplotene and diakinesis

At the diplotene stage the nucleolus detaches from its associated bivalents, and the bivalent chromosomes begin to separate as their centromeres pull them apart. They are still attached, however, at points called chiasmata (a single point is a chiasma), which are the sites of genetic crossing over. Chiasma means cross, and chromosomes having only one chiasma typically assume a cross-like appearance. Each chiasma acts as a sort of knot that holds the paired chromosomes together so that the chromatids do not separate. In normal human meiosis, there is usually at least one chiasma per bivalent. If no chiasmata are present, nondisjunction can occur, leading to aneuploidy. In males, diplonema marks the disappearance of the sex vesicle, and the continued end to end association of X and Y can be observed.

Diakinesis occurs at this point in males: the chiasmata appear to move toward the ends of the bivalents (terminalize), the nucleolus dissipates, and the nuclear envelope disappears. In human females, however, meiosis is halted before diakinesis in the ovaries of the unborn female fetus. The oocytes remain in this special diplonema stage, called dictyotene, as long as 50 years or until each oocyte is singly ovulated after puberty before reaching the diakinesis stage. Meiosis is never completed unless fertilization occurs. When diakinesis is finished, the cell is ready to move into metaphase I.

Metaphase I

Here the spindle is formed and the bivalents line up at the equatorial plane. In females, the spindle is off-center in the cell and determines, by its position, which of the daughter cells will inherit most of the cytoplasm at anaphase. The bivalent chromosomes are lined up so that maternally and paternally derived chromosomes randomly face one pole or the other. This will allow them to sort independently to the daughter cells; each daughter cell will contain a mix of both paternal and maternal chromosomes. Excluding crossing over, this shuffling of 23 pairs of chromosomes can produce about 8 million genetically different gametes.

The form of the individual bivalents in metaphase I depends upon the number of chiasmata present. Bivalents with one chiasma form a cross-shaped structure at diakinesis, which proceeds to form a rod-shaped structure at metaphase when the chiasma terminalizes. Two terminalized chiasmata form a ring-shaped structure at metaphase; three chiasmata create a figure eight; and four or more will appear with additional loops. The X and Y chromosomes sometimes appear separated in metaphase I and are then called univalents.

Anaphase I and telophase I

Whole chromosomes, centromeres intact, move to opposite ends of the cell during these stages. In the oocyte of the female, one of the two daughter cells receives most of the cytoplasm and becomes the secondary oocyte. The other cell, mostly nucleus, becomes the first polar body. In humans, the cycle proceeds directly to meiosis II without an intervening interphase stage.

Metaphase II

In human meiosis there is no true prophase II; the cells pass directly into the second meiotic metaphase. The 23 chromosomes, each composed of two chromatids, move to the equatorial plate (metaphase II). At this stage, the chromosomes appear somewhat spiraled and fluffy. Although only a haploid set of chromosomes is present, i.e., one of each human homologue, there is still a diploid amount of DNA because the replicated strands have not yet separated. In anaphase and telophase, the two chromatids finally separate and go to two daughter cells so that the end product of meiosis is four haploid cells, each with one complete but different set of genetic material. In females, the spindle is again off-center, giving rise to a very large cell called an ovum and a second polar body. The first polar body may also undergo meiosis II, creating two additional polar bodies. Of the resulting four haploid cells, the ovum is theoretically the only viable gamete. For a video demonstration of the processes of meiosis, search ‘meiosis’ on YouTube (www.youtube.com/watch?v=R_LUJSqeSrI). For example, this URL may be helpful to grasp the basic concepts: http://www.youtube.com/watch?v=R_LUJSqeSrI.

1.3 Recombinant DNA techniques

A breakthrough in the study of DNA occurred when researchers discovered bacterial enzymes that cut DNA at specific sequences [28]. These restriction enzymes often make staggered cuts in the two DNA strands, leaving short, single-stranded tails on the ends of both fragments. These single-stranded ends easily bind to complementary fragments by base pairing. Two fragments that have attached in this way can be permanently joined by adding DNA ligase, a repair enzyme that produces a recombinant molecule.

1.3.1 Bacterial-plasmid cloning

Further advances in this field took advantage of the fact that many bacteria contain plasmids, tiny circular DNA molecules, and that these plasmids can replicate autonomously in bacteria. Plasmids from the bacterium *Escherichia coli* (*E. coli*), which had only one recognition site for the restriction enzyme EcoRI, were cut by the enzyme; foreign DNA, also cut with EcoRI, was spliced in; and the plasmids were sealed with ligase. The hybrid plasmids were then transferred back into *E. coli*, where they carried out the instructions of the inserted DNA and reproduced with the bacteria’s own DNA [29]. Using these techniques, researchers were able to isolate bacteria that had acquired a gene of interest and then make an enormous number of copies (cloning), owing to the rapid reproductive rate of bacteria [8]. This technique found practical uses; for instance, large amounts of insulin could be made for use by patients with diabetes.

Other advances in the study of DNA and genetics were made when Sanger, Maxam, and others devised methods for determining the base sequence of a given DNA molecule [30,31]. This capability led to the sequencing of mitochondrial DNA and bacteriophage λ . Sanger’s dideoxy method was eventually used to sequence the entire human genome. Because restriction enzymes cut DNA at specific nucleotide sequences or recognition sites, the length of each fragment produced depends on the distance from one recognition site to the next. Harmless natural variations exist, such as the one at a point about 7000 nucleotides away from the beta-globin gene on chromosome 11. A recognition site for the restriction enzyme HpaI is present at that point in the DNA of some people, but not others. If the site is present, a short fragment containing the beta-globin gene, 7600 bp long, is produced. If absent, the beta-globin-negative fragment is 13,000 bp long. These normal variations have been named restriction fragment length polymorphisms (RFLPs).

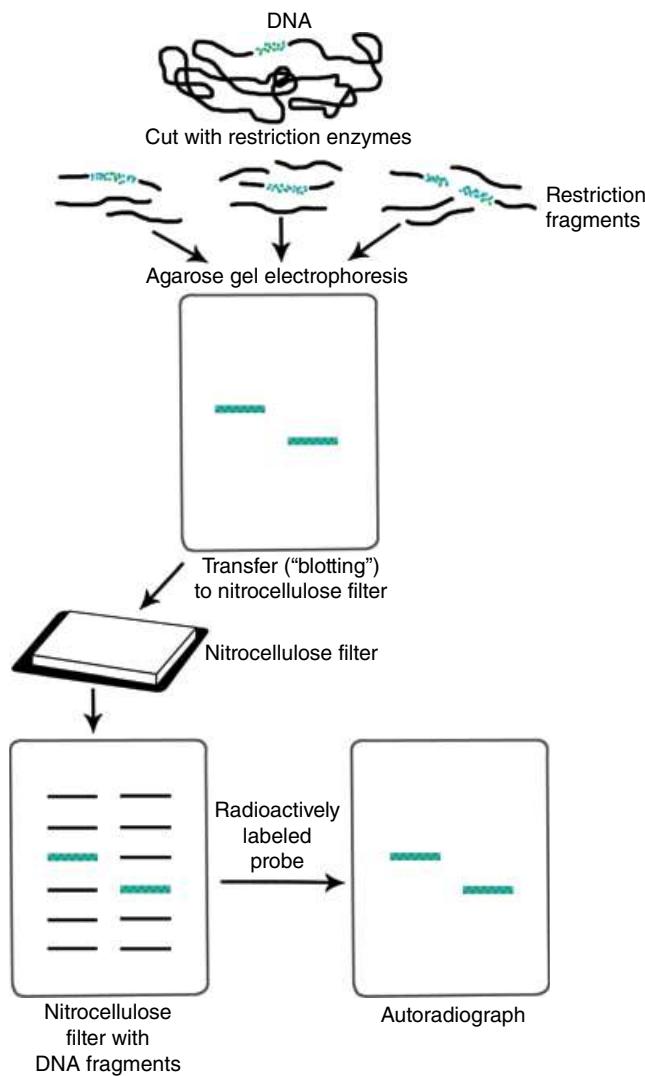
1.3.2 Electrophoresis

Fragments formed by restriction enzymes can be further separated by electrophoresis, a process in which the DNA fragments move through porous agarose gels (for large fragments) or polyacrylamide gels (for small fragments) under an electric field. Smaller fragments migrate more quickly, providing a visual way for determining fragment sizes by their positions in the gel. This separation comparison has proven useful in the study of heritable diseases like sickle cell disease [32].

Direct detection of the sickle cell globin gene has been demonstrated using the restriction enzyme MstII, which cuts within the beta-globin gene, as well as many other places. In the normal beta-globin gene, MstII cuts at the sequence CCTGAGG, producing two fragments of 1150 and 200 bp. The sickle cell mutation changes the sequence to CCTGTGG, thereby eliminating the recognition site; therefore, MstII produces only one 1350 bp fragment [28].

1.3.3 Southern blotting

Detecting whether a person has the two smaller fragments (normal) or the single 1350 bp fragment in sickle cell disease testing is complicated by the fact that there are so many fragments of similar sizes. This is overcome by using radioactively labeled probes in a technique called Southern blotting (see Figure 1.9).



Southern blotting. DNA fragments are separated according to size by agarose gel electrophoresis. They are then transferred to a nitrocellulose filter where they are exposed to radioactive probes that hybridize with complementary sequences. The radioactive signals are detected by autoradiography using x-ray film.

Figure 1.9 DNA fragments are separated according to size by agarose gel electrophoresis. They are then transferred to a nitrocellulose filter where they are exposed to radioactive probes which hybridize with complementary sequences. The radioactive signals are detected by autoradiography using X-ray film. Watson 1983 [28]. Reproduced with permission of John Wiley and Sons.

A probe is a short sequence of purified DNA that is complementary to the DNA of interest. In order for the probe to attach, the double-stranded DNA must be denatured by heat or alkali. The probe used to detect the sickle cell gene is a fragment of the cloned beta-globin gene, made radioactive so that it can be detected in the presence of large amounts of nontarget DNA.

DNA to be tested for the sickle cell mutation is cut with MstII. The resulting fragments are then separated by agarose gel electrophoresis and treated chemically to denature them. Next, the fragments are transferred (blotted) onto a nitrocellulose filter, to which they become bound. The transfer retains the pattern of fragments that was produced in the agarose gel. Next, the filter is exposed to the radioactively labeled probe. Once hybridized to its complementary DNA, the radioactivity can be detected by placing the filter next to X-ray film, which exposes the film to produce an autoradiogram [28,32].

If a band corresponding to a DNA fragment of 1350 bp shows up on the autoradiogram, it represents the sickle cell gene. If two bands of 1150 and 200 bp appear, they represent the normal gene. If bands representing both the longer and the two shorter fragments are present, the individual has inherited the sickle cell gene from one parent and the normal beta-globin gene from the other parent and is a carrier for the sickle cell trait [28].

1.3.4 Synthetic oligonucleotides

Because it is unusual for a genetic mutation to exist in a restriction site, other means of detection must be used. One option is to use synthetic oligonucleotides (oligo = few). These short molecules are engineered to match portions of a normal gene exactly. If there is a change in just one base, the hybrid molecule will be unstable and will denature easily. These oligonucleotide probes can therefore be used to detect genetic defects that involve a point mutation or change in a single base [28,32].

1.3.5 Polymerase chain reaction

Another useful tool available to molecular geneticists is the polymerase chain reaction (PCR), which allows small amounts of DNA or RNA to be amplified, producing millions, even billions, of copies [33]. This makes it possible to make, from tiny samples of DNA, amounts great enough to be analyzed using restriction enzymes, oligonucleotides, or direct sequencing. The low cost of this method, in addition to its flexibility and precision for amplifying a short stretch of DNA, has resulted in PCR testing largely supplanting the bacterial plasmid cloning method for many clinical and research applications.

1.4 The human genome

1.4.1 Genomic DNA variations

Genomic DNA variation can provide useful methods by which to identify individuals. For instance, RFLP analysis is sometimes referred to as fingerprinting. However, these variations may also play a role in human phenotypic variation and disease. The single nucleotide is the smallest unit of variation. Single nucleotide polymorphisms (SNPs), by definition, are present in at least 1% of the population and may account for a large fraction of human genetic variation. Recently, surprising observations regarding human genetic variation on a larger scale were made. First, alterations in DNA copy number of large segments of DNA were reported in normal individuals. These segments (copy number variations, or CNVs) ranged from kilobases to megabases of DNA. The size of these alterations was below the resolution of karyotypic analysis and above the resolution of most molecular techniques, thus their discovery relied on the technique called array comparative genomic hybridization (aCGH), which is a sensitive method to detect genomic DNA imbalances at a lower limit of statistical resolution of several kilobases. Fifty-six percent of the clones overlapped with known coding regions and some included one or more genes [34].

Further research has demonstrated that individuals carry their own unique CNV signatures, and that CNV databases from populations or ethnic groups carry variations and similarities. Similarity between groups with unusual phenotypic characteristics or disease, has led to some CNVs being recategorized as pathogenic rather than benign population polymorphisms. The DNA architecture lying immediately adjacent to a region is thought to convey susceptibility to rearrangements observed as CNVs. Certain DNA regions contain low copy repeats (LCRs) that may be in direct head-to-tail orientation or in inverted tail-to-tail orientation; these repeats may facilitate nonallelic homologous recombination (NAHR) during meiosis [35] resulting in duplications or deletions. Many of the chromosomal deletion/duplication syndromes share this etiology. For example, velocardiofacial-DiGeorge syndrome (VCF/DGS) is usually caused by a deletion that results from unequal crossover events between two 240 kb LCRs termed LCR22-2 and LCR22-4 on chromosome 22 long arm in band q11.2. These LCRs may also cause duplications in the same region. Similarly, LCRs flank the Charcot-Marie-Tooth/HNPP (hereditary neuropathy with liability to pressure palsies) region on chromosome 17p12. Duplications lead to Charcot-Marie-Tooth syndrome (MIM: 118220) and deletions cause HNPP (MIM: 162500). Depending on the size, CNVs may be observed using a number of different methods, including chromosome analysis, FISH, and chromosome microarray analysis. Although many larger CNVs have been described using chromosome and FISH analysis, most of the recent additions to the CNV databases, and much of what we know of CNV formation, have been uncovered using array technologies [36].

The sequencing of the human genome, first published in 2001, has opened up a new era in understanding genetics from a functional viewpoint [37,38]. One early surprise in this process was the relatively low number of genes resulting in a peptide or protein. The number is believed to be 21 - 30,000 rather than the expected 100,000. Only about 1% of the genome is comprised of exons. Introns account for another 25%.

A consortium of international scientists is seeking to identify all biologically functional elements in the genome (ENCODE project). The results of their study of the first 1% have revealed more surprises [39,40]. The majority of DNA is transcribed into RNA, and the transcripts overlap one another extensively, but about half are not constrained evolutionarily. A number of previously unknown start sites for DNA transcription have been identified. Regulatory sequences are symmetrically distributed around transcription start sites with no bias toward upstream regions. Chromatin accessibility and histone modification patterns are highly predictive of both the presence and activity of transcription start sites.

Although alternative splicing of transcripts has been known for some time, the ENCODE project has verified that splicing occurs for virtually all genes. Also, a given gene can code for more than one protein. The estimate is that the average protein-coding region produces 5.7 different transcripts. Exons may be spliced into transcripts from other genes, even from different chromosomes. So, while the absolute number of protein coding genes identified by the Human Genome Project was surprisingly low, through alternative splicing and post-translational modifications, the actual protein repertoire of the human genome is quite large. When this is superimposed on the different gene expression and protein interaction networks in particular cells and tissues, the ‘protein function’ repertoire becomes larger still.

References

1. Becker WM, Kleinsmith LJ, Hardin J. *The World of the Cell*, 6th ed. San Francisco, CA: Pearson Benjamin Cummings; 2006.
2. Lewin B, Lingappa VR, Plopper G. *Cells*. Sudbury, MA: Jones and Bartlett; 2007.
3. Pelengaris S, Khan M. DNA replication and the cell cycle. In: Pelengaris S, Khan M, Blasco M, eds. *The Molecular Biology of Cancer*, 2nd ed. Hoboken, NJ: Wiley-Blackwell; 2006, 109.
4. Brown R. On the organs and mode of fecundation in Orchideae and Asclepiadaceae. *Trans Linn Soc Lond (Bot)* 1833; 16: 685–745.
5. Watson JED, Crick FHC. A structure for deoxyribose nucleic acids. *Nature* 1953; 171: 737–738.
6. Watson JD, Crick FHC. The structure of DNA. *Cold Spring Harbor Symp Quant Biol* 1953; 18: 123–131.
7. DuPraw EJ. *DNA and Chromosomes*. New York: Holt, Rinehart and Winston; 1970.
8. Pines M. *The New Human Genetics – How Gene Splicing Helps Researchers Fight Inherited Disease*. Bethesda, MD: National Institute of General Medical Sciences; 1984.
9. Elgin SCER, Amero SA, Eissenberg JC, Fleischmann G, Gilmour DS, James TC. Distribution patterns of non-histone chromosomal proteins on polytene chromosomes: functional correlations. In: Gustafson JP, Appels R, eds. *Chromosome Structure and Function, Impact of New Concepts*. New York: Plenum Press; 1988, 145–156.
10. Lewin B. *Genes IV*. New York: Oxford University Press; 1990.
11. Barr MEL, Bertram EG. A morphological distinction between neurons of the male and female, and the behavior of the nucleolar satellite during accelerated nucleoprotein synthesis. *Nature (London)* 1949; 163: 676–677.
12. Lyon MF. Sex chromatin and gene action in the X chromosome of mammals. In: Moore KL, ed. *The Sex Chromatin*. Philadelphia, PA: WB Saunders; 1966, 7–15.
13. Levitan M. *Textbook of Human Genetics*, 3rd ed. New York: Oxford University Press; 1988.
14. Wagner RP, Maguire MP, Stallings RL. *Chromosomes: A Synthesis*. New York: Wiley Liss; 1993.
15. Lock LF, Martin GER. Dosage compensation in mammals: X chromosome inactivation. In: Risley MS, ed. *Chromosome Structure and Function*. New York: Van Nostrand Reinhold; 1987.
16. Thurman E, Susman M. *Human Chromosomes: Structure, Behavior, Effects*, 3rd ed. New York: Springer-Verlag; 1993.
17. Flejter WL, VanDyke DL, Weiss L. Bends in human mitotic-metaphase chromosomes including a bend marking the X-inactivation center. *Am J Hum Genet* 1984; 36(1): 218–226.
18. Brown CJ, Ballabio A, Rupert JL, Lafreniere RG, Grompe M, Tonlorenzi R, Willard HF. A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* 1991; 349(6304): 38–44.
19. Brown CJ, Lafreniere RG, Powers VE, Sebastio G, Ballabio A, Pettigrew AL, Ledbetter DK, Levy E, Craig IW, Willard HF. Localization of the X inactivation centre on the human X chromosome in Xq13. *Nature* 1991; 349(6304): 82–84.
20. Chow J, Heard E. X inactivation and the complexities of silencing a sex chromosome. *Curr Opin Cell Biol* 2009; 21: 359–366.

21. Brown CJ, Flenniken AM, Williams BRG, Willard HF. X chromosome inactivation of the human TIMP gene. *Nucleic Acids Res* 1990; 18(44): 4191–4195.
22. Verma RS, Babu A. *Human Chromosomes: Principles and Techniques*. New York: McGraw-Hill; 1995.
23. Mazia D. Mitosis and the physiology of cell division. In: Brachet J, Mirsky A, eds. *The Cell*, vol 3. New York: Academic Press; 1961, 77–412.
24. Conant JB. *Science and Common Sense*. New Haven, CT: Yale University Press; 1951.
25. Dubos RJ, Pasteur L. *Free Lance of Science*. Boston: Little, Brown and Company; 1950.
26. Lince-Faria M, Maffini S, Orr B, Ding Y, Florindo C, Sunkel CE, Tavares A, Johansen J, Johansen KM, Maiato H. Spatiotemporal control of mitosis by the conserved spindle matrix protein Megator. *J Cell Biol* 2009; 184(5): 647–657.
27. Wood KW, Chua P, Sutton D, Jackson JR. Centromere-associated protein E: a motor that puts the brakes on the mitotic checkpoint. *Clin Cancer Res* 2008; 14(23): 7588–7592.
28. Watson JD, Tooze J, Kurtz DT. *Recombinant DNA—A Short Course*. Scientific American Books. New York: WH Freeman; 1983.
29. Beaudet AL, Scriver CR, Sly WS. Genetics and biochemistry of variant human phenotypes. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic Basis of Inherited Disease*, 6th ed. New York: McGraw-Hill; 1989, 3–53.
30. Maxam AM, Gilbert W. A new method for sequencing DNA. *Proc Natl Acad Sci USA* 1977; 74: 560–564.
31. Sanger F, Coalson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J Mol Biol* 1975; 94: 441–448.
32. Watson JD, Hopkins NH, Roberts JW, Steitz JA, Weiner AM. *Molecular Biology of the Gene*, 4th ed. Menlo Park, CA: Benjamin-Cummings; 1986.
33. White TJ, Arnheim N, Erlich HA. The polymerase chain reaction. In: Stewart A, ed. *Trends in Genetics*, Cambridge: Elsevier Trends Journals: 1989; 5(6): E185–188.
34. Iafrate AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y, Scherer SW, Lee C. Detection of large-scale variation in the human genome. *Nature Genetics* 2004; 3(69): 949–951.
35. Lupski JR, Stankiewicz P. Genomic disorders: molecular mechanisms for rearrangements and conveyed phenotypes. *PLoS Genetics* 2005; 1(6): e49.
36. Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P, Maner S, Massa H, Walker M, Chi M, Navin NE, Lucito R, Healy J, Hicks J, Ye K, Reiner A, Gilliam TEC, Trask B, Patterson N, Zetterberg A, Wigler M. Large-scale copy number polymorphism in the human genome. *Science* 2004; 305(5683): 525–528.
37. Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, Fiegler H, Shapero MH, Carson AR, Chen W, Cho EK, Dallaire S, Freeman JL, Gonzalez JR, Gratacos M, Huang J, Kalaitzopoulos DE, Komura D, MacDonald JR, Marshall CR, Mei R, Montgomery L, Nishimura K, Okamura K, Shen F, Somerville MJ, Tchinda J, Valsesia A, Woodwark C, Yang F, Zhang J, Zerjal T, Zhang J, Armengol L, Conrad DF, Estivill X, Tyler-Smith C, Carter NP, Aburatani H, Lee C, Jones KW, Scherer SW, Hurles ME. Global variation in copy number in the human genome. *Nature* 2006; 444(7118): 444–454.
38. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, Ballew RM, Huson DH, Wortman JR, Zhang Q, Kodira CD, Zheng XH, Chen L, Skupski M, Subramanian G, Thomas PD, Zhang J, Gabor Miklos GL, Nelson C, Broder S, Clark AG, Nadeau J, McKusick VA, Zinder N, Levine AJ, Roberts RJ, Simon M, Slayman C, Hunkapiller M, Bolanos R, Delcher A, Dew I, Fasulo D, Flanigan M, Florea L, Halpern A, Hannenhalli S, Kravitz S, Levy S, Mobarry C, Reinert K, Remington K, Abu-Threideh J, Beasley E, Biddick K, Bonazzi V, Brandon R, Cargill M, Chandramouliswaran I, Charlab R, Chaturvedi K, Deng Z, Di Francesco V, Dunn P, Eilbeck K, Evangelista C, Gabrielian AE, Gan W, Ge W, Gong F, Gu Z, Guan P, Heiman TJ, Higgins ME, Ji RR, Ke Z, Ketchum KA, Lai Z, Lei Y, Li Z, Li J, Liang Y, Lin X, Lu F, Merkulov GV, Milshina N, Moore HM, Naik AK, Narayan VA, Neelam B, Nusskern D, Rusch DB, Salzberg S, Shao W, Shue B, Sun J, Wang Z, Wang A, Wang X, Wang J, Wei M, Wides R, Xiao C, Yan C, Yao A, Ye J, Zhan M, Zhang W, Zhang H, Zhao Q, Zheng L, Zhong F, Zhong W, Zhu S, Zhao S, Gilbert D, Baumhueter S, Spier G, Carter C, Cravchik A, Woodage T, Ali F, An H, Awe A, Baldwin D, Baden H, Barnstead M, Barrow I, Beeson K, Busam D, Carver A, Center A, Cheng ML, Curry L, Danaher S, Davenport L, Desilets R, Dietz S, Dodson K, Doucette T, Ferriera S, Garg N, Gluecksmann A, Hart B, Haynes J, Haynes C, Heiner C, Hladun S, Hostin D, Houck J, Howland T, Ibegwam C, Johnson J, Kalush F, Kline L, Koduru S, Love A, Mann F, May D, McCawley S, McIntosh T, McMullen I,

- Moy M, Moy L, Murphy B, Nelson K, Pfannkoch C, Pratts E, Puri V, Qureshi H, Reardon M, Rodriguez R, Rogers YH, Romblad D, Ruhfel B, Scott R, Sitter C, Smallwood M, Stewart E, Strong R, Suh E, Thomas R, Tint NN, Tse S, Vech C, Wang G, Wetter J, Williams S, Williams M, Windsor S, Winn-Deen E, Wolfe K, Zaveri J, Zaveri K, Abril JF, Guigó R, Campbell MJ, Sjolander KV, Karlak B, Kejariwal A, Mi H, Lazareva B, Hatton T, Narechania A, Diemer K, Muruganujan A, Guo N, Sato S, Bafna V, Istrail S, Lippert R, Schwartz R, Walenz B, Yooseph S, Allen D, Basu A, Baxendale J, Blick L, Caminha M, Carnes-Stine J, Caulk P, Chiang YH, Coyne M, Dahlke C, Mays A, Dombroski M, Donnelly M, Ely D, Esparham S, Fosler C, Gire H, Glanowski S, Glasser K, Glodek A, Gorokhov M, Graham K, Gropman B, Harris M, Heil J, Henderson S, Hoover J, Jennings D, Jordan C, Jordan J, Kasha J, Kagan L, Kraft C, Levitsky A, Lewis M, Liu X, Lopez J, Ma D, Majoros W, McDaniel J, Murphy S, Newman M, Nguyen T, Nguyen N, Nodell M, Pan S, Peck J, Peterson M, Rowe W, Sanders R, Scott J, Simpson M, Smith T, Sprague A, Stockwell T, Turner R, Venter E, Wang M, Wen M, Wu D, Wu M, Xia A, Zandieh A, Zhu X. The sequence of the human genome. *Science*. 2001; 291: 1304–1351.
39. International Human Genome Sequencing Consortium. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P, McKernan K, Meldrim J, Mesirov JP, Miranda C, Morris W, Naylor J, Raymond C, Rosetti M, Santos R, Sheridan A, Sougnez C, Stange-Thomann N, Stojanovic N, Subramanian A, Wyman D. Initial sequencing and analysis of the human genome. *Nature* 2001; 409: 860–921.
40. Birney D, Stamatoyannopoulos JA, Dutta A, Guigo R, Gingras TR, Margulies EH, Weng Z, Snyder M, Dermitzakis ET. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 2007; 447: 799–816.

CHAPTER 2

Cytogenetics: an overview

Helen J. Lawce¹ and Michael G. Brown²

¹Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA

²(retired), Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA

Chromosomes have attracted many microscopists not only because these sausage-like bodies represent vehicles of genetic material (and hence, are biologically important) but also because they are hypnotically beautiful objects.

T. C. Hsu, *Human and Mammalian Cytogenetics: An Historical Perspective*, 1979, Springer-Verlag

2.1 Introduction

Cytogenetics is the study of chromosome morphology, structure, pathology, function, and behavior. Chromosomes are best studied at mitotic or meiotic metaphase, although some studies, such as fluorescence in situ hybridization (FISH) methods, may utilize interphase cells. The metaphase chromosomes may be studied in spontaneously dividing tissues or in cells that have been stimulated to divide in culture. Spontaneously dividing cells may be found in bone marrow, lymph node, testis, chorionic villi of placenta, some leukemic blood, solid tumor, some pleural or ascitic fluid, fetal ascites or cystic hygroma fluid, and often in fetal or newborn blood. Cells that are cultured before cytogenetic study include all of the above (for study of other components that may not be spontaneously dividing), plus blood lymphocytes, amniotic fluid, skin, and other tissues containing fibroblasts.

Once dividing cells are obtained, mitotic arresting agents (such as Colcemid®, colchicine, or Velban) are used to collect the metaphase cells. Then the cells are processed (harvested). This is usually performed with suspensions of cells, although some cells are harvested “in situ” directly on the cultured cells in a Petri dish. During the harvest procedure, hypotonic solutions are used to increase cell volume, which spreads the chromosomes apart, and methanol–acetic acid is used to fix (preserve) them for study. Slides are prepared from fixed cells, either in suspension (dropped onto slides), or *in situ*, on the growth surface (fixed cells are allowed to dry on the flask, coverslip, or the slide on which they were cultured). Either way, cells are then stained with appropriate stains, observed, and analyzed. Computer-generated images are now used to arrange the chromosomes in pairs on karyograms (Figure 2.1). The chapters that follow discuss these methods in detail.

2.2 History of human cytogenetics

Cytogenetic methodologies have been developed gradually [1,2]. The earliest known observation of chromosomes was by Eduard Strasburger in 1875, using plant material, and by Walther Flemming in 1879–1889, using animal material. Flemming coined the terms *mitosis*, *chromatin*, *prophase*, *metaphase*, *anaphase*, and *telophase*. W. Waldeyer coined the term *chromosome* in 1888.

Although these workers in Germany first observed human chromosomes about 100 years ago, technical problems made counting the chromosomes quite difficult. Painter [3] published the human diploid number in 1923 as 48, and this was believed to be the true number for three decades. This was because chromosomes were difficult to spread out in the cell, and because the differences between primary constrictions (centromeres) and secondary constrictions (heterochromatic

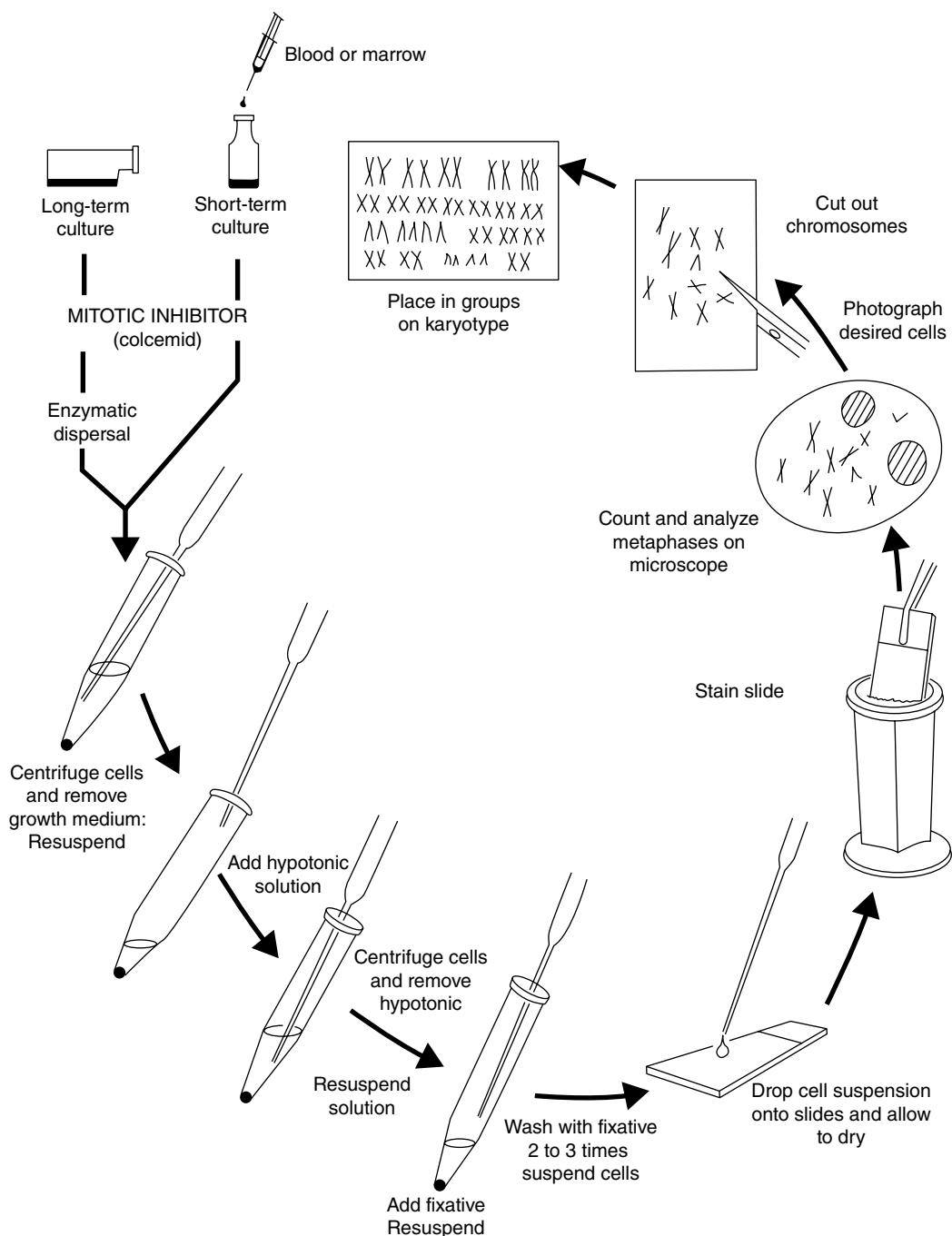


Figure 2.1 Schematic of chromosome preparation methods. Cutting chromosomes from prints has largely been replaced by computerized karyotyping systems. At the “Stain slide” step, the slide may be used for FISH instead of banded chromosome preparations. FISH studies can be performed on interphase cells at almost any stage, including on fresh, unharvested tissues (e.g., formalin-fixed, paraffin-embedded tissue sections or cerebrospinal fluid dropped onto slides and fixed *in situ*), or on fixed tissues and/or cells, whether harvested or not. Metaphase FISH can be performed on unstained cells from harvested cultures, or sequentially after staining chromosomes with banding methods or solid stain.

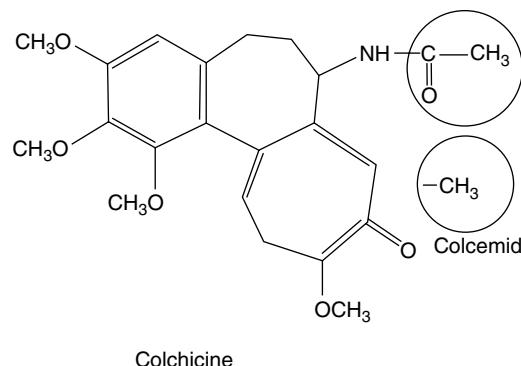


Figure 2.2 Colchicine and Colcemid®. Colcemid® is deacetylmethylcolchicine: the acetyl group of colchicine (upper circle) is replaced by a methyl group (lower circle).

regions of chromosomes 1, 9, 16, and the stalk regions of chromosomes 13, 14, 15, 21, and 22) were obscure. It was easy to interpret two chromosomes in association with each other as one chromosome, and, conversely, it was difficult to be sure that what appeared to be a large chromosome was not, instead, two smaller chromosomes in association. Even today, with much more sophisticated methods, it can still be a challenge, without some experience, to differentiate between a translocation and closely aligned telomeres, or between a centromere and a twisted or broken chromosome region, or a stretched heterochromatic region.

The other great difficulty was in obtaining dividing cells to study. It was not until tissue and cell culturing methods were sufficiently developed to grow fetal lung tissue that Tjio and Levan [4] were able to use this material in 1956 to get a clear look at the chromosome number for the first time. They used colchicine (Figure 2.2) to arrest cells in metaphase and then used hypotonic treatment [5,6] to swell the cells to a much larger volume, so that the chromosomes were less likely to be crowded together. However, the method used to spread the chromosomes onto slides was still the squash preparation, in which tissue was pressed between a coverslip and the slide. This method required harsh aqueous acetic acid fixatives in order to soften the tissues enough to squash flat, and created preparational artifacts, losing some chromosomes from many cells. Using these techniques for clinical purposes was also highly impractical, because of invasive tissue collection procedures. It was not yet possible to culture peripheral blood, and so the only tissues available for chromosome studies were bone marrow, testicular biopsies, and skin. When Nowell [7] discovered a way to stimulate blood lymphocytes to divide, and Moorehead et al. [8] described a method to spread alcohol–acetic acid–fixed lymphocyte chromosomes by air drying it became possible to examine human chromosomes obtained by noninvasive methods, with much less technical artifact than ever before. This, together with the discoveries of the chromosomal causes of Down syndrome, Turner syndrome, Klinefelter syndrome, and the Philadelphia chromosome in CML, led to the development of the science of clinical cytogenetics in the 1960s.

Metaphase chromosomes were originally stained with Giemsa or aceto-orcein, producing unbanded (“solid stained”) chromosomes, which could only be grouped by size and shape, but not identified individually, aside from chromosomes 1, 3, 9, 16, and Y, if they were normal. Research methods to identify individual chromosomes, including autoradiography, had met with limited success. Thus, many abnormalities either went undetected or could not be completely characterized.

In 1968, Caspersson et al. [9] published a fluorescence method (Q-banding) using quinacrine mustard stain that differentiated the pairs of chromosomes in lower animal and plants; this paper was largely ignored, however, until Caspersson’s group [10] showed that each human chromosome had a distinct pattern of bands using his method. Finally, many human chromosome abnormalities could be characterized by the patterns of the light and dark regions (bands), which allowed identification not only of each pair, but also of small chromosomal segments. Methods subsequently described included the much more popular G-bands, using trypsin and Giemsa, which did not require fluorescence, as well as reverse (R-bands) and constitutive heterochromatin (C-bands) stains (Figure 2.3). Other methods followed, as discussed in Chapter 6. These stains expanded the detection and characterization of many more cytogenetic abnormalities, especially for the field of oncology, where the discovery that specific chromosomal rearrangements were associated with certain neoplastic conditions led to a dramatic improvement in both diagnostic and prognostic capabilities for clinicians (see Chapter 11, Cytogenetic analysis of hematologic malignant diseases and Chapter 12, Cytogenetic methods and findings in human solid tumors) than what could be seen with solid staining. Chapter 6, Chromosome stains, discusses these and other staining procedures.

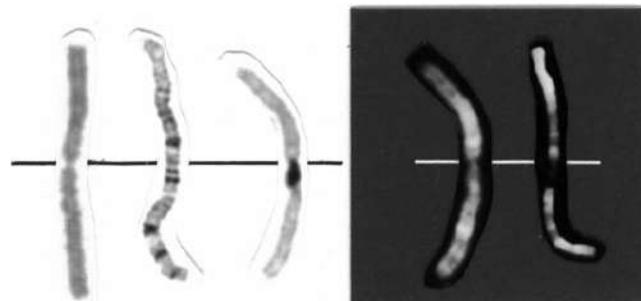


Figure 2.3 Comparing banding methods. A human chromosome 1, stained with (left to right) solid Giemsa, trypsin G-bands, C-bands, Q-bands, and R-bands.

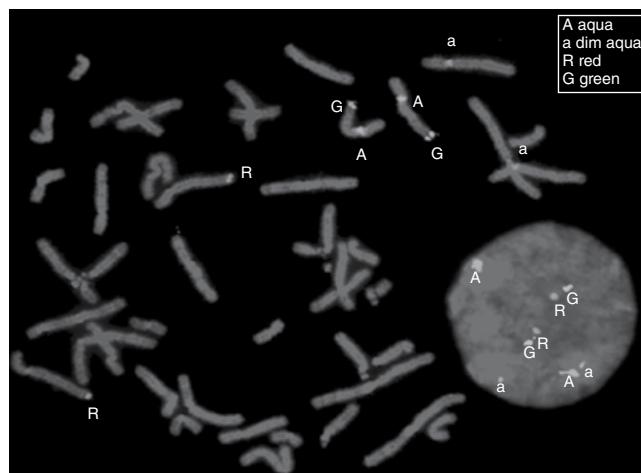


Figure 2.4 Fluorescence in situ hybridization on metaphase and interphase cells. FISH may be used on metaphase or interphase cells. This metaphase has been hybridized to three different colored fluorophores. The chromosome 4 long arm telomere is in red, chromosome 9 centromeric, alpha-satellite DNA is in aqua, and the chromosome 9 long arm is in green. Note that the signals can be enumerated in an interphase cell (right), as well as in the metaphase chromosomes. The small aqua on the B-group chromosome is due to similarities between the alpha satellite DNA between chromosomes 4 and 9. See insert for color representation of this figure.

In 1976 Jorge Yunis [11] revolutionized the field by demonstrating that short exposures to low concentrations of Colcemid®, along with cell synchrony methods, would yield elongated chromosomes with nearly twice the number of bands, termed “high-resolution” banding, that could then be used to detect smaller and more subtle chromosome abnormalities. Microdeletions, such as those responsible for Prader–Willi [12] and Angelman [13] syndromes on chromosome 15 long arm, DiGeorge/velocardiofacial syndromes on chromosome 22 long arm [14,15], and Miller–Dieker syndrome [16] on chromosome 17 short arm, were eventually discovered by using these methods.

In the 1980s, prenatal diagnosis by chorionic villus biopsy (CVS) became available to couples desiring earlier diagnosis than could be provided by amniocentesis. CVS could be successful at 10–12 gestational weeks, whereas amniocentesis becomes feasible at 14+ weeks since there is inadequate fluid volume present at earlier gestational ages. Knowledge of the cytogenetics of various neoplasms and correlation with diagnosis and prognosis of disease also became much better defined.

Also in the 1980s, the development of molecular cytogenetics sent the rapidly changing field into another revolution, uncovering for the first time the nature of cryptic rearrangements or changes, often visible only at the molecular level. The new FISH methods included whole chromosome probes (paints), single-copy probes, and centromere-specific probes, allowing many different kinds of questions to be answered, sometimes in the absence of metaphase cells (see Figure 2.4).

During the 1990s and early 2000s, FISH probes became commercially available for many different constitutional and acquired diseases. Specialized methods were developed to stain each chromosome a different color, such as SKY, M-FISH, and color-banded FISH probes, as well as comparative genomic hybridization (CGH) and panels for all subtelomere regions or all centromeres. Probes that could detect specific deletions and translocations in interphase cancer cells became commercially available in many forms for many different cancers, allowing for precise determination of disease diagnosis (e.g., *BCR/ABL1* translocation for diagnosing CML in certain situations; prognosis (e.g., *ATM*, *TP53*, 12 centromere aneuploidy, etc., for evaluating chronic lymphocytic leukemia (or CLL); response to treatment (e.g., *ERBB2*, also called *HER2/neu* gene amplification testing) for determining the therapeutic course for certain invasive breast carcinomas; residual disease (RD) status (e.g., *PML/RARA* rearrangement recurrence in acute promyelocytic leukemia (APL); and transplantation engraftment success (e.g., using X and Y centromere probes for detecting the presence and percentage of opposite-sex donor marrow cells).

Finally, comparative genomic hybridization was refined into chromosome microarray analytical tests, including mCGH or aCGH for array, allowing a high-throughput FISH analysis for hundreds or thousands of genes or chromosomal DNA sequences in a single assay (see Chapter 18, Genomic microarray technology for the cytogenetics laboratory). Table 2.1 shows a timetable of selected landmarks in the history of cytogenetics. No doubt the revolutions in this rapidly evolving field will continue to surprise us.

2.3 Cytogenetics methods

In this overview chapter we will discuss the general flow of activities within the cytogenetics laboratory, as well as the theories behind their processes, i.e., harvesting, slide-making, staining, chromosome analysis (Figure 2.1), and case reporting, as it applies to all specimens. The chapters that follow will discuss in more depth the methods that are used to process specific tissue types received by cytogenetics laboratories, such as, blood, bone marrow, tumors, amniotic fluid, chorionic villi, skin, and products of conception, as well as molecular (FISH) methods that can further interrogate the cells obtained from these tissues.

The methods used to study a tissue depend upon what the tissue is and what cytogenetic questions need to be addressed. Constitutional chromosome aberrations are typically studied in blood lymphocytes, but other tissues from different germ layers, such as skin and gonad, or tissue from placenta, may also be studied when looking for tissue mosaicism (see 2.6.2, Mosaicism). In addition, there may be occasions in which blood is not available (e.g., the patient is deceased), but skin or other tissue is still viable.

Acquired conditions associated with neoplasms must study the site of the malignancy, e.g., tumor, lymph node, metastatic site, bone marrow aspirate, bone core in the event of a dry tap, or neoplastic blood, if appropriate to the diagnosis. Situations may arise, however, when a second, non-neoplastic source may be required in order to determine whether a karyotypic abnormality is neoplasia-related or constitutional. For example, trisomy 21 in bone marrow from a leukemic child may be acquired or constitutional.

Tissues that are in suspension, such as amniotic fluid, bone marrow, and peripheral or cord blood, are easiest to culture, because they do not require dispersal before being set up in culture. Solid tissues, such as, chorionic villi, skin, solid tumors, and products of conception, however, will require some enzymatic dispersal before culturing for the best results.

2.3.1 Work flow

Specimen procurement

The first step in cytogenetic analysis is to obtain the correct tissue in the correct collection medium/anticoagulant in appropriate containers, at the correct temperature and in a timely manner. Some tissues must be received immediately, such as many brain tumors; others may be delayed for 1–3 days, such as the average blood sample. The tissue of choice depends on whether the referral is for constitutional or acquired disorders. All laboratories should create and provide standard specimen request forms for submitting physicians and laboratories so that the client will know what information is needed by the cytogenetics laboratory.

Collection containers must be sterile, and in most cases, are either standard stock (e.g., syringes, vacutainers, glass test tubes, urine collection cups) or are provided by the cytogenetics laboratory (sterile Petri dishes, plastic centrifuge tubes with or without transport medium). The technologist, however, should pay close attention to any suboptimal growth pattern that affects multiple patients drawn under similar conditions, as there could be unexpected toxicity in a lot or type of container being used by the physician or laboratory. Many types of specimens (e.g., solid tissues, such as chorionic villi; products of conception, solid tumors) must be kept moist during transport by using isotonic saline or various collection/transport media. Small samples of blood, bone marrow, or other fluids will survive much better in medium as well, since fewer cells dry onto the collection tube surface, and some of the nutritional and buffering requirements are met by the tissue culture medium.

Table 2.1 Major discoveries and landmarks in cytogenetics, 1865–2001

Year	Investigator	Discovery
1865	Gregor Mendel	Discovered principles of heredity
1867	Gustav Giemsa	Invented Giemsa stain mixture
1882	Walther Flemming	Described mitosis, used fixation, staining
1888	Heinrich Wilhelm Waldeyer	Named chromosomes
1893	Oscar Hertwig	Published <i>Cell and Tissue</i> , the first cytology textbook
1902	Theodor Boveri, Walter Sutton	Demonstrated presence of pairs (homologues)
1905	Nettie Stevens	Reported presence of Y chromosome in males
1914	Theodor Boveri	Theorized that chromosome changes could cause cancer
1921	John Belling	Introduced squash method for chromosome spreading
1922	Theophilus Painter	Published that humans have 48 chromosomes based on testicular sections
1937	Albert Blakeslee and A. G. Avery	Used colchicine for arresting metaphases
1941	Frits Zernike	Phase contrast microscope developed
1951	George Gey	Established HeLa cell line
1952	Arthur Hughes; Kyoko Makino and Kazuo Nishimura; T. C. Hsu	Described the use of hypotonic solution in cytogenetics
1953	James Watson and Francis Crick	Described double helix structure of DNA
1956	Jo Hin Tjo and Albert Levan	Established chromosome number of man as 46 using colchicine, 0.04–0.004 µg/mL for 20 hours, and hypotonic on embryonic lung cultures from four fetuses
1958	Charles E. Ford	First report of chromosome abnormalities in leukemia
1958	David Hungerford	Reported 0.075 M KCl improves chromosome morphology over sodium citrate hypotonic solution
1959	Jerome Lejeune	Discovered an extra chromosome 21 in Down syndrome patients
1959	Patricia Jacobs and John Strong	Described XXY in Klinefelter syndrome
1959	Charles E. Ford	Reported 45,X Turner syndrome
1960	J. A. Böök and Berta Santesson	Described triploidy in humans
1960	Klaus Patau	Described trisomy 13
1960	John Edwards	Described trisomy 18
1960	Peter Nowell	Described phytohemagglutinin A (PHA) effect in lymphocyte culture
1960	Paul S. Moorhead, Peter Nowell, W. J. Mellman, D. M. Battips, and David Hungerford	Described blood culture and harvest protocol using PHA, colchicine, hypotonic, 3:1 methanol–acetic acid fixative, Giemsa stain
1960	ISCN	First conference on chromosome nomenclature (Denver, CO)
1961	Mary Lyon	Described X inactivation
1963	Jerome Lejeune	Described deleted 5p in Cri-du-chat syndrome
1964	Patricia Farnes and Barbara Barker	Described pokeweed mitogens use
1968	Torbjörn Caspersson, L. Zech, E. Modest, G. Foley, and U. Wagh	Described Q-bands in <i>V. Faba</i> , <i>Trillium</i> , and Chinese Hamster
1969	Herbert Lubs	First described fragile X in families

Table 2.1 (Continued)

Year	Investigator	Discovery
1969	Joseph Gall and Mary Lou Pardue	First described <i>in situ</i> hybridization using radioactive probes
1969–1970		Amniocentesis becomes clinically used for cytogenetic diagnosis
1970	Torbjörn Caspersson, Lore Zech, C. Johansson	First Q-bands in humans described
1971	ISCN	Third meeting for human chromosome nomenclature (Paris, France). First meeting to describe banding for ISCN
1971	Adrian Sumner; S. R. Patil; Maximo Drets and Margery Shaw; Marina Seabright	Described various G-band methods
1971	Bernard Dutrillaux, Jerome Lejeune	Published R-bands in the USA
1973	Janet Rowley	Described Ph chromosome as a t(9;22), not a deletion of 22q using Q-bands
1974	P. Perry and Sheldon Wolff	Described sister chromatid exchange staining with BrdU and Giemsa
1974	David Cox, Virginia Niewczas-Late, Margaret Riffell, and John Hamerton	Described <i>in situ</i> harvesting method
1975	Samuel A. Latt	Described replication banding methods
1975	C. Goodpasture and S. E. Bloom	Described NOR silver staining of chromosomes
1977	David Peakman, Marilyn Moreton, Barbara Corn, Arthur Robinson	Described coverslip <i>in situ</i> harvesting method
1979	Jorge Yunis	Described high-resolution G-banding methods
1980	J. G. Bauman, J. Wiegant, P. Borst, P. van Duijn	First <i>in situ</i> hybridization with fluorescent fluorophores
1981	P.R. Langer, A. A. Waldrop, D. C. Ward	Described biotin-labeled probes for <i>in situ</i> hybridization
1981–1984	N. Wake	Improved methods for solid tissue culture using collagenase dissociation
1982–1983	Z. Kazy; R. H. T. Ward; B. Brambati and A. Oldrini; L. G. Jackson and J. M. Hahnemann	First reports of CVS use for prenatal diagnosis
1986–1988	Daniel Pinkel and Joe Gray	Developed interphase and metaphase FISH
Mid 1980s		Automated (computerized) capture/karyotyping systems developed
1987	Jack Spurbeck	First described robotic harvesting method
1989	Hermann-Josef Lüdecke	First described microdissection of chromosomes
1991	A. Kallioniemi, O-P Kallioniemi	Described comparative genomic hybridization (CGH)
1996	Evelyn Schröck, Thomas Ried	Described multicolor spectral karyotyping
1996	Affymetrix company	Introduced the first gene chips, also called microchips/microarrays
1999	ICGSE	Rough draft of human genome established
2000–2001	Various companies	Commercially available microarrays for high-resolution cytogenetic analysis

Specimens to be cultured must never be frozen or exposed to excessive heat, because live cells are required for cytogenetic preparations cell culture. The exception is cells cryopreserved in special freezing media to keep them viable. Refrigeration is helpful for some specimens, especially those, like products of conception, which may have a risk for contamination, but because the specimen could freeze by accident at only a few degrees colder than standard refrigerator temperatures, and some refrigerators are not well controlled, room temperature for specimen storage and shipping is often recommended.

Communication between the laboratory and referral personnel is very important each time a clinical sample is sent, in order to confirm that these specimen variables and requirements are met, and to obtain a complete patient name, diagnosis, reason for referral, birth date, billing information, and details for delivery. Many culture, harvest and analysis decisions depend on the reason for referral, and although this may sometimes be difficult to obtain, it is nevertheless crucial.

Unless otherwise required by state or federal regulatory agencies, all specimens received by the laboratory must have at least two identifiers. One is usually the patient name, and the other may be patient birth date, medical record number, and/or other identifying data. These identifiers match with the order requisition. If a specimen arrives without a patient name or unique identifier, many laboratories refuse it, and request another sample. If no other sample can be obtained, the laboratory should keep careful records of the circumstances, and note the deficiency on the final report. The sender should be alerted regarding samples with insufficient patient identification, and the referring MD, hospital unit or laboratory should provide a signed release form verifying and accepting responsibility for specimen identification before the specimen is fully processed. Under certain situations, however, a rejection or delay in processing may not be in the best interest of the patient (e.g., brain tumor), or when the specimen has been obtained via an invasive or irreplaceable procedure (e.g., amniotic fluid, bone marrow, or solid tumor tissue). Unless otherwise directed by your facility, the laboratory may choose to accept the specimen for processing if the person collecting the specimen can irrefutably confirm the specimen's identification, and the referring office or laboratory sends a release form to the cytogenetics laboratory. Careful records should be kept of the circumstances, and the deficiency should be noted on the final report.

Specimens received for genetic testing are ordered by the physician; therefore, in most cases the cytogenetics laboratory performing the test does not see the patient. This becomes a problem when informed consent forms are required, especially for prenatal genetic testing. Informed consent confirms that the patient was made aware of not just the reason for and what is expected from the proposed procedure, but also the risks involved, benefits projected and alternatives available. The physician is generally the one who explains the test to the patient, and the patient must sign the informed consent form before the procedure is preformed; however, a copy of this signed consent is not always forwarded to the cytogenetics laboratory. If the form is prepared by the physician, it may also not include information pertinent to the specific test being performed by cytogenetics; therefore, it is important that laboratory directors, in conjunction with the institution's legal staff, review the content of all informed consent forms for their specific tests in order to ensure that appropriate information has been covered, and that a copy be maintained for reference in the laboratory. If a copy of the signed form is not being sent to the laboratory with the specimen or faxed upon test ordering, laboratory personnel should devise some method by which the physician can indicate that an informed consent was signed for that particular procedure, and that, in the event that the laboratory will require a copy of the form for verification (e.g., laboratory inspection), the physician will provide the copy or scanned image of the document within a specified time. If the result of a test sent for constitutional chromosome analysis is abnormal or questionable, whether prenatal or postnatal genetic counseling must be recommended on the final report, in order to help the patient(s) understand their options and to answer any questions they may have. Neoplastic situations are generally handled by the oncologist, but they also may refer patients for genetic counseling if it would be helpful to the patient.

Specimen logging

Once the specimen has been properly received, it should be recorded into the laboratory log, whether this is computerized or in book form, and be given a unique, generally consecutive accession number. The accession number format is devised by laboratory management to meet all regulatory and state guidelines. Some institutions incorporate letters indicating specimen type, and a numeric prefix to indicate the year, for example, BM-15-001531 could indicate the 1531st bone marrow specimen received in 2015. The log or system worksheet should have at least the laboratory accession number and two patient identifiers, for example, patient's name, physician's name, birth date, sample type, and date of receipt in the laboratory. All paperwork and sample containers should be double-checked to ensure that each has been correctly recorded and labeled. The receipt date should be compared to the date of service, or the date the specimen was procured. The time of day the sample was received should be recorded as it may explain how specimens were subsequently cultured and harvested, and may help differentiate between multiple specimens on a patient, etc. Any unusual delay in specimen receipt and other variables, such as specimen condition, or any other observation that could compromise the viability of the specimen should be noted.

After the specimen is logged in, a culture and harvest schedule must be assigned according to specimen type and urgency of the results, as well as the day of the week and time of day that it is received in the laboratory. Spontaneously dividing samples, such as bone marrow or solid tumors, may be set up for a direct (uncultured) harvest, and may also be cultured for one or more days. Other samples may require culturing for several days to a week or two to stimulate growth, some with mitogens (e.g., blood lymphocytes), some following mechanical dispersal (mincing with scissors or scalpels or forcing tissue through a mesh screen, as for solid tumors and lymph nodes), or enzymatic disaggregation with collagenase and/or trypsin (e.g., solid tissues, tumors, chorionic villi) (see individual chapters for details). Some less urgent samples may be refrigerated at 4°C until they can be fit into the laboratory workweek schedule.

Once the sample has been cultured and is ready to process for cytogenetic analysis, a harvest protocol specific to the sample type and culture method (e.g., *in situ* versus suspension harvests) is performed.

2.3.2 Culture methods

With the exception of some sample types, such as STAT interphase FISH samples on directly harvested cells, some period of culturing will be necessary before the specimen can be harvested. This period varies from one day to several weeks, depending on sample type. For example, bone marrow cells would be cultured 0–5 days, whereas skin fibroblasts would require 1–3 weeks to grow enough to harvest. Other conditions, such as sample size, viability, and urgency of the result can also affect the culture duration. The proper culture duration may be critical to obtaining the results, because abnormal tumor cell lines may be lost over time or, as with some bone marrows, may appear only after a few days in culture. Culturing is performed using strict aseptic techniques, because microbes will cause culture failure.

There are two general categories of tissue culture:

1. Suspension cultures, such as bone marrow and blood, and some types of tumors, such as some neuroblastomas and most lymphomas, which are suspended in growth medium and do not attach to the culture vessel; and
2. Attached monolayer cultures, or anchorage-dependent cultures, such as amniocytes, skin fibroblasts, etc., which require adherence to a culture surface to grow.

These two growth patterns are often called short-term and long-term cultures, but these categories are less distinct now that “long-term” cultures are often harvested in 2–3 days (solid tumor attached cultures) to 6–7 days (*in situ* amniocytes). Lymph node and solid tumors may be set up in both suspension and attached cultures for specific conditions, such as with a differential diagnosis of lymphoma versus sarcoma, or when the tumor type requires both culture types (e.g., neuroblastoma or other round blue cell tumors). These cultures may be incubated in an open or closed system. Open cultures are able to exchange gases with the atmosphere surrounding the culture. Closed systems are sealed tightly and do not exchange gases with the atmosphere. Closed systems have less chance of microbial contamination entering the culture, but open systems allow the culture to release gas phase byproducts of metabolism, which may be toxic to the cells. Open cultures are advantageous for *in situ* culturing and harvesting methods, in which cultures are grown and harvested on coverslips or slides that are inserted into Petri dishes or commercial slide flasks (Figure 2.5).

The pH of the cultures, as indicated via a bicarbonate buffer in the media, can be maintained by

1. allowing the CO₂ and other metabolites from the cultured cells to adjust the culture pH (closed system);
2. gassing the culture flask or tube from a canister with the desired gas mixture and closing it tightly to prevent gas exchanges (closed system); or
3. providing the incubator with a constant, controlled flow of the desired gases from external gas tanks (open system).

Closed systems are often put into incubators with a controlled flow of gas as a backup against leaks in the flask. The usual gas mixtures are 5% CO₂ in ambient air, which is composed of about 15–18% oxygen, or 5% CO₂ in 2–5% O₂ with the balance (93–95%) inert N₂. The 5% CO₂ adjusts the culture medium to pH 7.25–7.40 via the buffer in the medium, and the low-oxygen formula of the 2–5% O₂ mixture has been shown to increase the growth rate of many cell types.

Some cell cultures are grown with no oxygen, and may be gassed only with nitrogen. An example is transformed cell lines from patients with Fanconi anemia, which are sensitive to the presence of oxygen, and may not grow in its presence. Media with buffering systems other than the CO₂–bicarbonate equilibrium are also available, for example, HEPES buffer. This type of buffer is useful for transporting cells or tissues to the laboratory when the CO₂ component is not available, and standard media would lose the pH. Human cell cultures derived from human tissue are best grown at the physiologic body temperature of 37–37.5°C, as growth curves fall off sharply over 38°C, often resulting in cell death. Incubators should have alarms to alert personnel to temperature extremes and minimum-maximum temperature recordings for monitoring daily fluctuations (see Chapter 20, Selected topics on safety, equipment maintenance, and compliance for the cytogenetics laboratory).

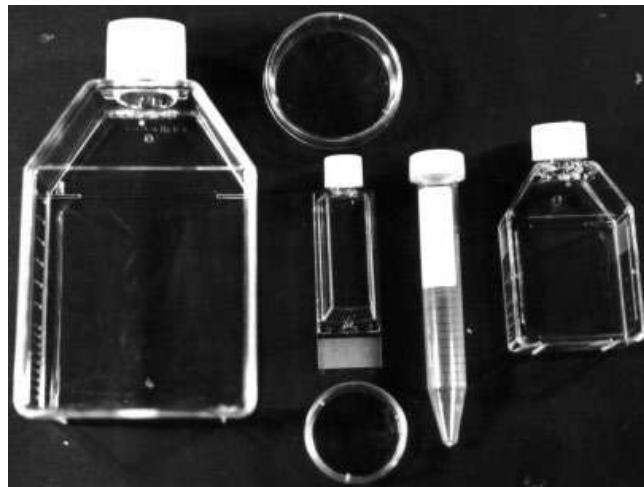


Figure 2.5 Tissue culture plasticware. Common tissue culture plasticware used by cytogenetic culture methods include the T-75 flask (a) and T-25 flask (f), which are appropriate for growing attached or suspension cultures, as are the chamber slide (c) and Petri dishes (b,d). The smaller Petri dish (d) also comes with a coverslip inside for *in situ* culture and harvesting. The 15-mL conical centrifuge tube (e) is used for growing (in culture) and harvesting blood, bone marrow, and other suspension cultures, and for harvesting attached cells that have been brought into suspension for a harvest.

Table 2.2 Tissue culture media commonly used for cytogenetics

Medium	Type of culture	Comments
RPMI 1640	Blood, bone marrow, solid tumor culture	Developed for lymphocytes but has been used for every application
RPMI 1603	Blood, bone marrow	Yunis recommended this medium for prophase; difficult to find
MEM or Eagle's MEM	All	Minimum essential medium
MEM Alpha	All	Enriched; available with ribonucleosides, deoxyribonucleosides
DME	All	Dulbecco's modified Eagle's
Ham's F10	All	Enriched medium with low phenol red dye
Ham's F12	All	Enriched medium with low phenol red dye
Medium 199 or TC199	Fragile X (historically), prophase	Low folic acid
Chang	Originally a prenatal medium with growth factors; now available specifically for amnio, marrow, and blood cultures	Can be used for POCs, tumor cell cultures as well
AmnioMAX	Amnio, CVS	Can be used for POCs, tumor cell cultures as well
McCoy's 5A	All	Highly enriched; good for transport medium
MarrowMAX	Bone marrows, Hem/onc bloods	Ready to use, fully supplemented with serum, a human stromal conditioned medium, antibiotics, and L-glutamine

There are scores of tissue culture media available. The most commonly used media are listed in Table 2.2. Basal medium is supplemented with serum for protein and growth factors. Most laboratories prefer fetal bovine serum. Newborn calf serum is cheaper, but may be toxic; colostrum-free newborn calf serum is less expensive and may be as effective as fetal bovine serum. Serum concentration in complete medium varies from 2% to 20%, with more serum yielding faster growth in some cases.

Some cells, however, are sensitive to the presence of fetal bovine serum and may not divide as well with high concentrations (e.g., neuroblastoma cultures, lymphocytes), and others may arrive with serum in them (e.g., blood samples) and may not require as high a concentration of serum in the culture medium as other specimen types do. Other media supplements include antibiotics, L-glutamine, and optional additions, such as selenium, insulin, and giant cell tumor conditioned medium (see specific chapters for culture methods). Once supplemented, the medium is called complete medium.

Cultures that are valuable or may be needed for future use may be frozen in medium with 5–10% dimethyl sulfoxide (DMSO) or glycerol to prevent ice crystal formation, and kept in liquid nitrogen until such time as they are needed.

2.3.3 Harvesting

Once cells are growing either spontaneously or in culture, they must be processed (harvested) to obtain metaphases for study. Harvesting cells for cytogenetic analysis is a stepwise procedure that may be and often is accomplished by automated instrumentation. However, the success of the final preparation will depend on the resourcefulness and skill of the technologist. Controlling and working around inevitable problems and variables, such as cell density (too high or too low), low mitotic index, disease-related poor chromosome quality, unusual cell types, and environmental changes often requires thought, imagination, and inventiveness.

The three constant features of the metaphase harvest protocol are mitotic arrest, usually with Colcemid®; hypotonic treatment, usually with solutions of KCl, sodium citrate, or combinations of these salts; and fixation, usually with 3:1 methanol-acetic acid solution. There are, however, several other variable options, which would depend upon the type of culture being harvested or the type of results most desired.

2.3.4 Removal of attached cells and centrifugation steps

After mitotic arrest and just before the use of the hypotonic and fixative steps, an optional step for attached cells intended for a suspension harvest is the removal of the mitotic cells by mechanical methods (rapping flasks sharply on bench top or scraping with a rubber policeman) or by enzymatic methods. The latter is commonly accomplished with trypsin and/or EDTA solutions. This mechanical/enzymatic removal would only be needed for cells that are growing attached to the surface of the flask or dish. An advantage to the mechanical removal is that mitotic cells come off before the nondividing cells, and such methods enrich the harvest for metaphase cells by leaving the interphase cells behind. Either way, the culture may be refed and reincubated for further study, providing the mitotic arrestant used is reversible. Colchicine, for example, binds irreversibly, and cannot be washed off like its synthetic analog, Colcemid®.

Another variable step is centrifugation, which is employed during suspension culture harvests to change from one solution to the next. Each solution, such as culture medium, hypotonic solution, and fixative, is removed following centrifugation. In contrast, in situ harvesting is accomplished by adding the harvesting solutions (hypotonic and fixative) to cells growing on coverslips or slides inside of Petri dishes or in chamber slides, and since cells are held in place by anchorage to the growth surface no centrifugation between steps is necessary. Some laboratories have modified the in situ harvest method to work with bone marrow suspension cultures in Petri dishes, because the cells usually settle to the bottom of the dish; if pipetting is done carefully, the cells are not disturbed.

Centrifugation is never complete, and a number of cells remain in the supernatant or are lost to damage from shear forces between cells or with the centrifuge tube walls. This is why the number of centrifugation steps should be kept to a minimum. It is also why the in situ harvesting method may yield more metaphases than do similar cultures from suspension harvests. The speed, or revolutions per minute (RPM), of centrifugation required for most cells to be recovered depends on the radius of the arm of the centrifuge. A centrifuge with a 10-in. radius yields higher gravities at 1000 RPM than a centrifuge with a 6-in. arm would at the same number of revolutions, because the larger radius travels farther (faster) per revolution. The equation for calculating the gravities for a given centrifuge at a given speed in revolutions per minute is

$$g = (1.118 \times 10^{-5}) \times R \times S$$

where g = relative centrifugal force (gravities), R = rotating radius in centimeters (distance from center of rotation to bucket center), and S = rotating speed (RPM).

Therefore, the gravities that a cell will experience at 1000 RPM in a centrifuge with a 4-in. (10 cm) radius is about 110, and the gravities that the same cell would experience in a centrifuge with a 6-in. (15 cm) radius at 1000 RPM is about 170. This may be important when one uses a new centrifuge with a different design, as the difference in gravities (centrifugal force) can make a difference in the yield, either by incomplete migration to the pellet (too slow) or by breakage due to shear forces (too fast).

Of the harvesting steps, the one usually considered the most important is the hypotonic solution because it affects spreading, chromatid width and separation, and, in bloods and bone marrows, the elimination of red blood cells. However, chromosome spreading and morphology are also controlled by Colcemid® concentration to a high degree [17]. Many so-called hypotonic problems in cell spreading are actually Colcemid® problems (Figure 2.6). Cell culture density and slide-making variables, including glass slide quality, are also equally important to spreading and may be responsible for spreading problems. Permutations of all the steps affect the quality of the final product.

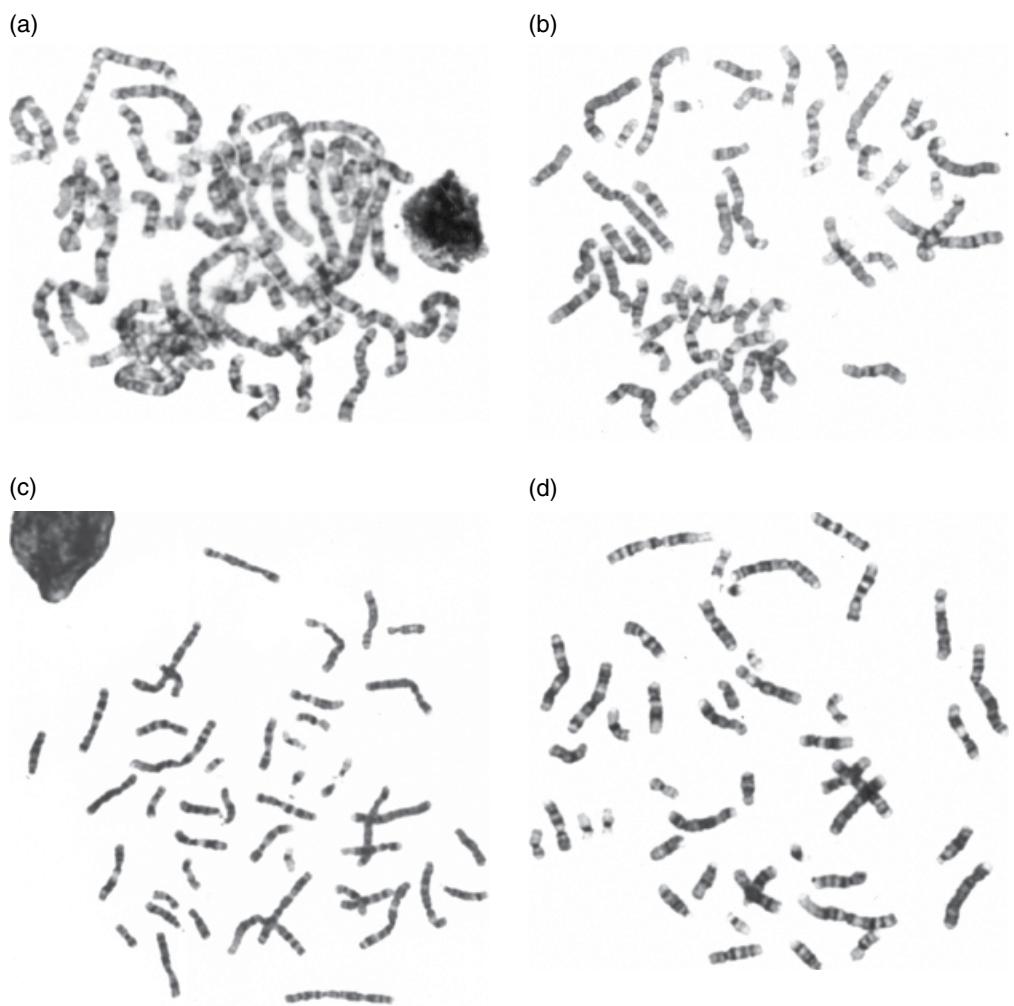


Figure 2.6 Colcemid® concentration effect on blood chromosome quality. The following examples show representative blood cells from a Colcemid® experiment using various concentrations of Colcemid® for 30 minutes. (a) No Colcemid® added. Mitotic index was very low, because mitoses were not accumulated. Metaphase chromosomes are bent, with chromosomes unable to spread out due to the effect of the spindle; (b) 0.02 µg/mL Colcemid®. Mitotic index on the slide was better, less chromosome bending is evident, and spreading is improved due to spindle poisoning; (c) 0.05 µg/mL Colcemid®. Chromosomes show better spreading and are straight, but are beginning to shorten and show fewer bands; (d) 0.1 µg/mL Colcemid®. Mitotic index was very high (not shown) because there are enough Colcemid® molecules to poison all of the microtubules of the spindles of all cells in the culture. Also, chromosomes are well spread and have few crossed chromosomes. However, chromosomes are highly contracted, and many small abnormalities, such as the Prader–Willi and DiGeorge deletions, would not be detectable at this band level.

2.3.5 Mitotic arrest: Colcemid®

The first step in most harvest procedures is to arrest cells in the mitotic stage required for standard cytogenetic analysis: metaphase. The effect of the most commonly used mitotic arrestant Colcemid® is to prevent formation of the spindle fiber apparatus, which would normally pull the sister chromatids to opposite poles for incorporation into the two daughter cells. Colcemid® also causes chromosome condensation, a process that becomes accentuated when increasing the time of exposure and the concentration. Modern cytogenetics laboratories strive for medium-length to very long chromosomes in order to increase the chance of detecting small rearrangements, so Colcemid® dosage and exposure are often reduced to a minimum. However, an understanding of the effects of Colcemid® can allow the technologist to vary the dosage and exposure time and still maximize the quality and quantity of mitotic cells in the harvest, depending on the culture and harvest type, the desired type of study, and other variables. The condensation effects which occur with longer time exposures seem to be greater with certain cell types, such as blood, bone marrow, and CVS, than with others (amniocytes, solid tumors), and may be at least partly related to cell cycle time and disease state. Also, some cell types are more sensitive to the concentration of Colcemid® than others. For many attached cultures, the slower growing cells seem to tolerate longer Colcemid® times. This condensation effect of Colcemid® is mitigated with the use of anticontraction agents, such as ethidium bromide (EB), 5-bromo-2'-deoxyuridine (BrdU), etc. (Figure 2.7; see Chromosome anticontraction methods).

The dose-dependent contraction effect of Colcemid® is well known [17–19], but increased dosage of Colcemid® also dramatically increases mitotic index, straightens chromosomes, sharpens chromatid edges, and increases chromosome spreading as it releases them from the mitotic apparatus [17–21] (see Figure 2.6). Experiments in our laboratory suggest that as Colcemid® concentration increases from 0.01 µg/mL to 0.1 µg/mL, there is a G-band coalescing effect, so that chromosomes with a long appearance do not have as many sub-bands as seen at the same physical length with lower dosages. See Table 2.3 for Colcemid® concentrations at various dosages.

The optimal concentration for each tissue type should be tested for every laboratory, and Colcemid® should be added with a micropipette to known volumes of medium in the culture in order to strictly control concentration. The concentration can be increased in the presence of EB or other anticontraction agents (see 2.3.9, Chromosome anticontraction methods later). In fact, EB causes chromosomes to spread poorly [22], and experiments in our laboratory led to the conclusion that spreading dramatically improves using 0.05 µg/mL of Colcemid® with EB over that seen with 0.02 µg/mL Colcemid® with EB (see Figure 2.7) in tumor cultures.

Colcemid® is a synthetic analog of colchicine, an alkaloid derived from autumn crocus (see Figure 2.2). Some laboratories still prefer colchicine, which is more toxic, and may therefore retard the cell cycle speed and effectively yield longer chromosomes. Some cultures may stop dividing and yield no metaphases due to the toxic effect of colchicine, although this is not common. If it is suspected that a culture is not as mitotic as it should be when harvested with colchicine, it is possible that Colcemid® would be a better choice. Velban (vinblastine sulfate) is also used for some or all tissues in some cytogenetics laboratories, and is a good substitute in tumor cultures and other difficult specimens when Colcemid® seems ineffective.

Often the mitotic arrest is blamed for contracted chromosomes, but other factors may be involved. In blood and bone marrow cultures, overinoculation may result in depleted medium and increased metabolic byproducts that cause irreversible chromosome contraction. Slide-making variables, such as cell suspensions that are too concentrated or contain excessive debris, result in crowded metaphases that appear more contracted than cells from slides made from more dilute suspensions of the same pellet. In our experience, specimens from compromised patients, such as leukemics, drug-treated patients, and neonatal intensive care patients, may always appear contracted in spite of our best efforts. The experienced technologist learns to use all of the tools available to ensure the best possible results, and Colcemid® is one of the most important.

2.3.6 Hypotonic treatment

The second major step in harvesting cells is treatment with a hypotonic saline solution to increase cell volume (Figure 2.8) so that the chromosomes have adequate space to spread out during slide preparation. Since the cell membrane is semi-permeable, water moves slowly into or out of the cell by osmosis, which equalizes the concentration on both sides of the membrane. Thus, if the concentration of the solution outside the cell is higher than inside (hypertonic), the cell will lose water and shrink. Alternatively, if the concentration outside the cell is lower than inside the cell (hypotonic), it will absorb water and swell. Hypotonic solutions work in this way, by creating a concentration gradient across the cytoplasmic membrane, so that water moves in by osmosis, though active transport may also be involved [19]. If the potassium pump is poisoned, the cells do not swell [19]. Various hypotonic solutions and their uses are listed in Table 2.4.

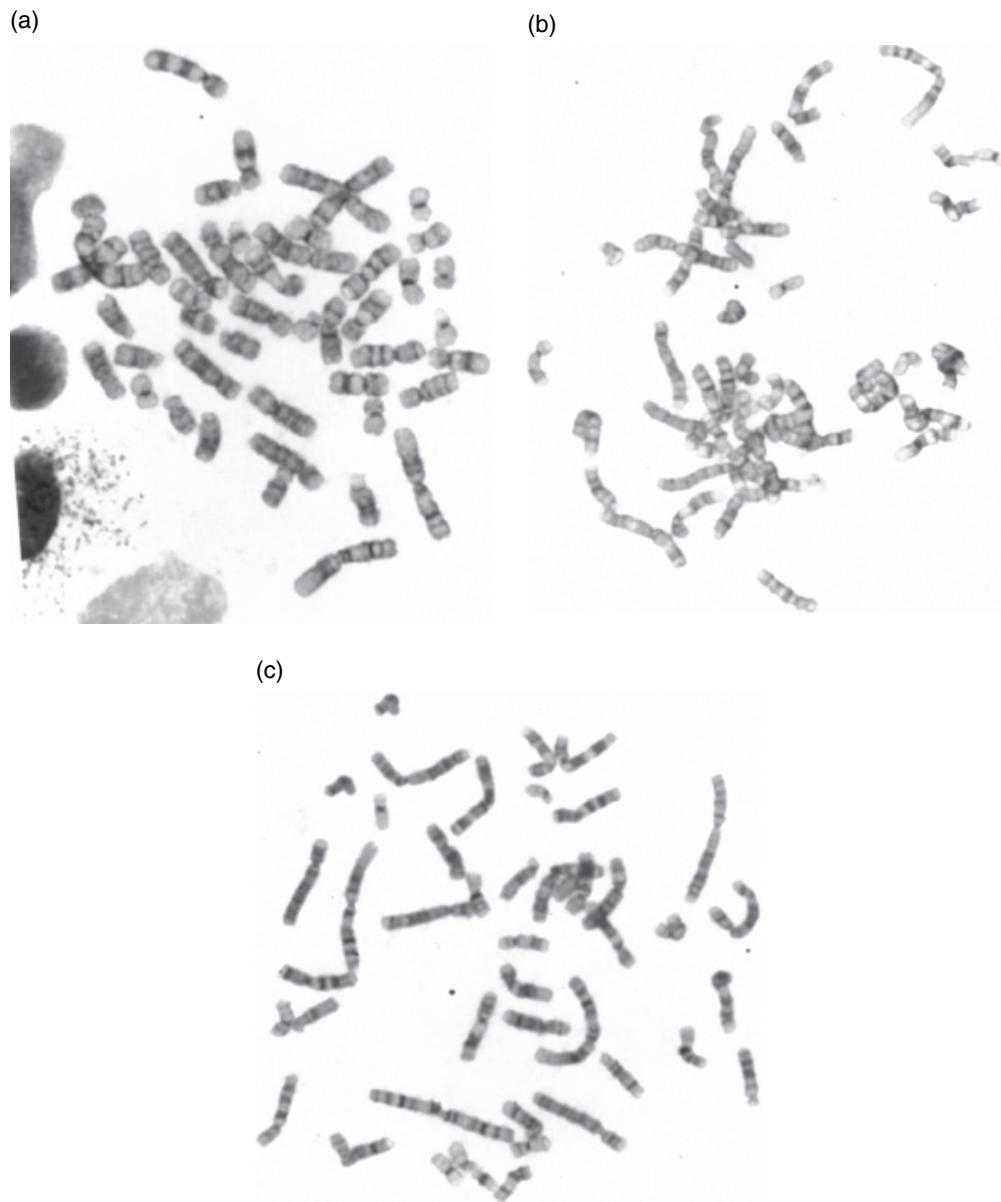


Figure 2.7 Colcemid® and ethidium bromide (EB) concentration effect on melanoma cells. Representative cells from an experiment on melanoma cells. (a) Harvested with 0.02 µg/mL Colcemid® and no EB. (b) Harvested with 0.02 µg/mL of Colcemid® and 5 µg/mL of EB. Chromosomes are longer than without EB, but tend to clump together and cross each other. (c) Harvested with 0.05 µg/mL of Colcemid® and 5 µg/mL of EB. This concentration of Colcemid® mitigates the clumping effect of the EB and reveals better band resolution for abnormal chromosomes than without EB.

Prewarming the hypotonic solution to 37°C may increase effectiveness by speeding up water transport across the cell membrane and possibly by softening the cytoplasmic membrane, which has a lipid component, giving it more stretching capability. The type of salts used in the hypotonic can affect the width and sometimes the length of the chromatids, Sodium citrate, for example, often yields wider chromatids than KCl, and Ohnuki's hypotonic usually yields longer chromatids than KCl.

Many cell types are sensitive to the hypotonic treatment, for example, solid tumors or acute lymphocytic leukemia (ALL bone marrows), with some being easily damaged by over treatment and some being resistant to swelling. In our hands, the best results are obtained when the hypotonic volume and treatment time are kept at the lowest effective level. We feel that most cells seem to respond well in the first 10–20 minutes of exposure. Problems due to large volumes of hypotonic solution (excluding *in situ*

Table 2.3 Colcemid® concentrations

μg/mL	Microliters to add (per 5 mL culture)	Syringe equivalents (25G needle)
0.05	5	½ drop
0.02	10	1 drop
0.05	25	2 ½ drops
0.1	50	5 drops
0.2	100	10 drops

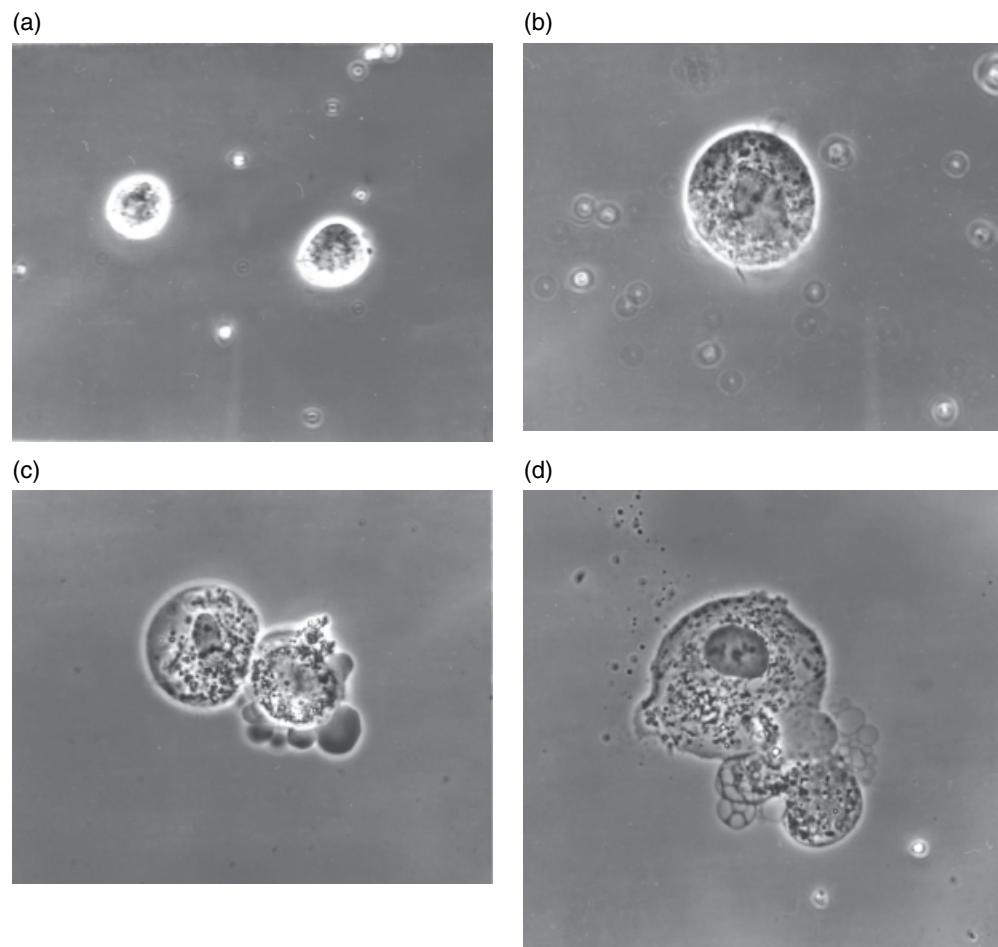


Figure 2.8 Hypotonic action on tumor cells in 0.075 M KCl. (a) Within 3 minutes of addition of hypotonic solution, cells have not begun to swell. (b) At 10 minutes of hypotonic duration, cells are double in volume, and membrane is stretched but still strong. (c,d) After 30 minutes and 45 minutes of hypotonic, respectively, cells have begun to lose integrity and collapse under the weight of the coverslip. A hole has opened in this cell (d) and cytoplasmic contents are streaming out. If this were a metaphase cell, with the nuclear envelope disassembled, some of the chromosomes could leak from the hole, leading to hypodiploidy or lowered mitotic index.

harvests) may include loss of mitotic cells due to increased fragility during centrifugation, as well as incomplete migration to the pellet. Long exposures to hypotonic may cause weak areas in the cytoplasmic membrane (see Figure 2.8), which, if they burst at any point, will allow some or all of the chromosomes to escape. This can lead to a (false) low mitotic index, scattered chromosomes, partial metaphases, or tight “imploded” cells. Holmquist and Motara [19] report that after a long hypotonic incubation period, the cells return to their original size.

Table 2.4 Formulas for hypotonic solutions

Solution	Formula	Usual harvest type
0.075 M KCl (0.56%) ^a	5.59 g/L	All
0.4% KCl	4 g/L	Neoplastic cells for increased spreading
CHS (0.4% KCl with EGTA and HEPES)	3 g KCl + 4.8 g HEPES + 0.2 g EGTA/L, pH 7.4	Cancer hypotonic solution
THC ^b	9 parts 0.075 M KCl, 1 part 0.25% Trypsin-EDTA, 0.08 µg/mL Colcemid®	Neoplastic cells: trypsin/hypotonic/Colcemid® to increase spreading, morphology
Sodium citrate ^c		
0.7%	7 g/L	All attached cell types, especially amniocytes
0.8%	8 g/L	
1.0%	10 g/L	
Sodium citrate and KCl mixtures	Many combinations of various concentrations of KCl, sodium citrate, dilute serum or medium	Amniocytes, fibroblasts
Dilute Hanks' BSS	1 part Hanks': 3–6 parts distilled water	Attached cell types
Dilute serum (calf, fetal bovine)	1 part serum: 3–6 parts distilled water	Attached cell types
Ohnuki's hypotonic	4.1 g KCl in 1 L water+ 2.33 g NaNO ₃ in 500 mL water+ 0.9 g CH ₃ COONa ^d (NaC ₂ H ₃ O ₂) in 200 mL water, or 5 mL 55 mM sodium nitrate, 2 mL 55 mM sodium acetate, 10 mL 55 mM potassium chloride	

Many methods call for addition of a few drops to several milliliters of fixative to the hypotonic at the end of the incubation period. The *in situ* harvest method requires this “prefixative” step to help prevent mitotic cells from becoming dislodged during addition of the first fixation. This prefixation begins the process of hardening the cells and preserving the chromosomes, and makes cells more resistant to damage from centrifugation and the shock of the pure fixative. It also encourages the lysing of any red cells present, and since the lysed cells do not migrate to the pellet, the result is a cleaner cell preparation after centrifugation. However, nucleated red cells, as found in some blood samples (e.g., newborn blood), as well as blood from lower vertebrates (birds, reptiles, and fish), do not lyse in hypotonic or fixative.

2.3.7 Fixation

The third constant feature of chromosome harvesting is fixation of the cells. This process removes water from the cells, killing and preserving them, hardening membranes and chromatin and preparing the chromosomes for the banding procedure. Banding patterns are not possible in formalin-fixed chromosomes, but the gentler 3:1 methanol–acetic acid changes the chromatin structure less, or in a different way, allowing subsequent banding. The first fixative may create turbulence at first when added to the remnant hypotonic solution. During this period of turbulence, the fixative is usually added slowly, or metaphase cells can be lost to breakage. Then the fixative is added more quickly. It is important to gently but thoroughly mix the cell pellet into suspension to prevent irreversible cell clumping. Once in the first fixative, the cells become stronger (are hardened), and subsequent fixations may be added much more quickly.

After first fixation, cells may be left to stand for some period of time to allow them to harden before additional manipulations. Cold fixative may improve chromosome morphology, and many protocols call for 1–24 hours for first fixative and/or final fixation in the refrigerator or freezer. Once cells are in the first fixative, they may be stored for days or weeks or more before slides are made. The composition of methanol–acetic acid fixative will change with time, at first becoming more acidic and eventually becoming contaminated with acetates as a reaction takes place between the acid and the methanol; this is the reason that so many protocols require freshly made fixative. Fixative also absorbs water from the air, which diminishes its fixative properties. This deterioration of fixative also occurs in stored cell pellets. Storing fixed cell pellets at low temperatures

decreases the fixative deterioration, and the cells are preserved better for longer periods. The Mayo Clinic Cytogenetics Laboratory is able to use very old cell pellets (up to 8 years) from bone marrow and phytohemagglutinin (PHA)-stimulated blood cultures which were kept in a -70 °C freezer in a 1.8-mL cryotube.

Certain types of plastic tubes are broken down by fixative, and the byproducts can ruin the cells. The experience of our laboratory and of other laboratories is that polystyrene tubes start to break down within a few days of exposure to fixative, whereas polypropylene tubes seem to resist fixative well.

For suspension-type harvests, the first fixation may be the final step in the harvest before cells are centrifuged out and slides are made, if the pellets are small, clean, and free of red cells (e.g., amniocytes, solid tumors). Additional fixation steps are required for large or red blood cell (RBC)-contaminated pellets to get rid of debris that would interfere with slide-making and banding. Cells with red cell components are usually washed with two additional changes of fixative before slides are prepared.

When slides are made, if the cells do not spread well, additional fixation steps may also be helpful, especially if there is still a brownish tinge to the supernatant from remaining, incompletely lysed red cells.

2.3.8 In situ harvesting

Cox et al. [23] described the in situ harvest method in 1974 using colonies of amniotic fluid that were grown and harvested in Petri dishes. The sides of the dish were removed and the cell growth surface was taped to a slide for microscopy. Peakman et al. [24] modified this method to growing cells on coverslips inside Petri dishes. This way the coverslips are harvested in situ ("in place") in the dishes, and removed and attached to slides for microscopy. They developed the method to deal with maternal cell contamination, but the method became very popular due to the improved turnaround time and the advantages of clonal analysis.

The advantages to harvesting cells on the growth surface (usually on a coverslip in a Petri dish or a special slide flask with removable top) include the following:

1. Harvests may be performed days earlier than with suspension harvests, improving turnaround times.
2. Cultures may grow better on the glass surface than on plastic ware. This is sometimes true for prenatal and tumor specimens; however, some specimens (e.g., very bloody amniotic fluid specimens) may prefer a flask environment because it dilutes out the RBCs and improves amniocyte contact with the culture ware.
3. Clonal analysis may be performed to rule out pseudo-mosaicism and culture artifact. There are one to three viable amniocytes per milliliter of amniotic fluid, with generally less viable cells available as the gestation advances beyond 25 weeks. Each amniocyte tends to grow out a single colony, so chromosome aberrations that are confined to part of the colony must have occurred after colony formation. Such cells are therefore spurious artifacts of culture, and may be discounted, in most cases. Aberrations that occur in the entire colony may, on the other hand, represent true mosaicism, especially if multiple colonies from multiple, independent cultures display the same whole-colony abnormality. Clonal analysis applies these ideas to form rules for studying in situ harvested cultures, examining a certain number of colonies rather than a certain number of metaphases, ignoring partial colony mosaicism.
4. Morphology of the parent colony is available for correlation with karyotypes. This may be especially useful in solid tumor studies, to distinguish fibroblastic stromal cell colonies (normal cells) from tumor colonies with unusual morphology.
5. Since they are usually inside culture ware with removable lids, in situ harvests may be automated [25] (see 2.3.13, Automatic harvesting devices and slide-making chambers/drying chambers).

Typically, in situ harvests are performed on attached cultures, since the mechanism that aspirates the harvest fluids would remove suspended cells with the discarded materials. However, the Mayo Clinic laboratory has success with an automated harvest method even with bone marrow suspension cultures, as the desired cells are settled to the bottom. About 10–20% of the bone marrow cells are removed during the harvest, but the metaphases are not selectively lost. Experiments in that laboratory suggest that significantly more bone marrow metaphases are recovered with the in situ than suspension harvests because the fragile metaphases are lost in the centrifugation steps.

Drawbacks to the in situ system include the following:

1. It is sometimes difficult to get attached cultures at optimal densities at a convenient time. Metaphase cells in the center of the colonies may not spread well so it is important to have rounded up mitotic cells around the periphery of the colonies. There is often a 2-day window in the culture duration where conditions are optimal. It is important, however, not to harvest all cultures on the same day for quality control.
2. The coverslip is delicate and can be broken or dropped face-down, scratching the cells.

3. There may be a higher chance of mixing up cultures because the coverslips themselves are usually not labeled. Many protocols require labeling the bottom of the Petri dish because the top can be switched. Some laboratories have been able to label the coverslip using a diamond-tip or other marking pen. Slide flasks (flaskettes) are more easily labeled than coverslips, and can be labeled both on the plastic flaskette top and on the label of the bottom, which will become the microscope slide when the harvest is complete and the top is removed.
4. Metaphase quality is limited by the procedure: only one attempt per culture can be made to spread cells and optimize chromosome morphology. Often, no backup culture for a specific coverslip is available if the harvest or spreading maneuvers are unsuccessful. Most laboratories have a mechanism for a backup suspension culture in case of need for FISH, extra counts, etc., using either cells from the Petri dish surface that remain after the coverslip has been removed, or a separate flask culture set up at the same time as the dishes.
5. Small Petri dishes can dry out faster than flasks in low humidity situations.

If growth is too dense when checking for harvest, some culture coverslips can be trypsinized or scraped so that there is sufficient surface for new growth, and then fed with fresh media. If time permits, the culture can rest before being inoculated with an overnight mitotic inhibitor for harvest the next morning, or left in culture until the next morning, when it would then be evaluated for harvest. Because colonies have been disrupted, and lifted cells could have replanted at a secondary location, the resulting culture can be used for cell counts, as in the flask method, but not for colony counts.

For poor-growing or at-risk cultures, it is important to check for colony growth around the inner periphery of the dish, off the coverslip surface, before adding Colcemid®. If there is any colony growth off the coverslip that is not an extension from a colony on the edge of the coverslip, the coverslip can carefully be removed to a new Petri dish for harvesting. The colonies growing off the coverslip in the original vessel can be trypsinized and replanted onto a new coverslip or flask, and be used as a backup for counts, if needed. Even though these cells can no longer add to the colony count, the number of colonies that were present before trypsinization should still be noted in the specimen worksheet.

Routine setup of a backup flask culture (*in situ* or flask) is a good policy, both as an insurance policy against harvest problems and for other uses, such as sending for DNA analysis, freezing cells in liquid nitrogen, etc.

In situ harvest steps include:

1. Addition and incubation of Colcemid® or other mitotic arrest solution.
2. (a) (Optional) Removal of half of the culture medium and addition of an equal volume of hypotonic solution, and/or (b) Removal of all culture medium. Addition of hypotonic solution for a period of time.
3. Usually, addition of an amount of fixative (cold or room temperature) equal to the hypotonic for a brief incubation to help adhere the mitotic cells to the slide or coverslip.
4. Removal of hypotonic solution and (usually slow) addition of first fixative, which is allowed to stand for a few minutes or more to harden and adhere metaphases.
5. One to three additional changes of fixative to get rid of all water possible. Then the cells may be left in fixative until it is convenient to dry them for chromosome preparations.
6. After harvest, coverslips are mounted (specimen side up or down) and the slide is labeled. The coverslip may be mounted with the cells down (between slide and coverslip), or with the cells up (so that the coverslip need not be removed for sequential staining of cells).

The critical steps for the *in situ* harvesting methods are:

1. Gentle treatment of cultures and often gentle addition of hypotonic and first fixative to retain loosely attached mitotic cells, and to prevent false low mitotic index from dislodged, lost cells, or cell movement due to dislodging and reattachment elsewhere, away from the parent colony.
2. Consistency in the amount of fluid left on the culture before the next solution is added. If much culture medium is left behind, it makes the hypotonic less effective. Large amounts of residual hypotonic make the first fixation less effective at removing water. In the final drying step (equivalent to slide-making), it is essential to remove all of the fixative to prevent waves of drying fixative from dislodging cells or breaking them open.

Cell drying will be discussed later in the chapter.

A modified *in situ* harvesting method may be used for harvesting very small numbers of cells from uncultured specimens for FISH studies. For example, bladder washings to be used for interphase FISH may be concentrated by centrifugation, placed on a glass slide in a small drop of fluid such as medium or saline, and harvested on the slide by draining the drop of medium off, replacing it with a drop of hypotonic, incubating for a few minutes, adding fixative, draining off the excess fluid, and fixing cells again. Many of the cells remain on the slide if the fluid is drained carefully. Certain types of cells (e.g., cerebrospinal fluid) may also be prepared for interphase FISH in a similar fashion, omitting the hypotonic step, and pre-treating the slide preparations with protease such as pepsin in order to remove the protein from the cytoplasm.

2.3.9 Chromosome anticontraction methods

As chromosomes progress through the stages of interphase and mitotic prophase toward metaphase, they condense from long, string-like structures into shorter and shorter bodies. As they condense, the banding patterns coalesce, with sub-bands merging into bands and major bands merging (see Figure 2.9). This serves to package the chromatin into neat, small units for distribution to the two daughter cells, where they return to the decondensed state as soon as possible. Most metabolic functions take place in interphase.

In the early 1970s, cytogenetic analysis was usually performed on mid-metaphase chromosomes that contained 300 or 400 bands per haploid set. In 1976, the first of several techniques to obtain elongated chromosomes with 500–2000 bands per haploid set was reported by Yunis [11]. The technique involved increasing the proportion of late prophase and early metaphase cells by synchronizing the cells with an amethopterin (methotrexate) block during synthesis, followed by a thymidine release and much lower Colcemid® concentrations than had been traditional until that time. Released cells in synchrony can then proceed from the S stage of the cell cycle into early metaphase, when they are harvested.

This technique led to the discovery of several very small chromosome deletion syndromes [12–16,26–29] and was useful in defining the exact nature and breakpoints of other chromosome aberrations. The subsequently developed techniques with varying synchrony chemicals and DNA-intercalating anticontractants were also used to improve mitotic index, chromosome morphology, and banding quality in all stages of metaphase for blood, bone marrow, solid tumor culture, amniotic fluid, and CVS cultures. Yunis and colleagues [30,31] and some others have used the techniques to find new chromosome abnormalities in leukemic bone marrow specimens and other human neoplasms [32,33]. Variations of the synchrony method were introduced by several authors [31,34–37], and chemical additives to prevent chromosome contraction, used alone or in combination with synchrony, were developed [34,37–44]. Techniques for all types of specimens were consequently developed using shorter exposures to lower concentrations of mitotic-arresting solutions. Most laboratories now offer prometaphase, prophase, or both types of chromosome analysis for blood cultures. This produced familiarity with and appreciation for the long chromosomes with more intricately detailed banding patterns and a trend toward using longer chromosomes in all cytogenetic studies resulted.

There are two main ways to utilize chromosome anticontraction methodologies: to obtain a high-quality regular study (e.g., bone marrow, amniotic fluid, and tumor cultures) or to obtain prophase and prometaphase chromosomes (Prader–Willi, Angelman, DiGeorge, Miller–Dieker syndromes) (Figure 2.10a,b). Our laboratory has also used a synchrony method to enrich solid tumor cultures for abnormal cells.

For focused prophase and prometaphase studies, where specific chromosome regions are being questioned by the clinician, the time required to complete the study is not much longer than for a regular study, if the preparation quality is good. Often, however, the study is not focused, and each chromosome pair must be examined on uncrossed, unobscured homologues. It is best to analyze a few relatively shorter cells because some inversions and deletions may actually present better on the shorter chromosomes (Figure 2.11).

The quality of very long chromosomes is dependent on good slide-making and staining methods and optimal photography or imaging. The most common technical problems are slide-making issues, overtrypsinization, overstaining, unsharp image, poor Köhler illumination (see Chapter 14, Köhler illumination), use of the wrong contrast photographic paper or film, or computer imaging problems.

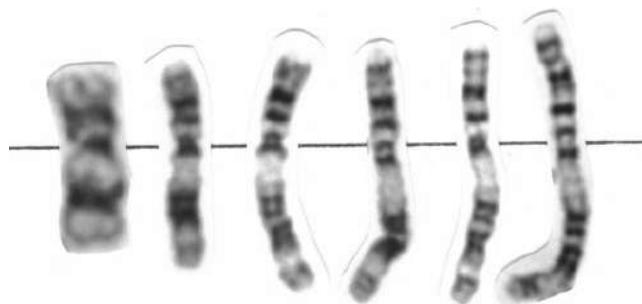


Figure 2.9 Forming metaphase bands. G-banded chromosome 11 at various band levels to illustrate how prophase and prometaphase bands (right) coalesce to form metaphase bands (left).

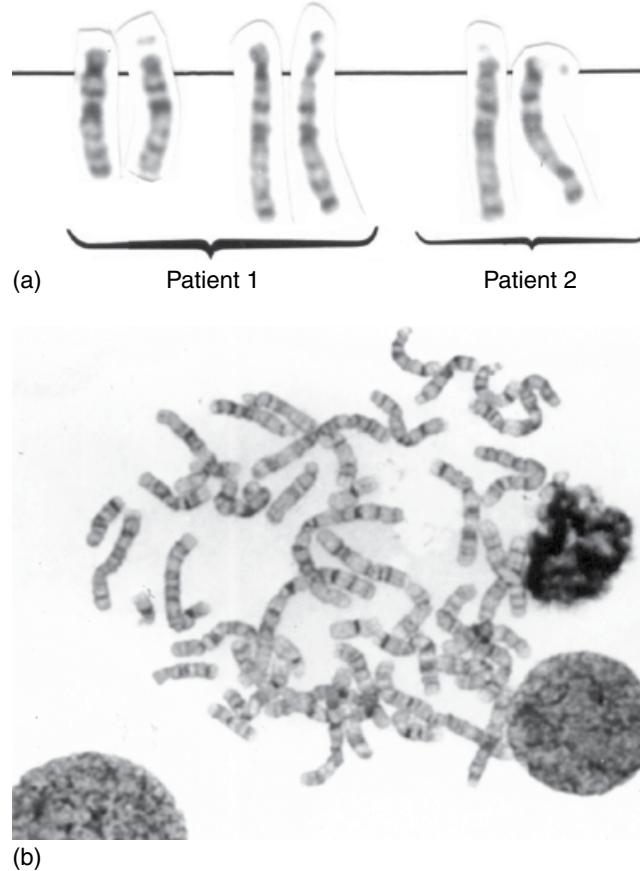


Figure 2.10 Two uses of anticontraction chemicals. (a) To detect microdeletions and other small aberrations: two patients with Prader-Willi (deletion of chromosome 15) were used. *Patient 1* (left-hand pair): Band-level resolution is not high enough to determine the presence or extent of the deletion for chromosome 15. *Patient 1* (right-hand pair): Chromosomes 15 from the same patient are at a high enough band level to determine the presence of a deletion at band 15q11.2. *Patient 2*: This deletion is from band 15q11.2 through the proximal q13 band (called q13.1). Note: Deleted chromosomes for both patients are placed on the right. Both deletions were confirmed with FISH. (b) A second use of anticontraction chemicals is used to obtain a higher quality preparation in samples that are normally poor, such as bone marrow and tumor specimens. This abnormal bone marrow was harvested with ethidium bromide and shows improved quality.



Figure 2.11 Advantages of short chromosomes. Relatively short chromosomes are also helpful in certain situations. (a) A pericentric inversion of this chromosome 6 (left) at the 700 band level is difficult to visualize because the centromere is not strongly constricted; whereas at the 550 band level (right), it is much easier to visualize. (b) Smith Magenis syndrome is caused by a small deletion in the proximal short arm of chromosome 17. At higher band levels (left), the abnormality seems to be more subtle than at shorter levels (right). This deletion was confirmed with FISH.

There are several methods of determining how many bands per haploid set (BPHS) a cell contains [45–50]. Band counting is still quite subjective, however, and does not give information on how crisp the patterns are, how many overlapped and bent chromosomes are present, or whether the cell displays the abnormalities in question. Most laboratories report the band level of the karyotypes in the final report, which is very important because it gives some idea of the degree to which the study answered the questions of the referring physician. Late metaphase chromosomes are at <400 BPHS level, mid-metaphase is 400–549 BPHS, early metaphase is 550–699 BPHS, late prometaphase is about 700–849 BPHS, early prometaphase is about 850–999 BPHS, and prophase is at or more than 1000 BPHS [47] (Figure 2.12). Usually, in our laboratory, neoplastic

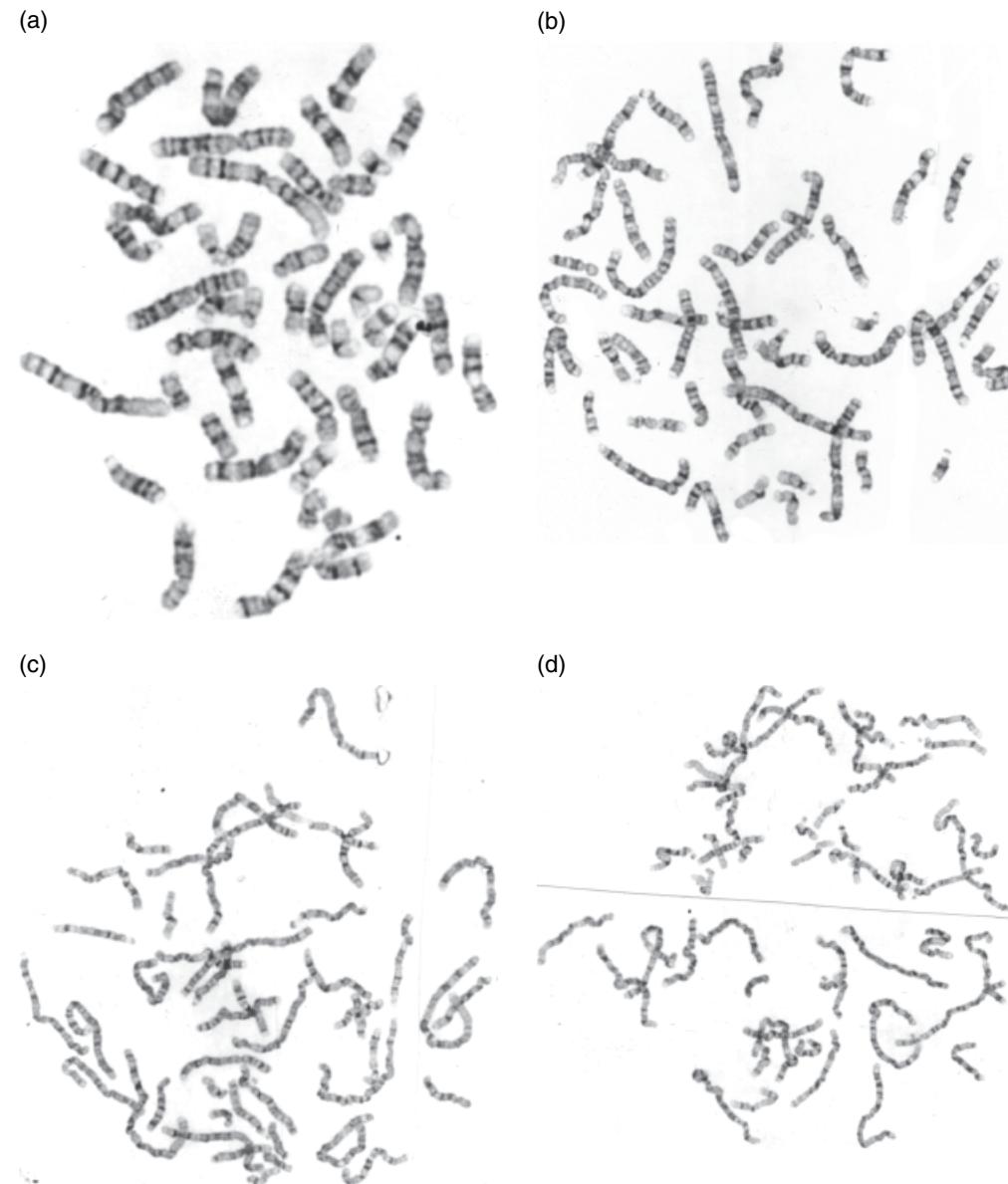


Figure 2.12 Metaphase cells at various band levels. (a) About 400–550 bands per haploid set (BPHS). This length is acceptable for certain types of studies, such as malignancies. (b) About 550–700 BPHS. This is a level most people feel comfortable with for analyzing cells at the microscope, and most abnormalities will be evident at this stage. (c,d) 700–850 BPHS is generally suitable for microdeletions greater than 3~5 Mb, and other small aberrations.

Table 2.5 Chemical agents used to produce cell synchrony

S-block agent	Release agent
Amethopterin (methotrexate), 10^{-7} M, 16–18 hours ^a (MW = 454.4)	Thymidine, 10^{-5} M, 5–6 hours ^a (MW = 242.2), OR BrdU, 12 µg/mL, 5.5 hours ^b
5-fluorodeoxyuridine (FdUrD or FUdR) 0.1 µM (bone marrows) ^c 3.3×10^{-7} M, 16–18 hours ^d (MW = 246.2)	Thymidine 3.3×10^{-3} M ^c 3.3×10^{-5} M, 5 hours ^d (MW = 242.2), OR BrdU as for amethopterin
Thymidine excess 300 µg/mL 16–18 hours ^e	2-deoxycytidine, 10 µM, 4 hours 15 min ^f (MW = 227.2) OR BrdU 50 µg/mL, 5–8 hours ^e , OR just rinse and feed OR no rinse or release ^e
BrdU excess 10 µg/mL, 15–17 hours ^g 200 µg/mL, 15–17 hours ^h	Thymidine, 0.3 g/mL, 6–7 hours OR just rinse and feed 6 hours ^c

^a From Reference 11; ^b Reference 40; ^c Reference 52; ^d Reference 52; ^e Reference 53; ^f Reference 37; ^g Reference 41; ^h Reference 35; ⁱ Reference 56.

specimens are in the late to mid metaphase range, amniotic fluid, and fibroblast cultures are in the early metaphase to prometaphase range, and bloods vary from early metaphase to early prometaphase. This variability reflects the quality that each type of tissue is capable of attaining as a result of intrinsic chromosome characteristics in various cell types [17]. Tumors and CVS cultures, for example, often seem to have short chromosomes under the most ideal conditions. If the resolution is unsatisfactory, it may be necessary to repeat culturing and analysis.

There are two basic methods of obtaining prometaphase and prophase chromosomes: (i) cell synchrony and (ii) additives to prevent contraction. They may be combined into still other permutations of synchrony and additive methods that may be synergistic. In theory, the synchrony method works by stopping cells in synthesis, collecting a large population of cells ready to begin division together. When the cells arrive at an early stage of metaphase together, they are harvested and put on slides. By timing the release period properly, one can obtain a large number of early metaphase and prophase cells.

The chemicals used to block synthesis include amethopterin (methotrexate), 5-fluorodeoxyuridine (abbreviated FdU, FUdR, or FdUrd), 5-bromo-2'-deoxyuridine excess (abbreviated as BrdU, BUdR, or BrdUrd), or thymidine excess. These chemicals are added to exponentially growing cells (e.g., 2–3-day stimulated blood cultures, bone marrow the first or second day of culture, or young primary or early subcultured monolayer cultures.) The block is released with thymidine (for methotrexate or BrdU excess type blocks), BrdU (for methotrexate), or 2-deoxycytidine (for thymidine excess) (Table 2.5). Drouin et al. [51] report that release time is about 30 minutes longer for BrdU than for thymidine.

2.3.10 Mechanism of action of synchrony chemicals

Amethopterin (methotrexate) interrupts the purine pathway by inhibiting dihydrofolate reductase, which interrupts the purine pathway. Tetrahydrofolate, the active form of folic acid, is a coenzyme that carries one-carbon groups for transfer reactions, such as synthesis of amino acids, purines, and thymidine. This includes the conversion of dUMP to dTMP via thymidylate synthetase. The removal of amethopterin and the addition of thymidine allow synthesis of dTMP via a thymidine kinase in the salvage pathway. 5-Fluorodeoxyuridine is also used to synchronize cells and has an effect similar to that of amethopterin, acting as an antagonist to thymidylate synthetase. Thymidine releases FdU cell blocks in the same way that it releases amethopterin blocks. Webber and Garson [52] report using FdU to synchronize bone marrow cultures, and Gibas et al. [53] developed a 24-hour CVS technique using FdU synchrony to improve chromosome morphology.

BrdU, an analog of thymidine, can also be used to release cells from amethopterin-type blocks. It will produce a greater number of bands, because it also inhibits chromosome condensation [43], and it can be used to produce RBA reverse chromosome bands in the preparation [35,54]. However, Yunis [33] reports that using BrdU as a releasing agent can select against some cancer cells and in high concentrations (>10 µg/mL) can produce differential stretching of chromosomes, which leads to artifactual homologue discrepancies. Numerous other methods produce synchrony [51] (see Table 2.4).

Excess thymidine inhibits DNA synthesis by feedback effects on the synthesis of other nucleotide precursors.

Excess thymidine inhibits DNA synthesis by feedback effects on the synthesis of other nucleotide precursors. These blocks can be released by washing cells and reculturing with ordinary medium, by adding the nucleoside 2-deoxycytidine [52], or by washing and incubating in BrdU. The advantage to this last method is that it requires no toxic chemicals. The method Sutherland reported for inducing fragile X with excess thymidine and no release [55] has also been adapted for obtaining longer chromosomes [56]. Excess BrdU probably works like an excess of its analog, thymidine. Because the block is not complete, some laboratories use the high thymidine method with no release step, and subsequent harvests may have reduced mitotic indices but long chromosomes. Also, releasing the block by washing off excess BrdU and incubating in normal medium can produce G-bands and R-bands in the same preparation with acridine orange staining or FPG (fluorescence plus Giemsa) methods [35]. Eichenbaum and Krumins [41] use this technique for amniotic fluid cultures. Cold synchrony has also been used for blood and bone marrow preparations [57]. Yu et al. [58] use a cold synchrony for 24-hour CVS preparations. Sha et al. [36] use a one-step S-block method for prenatal diagnosis in amniotic fluid cultures using a 3-hour pre-harvest exposure to high concentrations of BrdU, thymidine, and Colcemid® (200 µg, 0.3 µg, and 0.25 µg/mL, respectively).

The timing of the block is usually 16–20 hours. Less time may not yield enough blocked cells, and more time may cause cell death owing to the prolonged absence of thymine [59]. At the end of the S block, the old procedure was to centrifuge and wash cells in medium, and then reculture the cells in medium supplemented with agents that would facilitate release, such as thymidine. We do not find the washing steps critical, and Barnes and Maltby [60] report a similar observation. Lew [61] reports a release method for amethopterin-blocked cells in which thymidine is simply added to the blocked cultures. This is the thymidine release method our laboratory uses. Webber and Garson [52] report that washing after FdU synchrony is unnecessary. Wheater and Roberts [37] report that a thymidine block may be released by simple addition of 2-deoxycytidine, without washing. They believe that the washing steps can adversely affect mitotic index and chromosome length. It is important that medium used to reculture blood cells not have phytohemagglutinin because PHA is unnecessary at this point in the culture and can cause red cell agglutination.

Note that cells are arrested at whatever point they were stopped in the 6- to 9-hour synthesis period. Holmquist and Motara [19] report that the block seems to occur at the boundary between the early- and late-replicating chromosome synthesis periods, whereas Camargo and Cervenka [62] and Richardson et al. [63] report that arrest occurs at the late G/G2 stage. Drouin et al. [51] report that methotrexate, high thymidine concentration, and FdU may all cause accumulation of cells both at the G1/S interphase, and at the R/G transition (point at which R-positive bands stop replication and G-positive bands begin replication, about one half to two thirds of the way through the S phase). When released, they must go through the remainder of that S period and then continue through the G2 period, which takes another 2–5 hours. This variation in the arrest point explains why cells are not more precisely synchronized.

High thymidine concentrations do not prevent entry into S phase, but they inhibit DNA synthesis and prolong the S-phase period. Different cell types have completely different S and G2 times, so that solid tumor cells will require different (usually longer) release times than blood cultures. For blood cultures in our laboratory, we use 4.5 hours release time, which is shorter than what we used several years ago with a cooler incubator (37°C versus 37.5°C). Blood culture release time varies from 4.5 hours to 5 hours and 10 minutes [64]. In our laboratory, solid tumor cultures are released for 6–7 hours, with Colcemid® added for the final 2–4 hours. Morris and Fitzgerald [65] report that leukemic marrows have different cell cycles than normal hematologic specimens and that release times for these marrows may be disease- and patient-specific. Webber and Garson [52] recommend 7–8 hours release time for leukemic marrow cultures, and Gibas et al. [53] use a 7-hour release time for FdU-synchronized CVS samples. Barnes and Maltby [60] suggest that culture variables, such as serum type, may affect cell cycle time.

There are reports of synchrony of solid tumor [33,65] and CVS 24-hour cultures [53], but synchrony is underutilized for many monolayer-type cultures. We have seen in our laboratory two testicular stromal tumors with clonal abnormalities found only in synchronized cultures. The finding of abnormal clones only in synchronized cultures has been true in our laboratory for other tumors as well; the synchrony method reveals abnormal clones that are not as common or are not present in other harvests. There may be some abnormal periodicity in the abnormal cells, or the synchrony may simply enrich the culture for abnormal cells by collecting them all night during the block.

One disadvantage of synchrony techniques is the fact that cultures to be harvested on Monday must be synchronized on Sunday afternoon or evening, when many clinical laboratories are not open. By varying bone marrow and blood culture setup times and blood culturing periods (72–96 hours), most technologists can accommodate synchronized samples in the work schedule. Blood cultures may be stored at refrigerator or room temperature for a day or more before culturing. Bone marrow or suspension tumor specimens are best stored in culture medium in the refrigerator until the day before harvest and then cultured overnight with amethopterin or another synchrony agent. Our laboratory makes use of an on-call technologist for the weekend, and one of the duties of the technologist is to add amethopterin on Sunday to cultures that require synchronization.

Yunis [33] and Webber and Garson [52] suggest that for best results, bone marrow cells should be incubated for 3–8 hours before the addition of amethopterin so that the cells tolerate the blocking agent better. This is not always practical, however,

and we do not always follow this practice for bone marrow samples received late in the day; they are simply put in culture and immediately synchronized. We suggest performing an unsynchronized culture for all samples. This ensures results and controls against unforeseen technical problems, and it is especially important in bone marrow cultures because they tend to vary widely in cell cycle and in their response to cytotoxic agents.

The shelf life of stock solutions of amethopterin and thymidine, in our experience, seems to be almost indefinite at refrigerator temperatures. Gloves should be worn with all anticontraction chemicals, because they all interact with DNA and may be dangerous.

2.3.11 Additives to prevent chromosome contraction

Numerous chemical agents bind to or intercalate into DNA or chromatin and, when added for periods before fixation, are capable of preventing normal chromosome contraction during metaphase [35,36,38,40,42,66–72,74,75]. Some of these agents show a preference for certain areas of the chromosome. BrdU and 5-azacytidine preferentially bind to G–C-rich areas, and Hoechst 33258, DAPI, and distamycin A show a preference for A–T-rich regions. These chemicals inhibit contraction differentially rather than lengthening the chromosome homogeneously [42].

Actinomycin D (dactomycin), acridine orange, and ethidium bromide seem to bind uniformly and prevent contraction of chromosomes homogeneously, although Schollmayer et al. [54] found differential condensation using acridine orange. Because long-term cultures are considered more difficult to synchronize, ethidium bromide and actinomycin D techniques have been developed to elongate chromosomes of fibroblasts [39], pleural effusions [73], and amniotic fluid cultures [76]. Some of these agents decrease mitotic index, but this disadvantage may be offset by increased metaphase quality. Agents may be added to the culture before or at the time of addition of the mitotic inhibitor or during the hypotonic period (Table 2.6). 9-Aminoacridine has also been shown to intercalate chromatin and yield longer chromosomes than treatment with ethidium bromide [75].

Table 2.6 Chemical additives used to produce elongated chromosomes

Ref. no.	Author	Tissue type	Chemicals used	Concentration	Exposure time	Mitotic arrest
74	Dewald and Dines	Pleural effusions	AMD ^a	5 µg/mL	1 h	Last hour
43	Yunis J. ^b	Lymphocytes	AMD	5 µg/mL	1 h or more	Colcemid®, last 10 min
42	Yu et al.	Human fibroblasts	AMD	2 µg/mL	1 h	Velban 0.01 2 g/mL, last h
40	Ikeuchi T.	Lymphocytes	EB ^c	10 µg/mL	2.5 h	Colcemid®, last hour
76	Hoo et al.	Amniocytes	EB	5 µg/mL	4.5 h	Colcemid®, 0.6 µg/mL, last 1.5 hour
68	Latos-Bielenska and Hameister ^b	Lymphocytes	EB AMD AMD + EB Hoechst 33258 Hoechst 33258 + AMD Hoechst 33258 + EB Hoechst 33258 + EB + AMD	2.5 × 10 ⁻⁵ M (MW = 394.3) 2 µg/mL 2 µg/mL; 2.5 × 10 ⁻⁵ M 60 µg/mL 60 µg/mL; 2 µg/mL 60 µg/mL; 2.5 × 10 ⁻⁵ M 60 µg/mL; 2.5 × 10 ⁻⁵ M; 2 µg/mL	2 h 2 h 2 h 2 h 2 h 2 h 2 h 2 h 2 h 2 h	Colcemid®, 0.7 µg/mL, 7 min Colcemid®, 0.7 µg/mL, 7 min

^a Actinomycin D.

^b Used in combination with an amethopterin/thymidine or BrdU cell synchrony technique.

^c Ethidium bromide.

2.3.12 Combination of synchrony and additives for longer chromosomes

Several successful methods have been developed for chromosome elongation using a combination of synchrony and anti-contraction chemical additives. Yunis [33,40] reported use of amethopterin blocks followed by release with thymidine or BrdU and adds prefixation agents, such as, acridine orange or actinomycin D, to peripheral blood and tumor cell cultures. BrdU release with 1 hour or more of actinomycin D (AMD) inhibition yielded up to 2000 BPHS [43], and Yunis hypothesizes a synergistic effect between BrdU and AMD on elongation. Little or no decrease in mitotic index, no differential contraction, and few chromosome breaks are reported with this technique. Yunis prefers a high concentration of BrdU [40] in order to achieve more bands, and recommends a slightly longer release (5.5 hours). Perhaps with the use of combination techniques, the release times are less critical owing to the anti-condensation action of the added agents. Latos-Bielenska and Hameister [74] use various combination techniques (synchrony plus BrdU, Hoechst 33258, ethidium bromide, and AMD) successfully in a clinical laboratory. They recommend using several techniques for each patient to allow for patient response variables. Our laboratory uses a combination of amethopterin synchrony and ethidium bromide on peripheral blood cultures routinely with great success.

Other methods to enhance chromosome elongation and quality are harvesting manipulations, such as special hypotonic solutions (e.g., Ohnuki hypotonic and cancer hypotonic solution; Table 2.4), reduced time and concentration of Colcemid® to reduce band coalescence, and cold or higher alcohol content fixatives.

2.3.13 Automatic harvesting devices and slide-making chambers/drying chambers

The high workloads of modern cytogenetics laboratories made the automation of the harvesting and slide-making [25] steps important. Besides saving time, they also contribute to the consistency of cytogenetic preparations. There are several automated harvesting machines available, and a number of slide drying cabinets that control humidity and air temperature for optimizing the conditions for each sample type.

2.4 Slide-making

Once cells have been well-fixed in 3:1 methanol–acetic acid, they are dropped onto glass slides and dried using specific conditions for optimal chromosome spreading and morphology.

Although most of the variables for good slide-making are well understood, there is still a craft-like aspect to the procedure, making it impossible to verbalize all the necessary skills. One must simply practice until the intuitive part of the skill is mastered, and there will always be some technologists with a better feel for it. Laboratories may find slide-drying chambers both useful and cost-effective, because they allow environmental conditions to be set to specified requirements. Meanwhile, manual slide-making with adjustments for ambient conditions, whatever they may be, is still a necessary skill in many laboratories, and an understanding of manual slide-making is the basis for making slides under any circumstance.

2.4.1 History of slide-making

In the early days of slide-making, before banding methods, we were much less constrained in our methods to get chromosomes to spread out, because nonbanded chromosomes always stained well. In the 1950s and early 1960s, chromosomes were spread by squashing the cells (Belling, 1921, using plant material) [77] between slide and coverslip. If done with a stain such as aceto-orcein, a temporary wet mount preparation was obtained. Alternatively, the cells could be squashed in 50% or 60% aqueous acetic acid, and then the coverslip removed. The resulting slide could be stained with Giemsa. Some of the material, however, stuck to the cover glass, and even the elaborate methods devised to cope with this problem, such as, freezing slides on a block of dry ice or plunging the squashed, coverslipped slide in dry ice-cold methanol before popping the coverslip off with a razor blade [78], were not always helpful. Partial metaphases were the rule. The squash method was still being used for intact tissues (e.g., solid tumor direct preparations) in the late 1960s. It lost favor because the aqueous acetic acid fixatives that had to be used to soften cell membranes precluded banding patterns.

The air-drying slide-making methods we have today evolved in two steps. The first was when Rothfels and Siminovich accidentally allowed some *in situ*-harvested slides of monkey cultures to dry out by evaporation; they found that the chromosomes were spread out in a single plane without mechanical force [79]. Nowell [7], who described the use of PHA for lymphocyte culture, still used the squash method of slide preparation. It was Moorhead et al. [8], who modified Rothfels and Siminovich's air-dry techniques for lymphocyte cultures to give the slide-making method we still use four and a half decades later.

The fixative used by early workers was 3:1:1 methanol–acetic acid–chloroform and was known as Carnoy's fixative. The chloroform was found to be dispensable for cytogenetic purposes and was no longer used in fixatives after the 1960s. Three to one methanol–acetic acid is sometimes called modified Carnoy's fixative since it has no chloroform.

Early slide drying methods involved manipulations to improve spreading such as flaming cell preparations. One method was to dip slides in 95% ethanol, drop the fixed cell suspension onto the alcohol, and pass the slide through Bunsen burner or alcohol flame. Another was to drop slides in dry-ice-cooled water, remove the slide and then drop cells on the slide, ignite the fixative in a flame, and dry the slide on a hotplate. The idea of flaming is to effectively boil the cell open to spread the chromosomes. Some of these methods denature chromosomes and preclude banding, but if done very gently, flaming is still a method that can be used for certain extreme problems, such as spreading tumor cells with large ploidy numbers and refractory cytoplasm. However, in the early 1970s, to accommodate the new banding methods, most laboratorians learned to use air-drying methods instead of flaming. Slides were made by dropping the fixed cells onto wet slides and allowing them to dry by evaporation.

At this point in the development of air-drying techniques an inexplicable variability in chromosome spreading became apparent: something occasionally went wrong with the slides, with the chromosomes perhaps spreading well one day but not the next day. John Melnyk at the City of Hope in Duarte, California, was one of the first to uncover the reason. He was attempting to design an automatic slide-making machine, and he used compressed air to blow air on the cells to cause evaporation of the fixative. He found that some tanks of air worked better than others and traced the poor result to low water content in the compressed air in the tanks that had been filled with air on a dry day. When the cells dried too fast in the presence of the dry airflow, there was insufficient relaxation of the cytoplasmic membrane for the chromosomes to flatten out and move out from the center of the cell. The result was a pile of chromosomes and a crinkled, film-like membrane over them. City of Hope is located in a southern California desert environment, and the worst days for slide-making were when a dry desert wind blew.

Technologists in the early to mid-1970s were also discovering this correlation in slide-making with humidity, often from the other perspective. In the eastern, southern, and midwestern regions of the country, the humidity became so high at times that the cells dried too slowly, causing poor banding, tight cells, cell loss due to scattering chromosomes, and other difficulties. Consequently, various methods were developed to improve air-drying methods for slide-making, and most of these deal with relative humidity, which is linked to air temperature. (Warmer air is capable of holding more moisture than cold air. Thus, relative humidity is not a straight percentage but rather is the percentage of water present in the air compared to what air at that temperature is capable of holding.)

These correlations have been explored in controlled experiments by Spurbeck et al. [80], who measured cell spreading (cell volume) in relation to increasing humidity and temperatures, and found a positive correlation between increased humidity and spreading at a set temperature, up to a certain threshold. Beyond a certain optimal humidity, cells begin to lyse, spilling chromosomes out, and only the resistant, small (tightly spread) cells remain intact. This fits well with the observations made by technologists on a more intuitive level. The authors also reported that warmer air yields more spreading due to the ability of warm air to hold more moisture. However, warm air created in winter by building heating systems will drive moisture out of the air, and therefore, these warm air conditions will usually yield less spreading.

2.4.2 Theory of slide-making

Air drying of cells that are fixed in 3:1 methanol–acetic acid is based on the theory that chromosomes, which are contained in cells that are much enlarged and have much thinner cell membranes than before the harvest, will be supported by the layer of fixative on the slide in the first few seconds after application to the slide. Then, as the fixative evaporates, the layer of fixative becomes thinner and the meniscus pushes down on the top of the cell, enlarging the area of the cell and pressing the metaphase chromosomes between the upper and lower membranes, spreading them out (see Figures 2.13 and 2.14). This physical relaxation and collapse of the cell membrane takes some time, but this slow process stretches the chromosomes, thus determining to some extent what the banding level will be [81]. If the fixative dries before the cytoplasm has relaxed and cells have spread out, there will be a visible cytoplasmic background and the cells and chromosomes will be thicker (darker on the phase contrast microscope and after staining), shorter, and often poorly spread out [80]. The thick cytoplasmic covering will usually prevent good staining or *in situ* hybridization, and the stained chromosome morphology will be unsatisfactory in many cases (see Figure 2.15). Well-spread metaphases also have a cytoplasmic membrane intact over the cells, but it is very thin and requires special stains to visualize, such as 0.2% fast green [17]. If drying takes a very long time, the weakened cytoplasmic membrane may develop holes or may rip open, spilling some or all of the chromosomes out and losing the metaphase to analysis or causing pseudohypodiploidy (see Figure 2.13). On such preparations, it is not uncommon to see cells that are poorly spread, as well. This may be due to the rolling of the cells in the layer of fixative, which has currents and other forces causing cell motion. A cell that is rolling at the time of final drying has not relaxed and will appear tightly spread [80] (see Figure 2.13). This theory has been systematically developed, explored, and supported by the experiments of Spurbeck et al. [80]. The cell rolling can explain occasions in which the opposite occurs: very high humidity may sometimes yield a cytoplasmic background that can be alleviated by decreasing drying time (our technologists call this a “backward day”). Low humidity has been reported to cause scattered chromosomes [82], an exception to the paradigm. We conclude that slide-making is an empirical

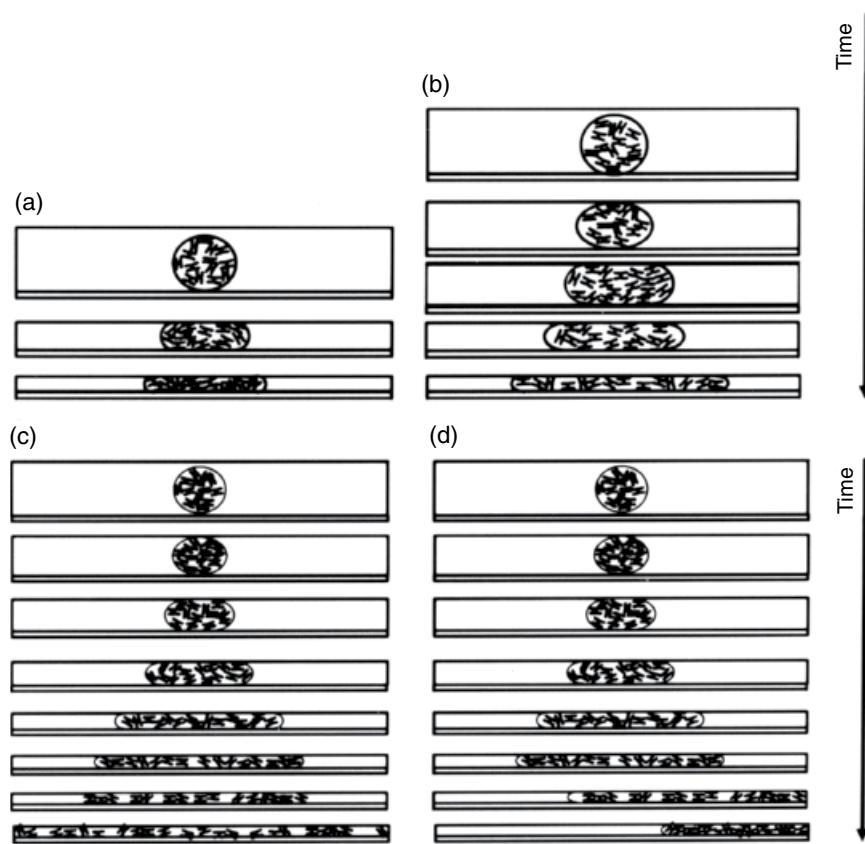


Figure 2.13 Chromosome spreading and drying time. Metaphases spread as a function of the duration of drying time. This figure illustrates a side view where the top of the fixative solution is represented by a single line, and the slide surface is represented by a double line. Time is represented from top to bottom. (a) Metaphases that dry too fast are often tight with many overlapping chromosomes. (b) Metaphases that dry at the optimum rate (for a top view, see Figure 2.14) have few overlaps and are not broken. (c) Metaphases that dry too slowly are characterized by both broken metaphases and (d) by tight, “rolled” metaphases. Courtesy of Jack Spurbeck. Reprinted from Spurbeck J. Dynamics of chromosome spreading. In: Spurbeck JL, Zinmeister AR, Meyer KJ, Jalal SM. *Am J Hum Genet* 1996; 387–393, © 1996, John Wiley and Sons, with permission.

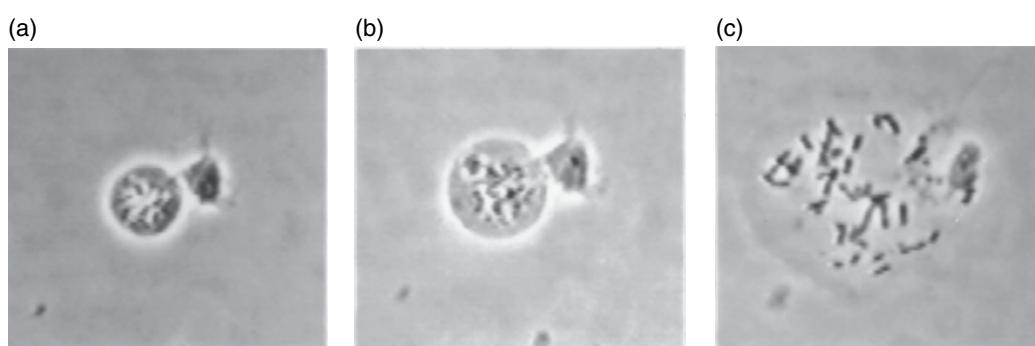


Figure 2.14 An amniocyte cell during the drying process. Videotape images of an amniocyte during the drying process at early (a), intermediate (b), and near-optimum (c) stages. Courtesy of Jack Spurbeck. Reprinted from Spurbeck J. Dynamics of chromosome spreading. In: Spurbeck JL, Zinmeister AR, Meyer KJ, Jalal SM. *Am J Hum Genet* 1996; 387–393, © 1996, John Wiley and Sons, with permission.

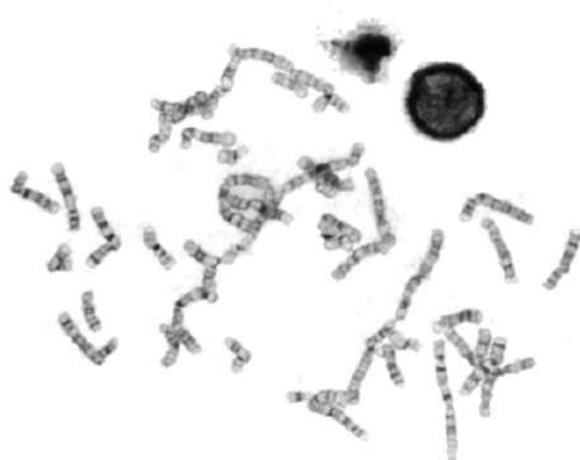


Figure 2.15 G-banded metaphase from poor slide-making. This picture demonstrates a G-banded metaphase that dried too fast and is encapsulated in the cytoplasmic membrane. Chromosomes did not have time to spread out completely, and trypsin digestion is uneven due to interference by proteins in the thick membrane over the chromosomes.

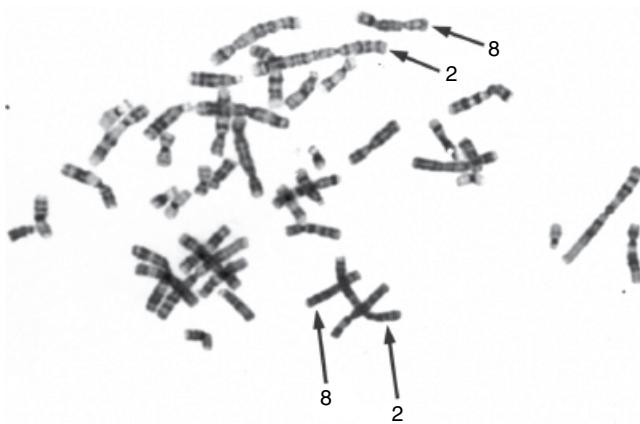


Figure 2.16 Differential drying. One side of the cell has dried at an optimal rate, and the other side has dried too fast, creating more contraction of chromosomes on that side. Note, for example, the two chromosomes 2 and the two 8s (arrows).

and qualitative skill, at least by today's level of understanding. There may be effects by other environmental factors yet unknown. Barometric pressure is probably not one of these factors, because Spurbeck et al. did test for barometric pressure effects, and did not find it to be an important variable (in the range of 29.74–30.03 inches of mercury). Claussen et al. [83] did some work on fixed cells drying on slides under different atmospheric conditions, and found that spreading involves significant water-induced swelling of mitotic cells during evaporation of the fixative from the slide.

Once the methanol–acetic acid-fixed chromosomes are dried onto a glass slide, they stick fast until physically scraped off, and staining may be accomplished without losing cells from the slides. Further changes in spreading are not possible after cells dry, although Claussen et al. [84] has discovered that chromosomes may be stretched with a micromanipulator to exhibit more bands. Cells fixed in other fixatives, such as formalin-fixed paraffin-embedded cells, do not stick to glass upon drying unless the glass is treated by coating (e.g., silanizing) or the glass is positively charged. Such preparations are often used for interphase FISH studies.

The length of chromosomes is somewhat affected by slide drying, as evidenced by cells that have dried differentially, with various band levels on different sides of the cell (see Figure 2.16). Differential staining is usually due to differential drying

speeds on opposite sides of the cell, and can be caused by debris nearby, uneven heating, water remaining in the vicinity of the cell, oil on the slide, or draw lines on the microscope slide created in the manufacture of the glass, among other variables. Drying can also differ from the inside to the outside of the cell for the same reasons. Unstained chromosomes can be viewed using a phase contrast microscope, and most slide-making protocols call for an assessment of spreading and chromosome spreading, morphology, and contrast using such a microscope, or using an ordinary light microscope with the condenser adjusted to view shadows of the unstained cells. Chromosomes may appear gray to black, depending on drying conditions, and the resulting banding quality will reflect the contrast seen in the unstained preparations [85] (Figure 2.17). We feel that this phase contrast microscopic assessment is one of the most important parts of the harvesting process.

The goal of the slide maker is to obtain chromosome slide preparations that have the following characteristics on the phase microscope:

1. Mostly medium gray to dark gray chromosomes. Light gray chromosomes yield trypsin-sensitive cells with poor contrast between bands, and very black phase contrast may have increased cytoplasmic background and be resistant to trypsin, although these banding guidelines may be different in some laboratories.
2. Little chromosome scattering (overspread, broken metaphases) because this can cause diagnostic problems when mosaicism is suspected or can mask true mosaicism at a low percentage.
3. Minimal number of chromosome overlaps, for accurate counting and band analysis.
4. Very thin or absent cytoplasmic background, if possible, so that trypsin G-banding will be optimal (see Figures 2.12 and 2.14).
5. Proper cell density on the slide: too few cells on slides can result in preparations that are time consuming to scan for metaphases. Slides that are too dense may interfere with spreading and G-banding, and FISH preparations from these slides may be difficult to interpret due to crowded signals and confusion as to which cells have been scored in a given field.
6. Chromatids that are together, not split apart. This may also be a function of the Colcemid® and hypotonic steps.
7. Absence of debris, such as glass chips from pipettes or slides, glove powder, diatoms from pipette bulbs, or bits of tissue from explants, which will interfere with proper slide drying.

2.4.3 Slide-making variables

Normally, on a good day, one should be able to drop fixed cells onto a clean, wet slide at a 20–30° angle lengthwise on a paper towel, drain the excess water off, maybe flood the slide with a little fixative to improve water removal and produce a consistent drying surface, and dry the slide flat or at an angle to get good preparations. If this does not do the trick, manipulations to improve spreading and morphology are numerous, and include the following variables. (Note: if an entire harvest yields consistently poor quality preparations, regardless of specimen type or environmental conditions, there may be a problem with the culturing or harvesting conditions. This could include too large an inoculum of cells in the culture, or hypotonic or fixative that was made incorrectly or contaminated with some other chemicals.)

2.4.4 Wet versus dry slides

Some laboratories prefer to drop fixed cells on wet slides, which facilitates spreading due to the immediate retraction of the water meniscus as soon as the acid alcohol hits it. Holmquist and Motara [19] report that by dropping cells onto wet slides, the energy of dehydration from fixation is returned as a change in the free energy of mixing between fixative and water, which spreads the cells. Others prefer to use dry slides, and under good environmental conditions, this method should work [80]. If wet slides are used, the retreating water should be eliminated by immediate draining onto paper towels, blotting with KimWipes, and flooding with fixative. Residual water spots cause spreading and staining irregularities due to localized drying time variations. For coverslips in dishes, it is crucial to completely dry the edges of the coverslip by diligent withdrawal of residual fixative. This ensures a consistent drying milieu across the coverslip and reduces cell breakage from waves of drying fixative.

Wet slides may further facilitate spreading control by the use of different temperatures of water coating to speed up or slow down drying time. Cold, wet slides will slow drying, increasing spreading, whereas higher room temperature or warm wet slides will accelerate drying time. The thickness of the water film may be varied by draining the slide on a paper towel more or less thoroughly, and this can help control spreading by using a thicker film of water on very dry days. Also, extremely fragile metaphases, such as acute lymphocytic leukemia bone marrow specimens, may yield more metaphases with very thin water layers, or even just by coating slides with moisture from the breath.

A method to ensure complete removal of water from the slide and uniform drying across the slide is to flood the slide with a few drops of fixative after the cells have been applied to the wet surface. This also may aid spreading by exerting pressure on the upper cell membrane to encourage the flattening or relaxation of the membrane to cause more spreading. Some laboratorians use slides that have been coated with fixative instead of water.

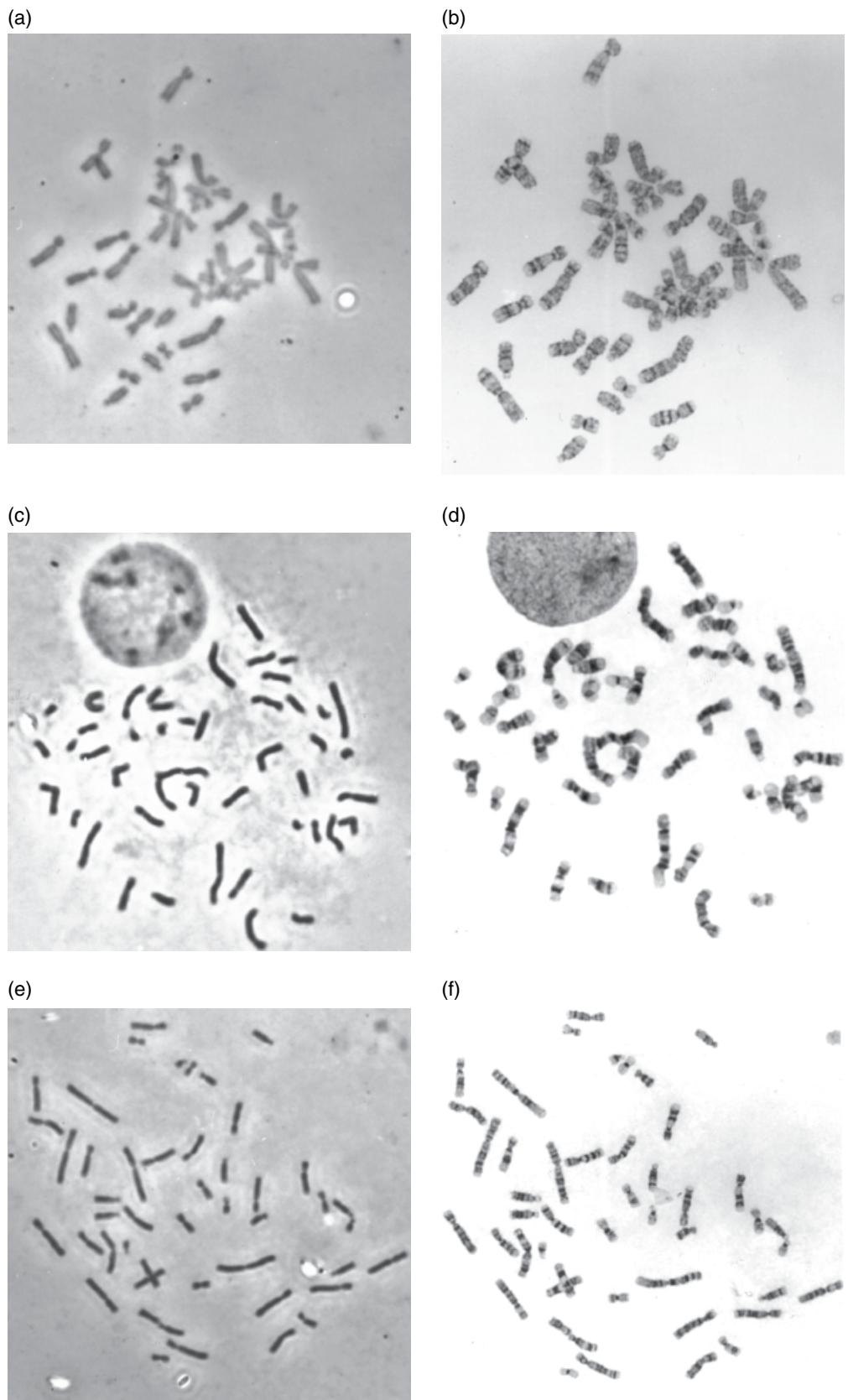


Figure 2.17 G-band quality comparison. Three cells are shown with different phase contrast images and their subsequent G-banding quality. (a) Too gray on phase contrast image. (b) Subsequent G-bands also lack contrast. (c) Too dark on phase contrast. (d) Subsequent G-bands are harsh, with high contrast. (e) Nice, medium dark cell on phase contrast. (f) Subsequent cell shows good contrast and chromosome morphology.

2.4.5 Angle of the slide

Some technologists drop cells onto horizontal slides, but they are in the minority. Most technologists allow the cell suspension to travel after it hits the slide, by angling either along the long axis (e.g., label end up or down) or along the short axis (e.g., one long edge down). Spreading may improve when cells travel and roll as the fixative level first becomes thin, although this is undocumented and does not apply to *in situ* harvest slide drying. The effect of dropping cells on slides angled along the long axis is that the water and fixative drain from the upper end of the slide to the lower end and pool there; this may cause faster evaporation at the upper end and drying may be uneven, both between cells at each end and within cells, giving different banding responses to trypsin from cell to cell and even within a metaphase (see Figure 2.15). Slides made with one long edge down tend to be more uniform, especially if the angle is kept at 20–30°, and cells are placed in the upper one-third of the slide and allowed to move downward. Cell suspension drops should not pool at one edge of the slide but should be in uniform circles of spreading that are about 20 mm in diameter and well centered on the short axis. Greater tilt angles may speed up the drying process at the upper end of the slide compared to the lower end, giving a somewhat uneven, inconsistent slide. Uniform drying can be achieved by tilting slides at one angle for part of the drying time and another for the final drying. Changing the angle of the slide and rapping the slide edge on the bench top are said to improve cell spreading for some technologists.

2.4.6 Ambient humidity and temperature

It is important to remember that relative humidity is the percentage of moisture actually in the air compared to its maximum potential saturation at that temperature; therefore, since warm air is able to hold more moisture than cold air, a relative humidity value of 50% means a much lower absolute moisture content for cold air than for warm air. Thus, slides spread differently according to air temperature, given the same relative humidity. Higher ambient temperature usually (but not always) translates into better spreading. For drying cells from *in situ* harvests, it is humidity that affects results over all other variables, with best results obtained at 5–10% higher humidity than that which would be ideal for suspension harvests. In dry air, fixative evaporates fast, whereas in moist air, evaporation occurs slowly. Neither fast nor slow evaporation is ideal, because either can result in poor phase contrast and spreading, scattered chromosomes, or visible cytoplasmic background around the metaphase. Drying time should be roughly 30–45 seconds, based on our experience. In dry climates, it is necessary to slow drying times, and in humid climates, to speed them up. Some ways to provide humidity are to dry slides in front of a humidifier, on top of wet paper towels, or over steam in a sink. In very humid, damp climates, a technique that encourages drying is needed, such as the use of 6:1 methanol-acetic acid fixative, as reported by Yunis et al. [64]. Note that his work was performed in Minnesota, where humid summers are not uncommon. Some areas of the country experience drastic climate changes during the year, and the technologist must become adept at handling all types of conditions. The most difficult situation is accommodating for rapidly changing weather conditions when making slides. Consistency is very difficult in such circumstances, and it may be best to leave slide-making until conditions stabilize.

The ideal relative humidity for slide-making can vary owing to differences in temperature, hygrometer accuracy, technique, and specimen type. In our experience, harvests from attached monolayer cultures tend to have more fastidious moisture requirements for good drying than the blood and bone marrow specimens, and the *in situ*-harvested samples seem to spread better at high humidities (e.g., 50–55%). Lundsten and Lind [86] suggest 20°C and 45% humidity, with the optimum range between 40% and 50%. Spurbeck et al. [80] find 50% humidity and 25°C to be the optimum setpoints, and they lower the humidity to 35% if chromosome scattering is a problem. Yu et al. [58] recommend 25 seconds of drying time for high-resolution preparations. The experienced technologist can usually tell if the humidity is not optimal by simply observing the drying time of the slide and the phase contrast image that results. Laboratory technologists have found ways to deal with environmental conditions that affect slide-making. For example, laboratories in the East and Midwest often use dehumidifiers in their slide-making area to compensate for high humidity. Humidifiers are also useful in many parts of the country, especially in winter, when the air is dried by heating systems. These may range from one or two small baby room humidifiers available in department stores, to the large, commercial ultrasonic room humidifiers with humidity controls (humidistats) that automatically control humidity in entire rooms. Lundsteen and Lind [86] report the use of a specially designed “climate room” for slide-making. Another way of compensating is to have a slide-making cabinet, as it is easier to control the temperature and humidity in a small area than it is in a large room. There are commercially available slide-making cabinets that are very popular. In regions such as the American northwest, it is rare that the air is saturated with moisture as it may often be in other parts of the country, and simply warming slides and/or increasing airflow is usually sufficient to get good preparations.

Other sources of humidity are breathing on slides as if to fog the glass, which gives a different effect than blowing to create air flow; wetting a pad of paper towels on which to place the drying slides (a microclimate just above the toweling is more moist than the ambient air), or if that does not work, placing a slide box lid over the top of the drying slide on the moist towel; using wet paper towels on a hot plate; placing wet cheesecloth over air flow vents in a hood to control humidity

inside the hood; and placing wet paper towels with an inverted Petri dish over *in situ*-harvested coverslips. Several laboratories recommend leaving the back of the slide wet rather than drying off excess water droplets. It is sometimes useful to flood the drops of cells with 6:1 methanol–acetic acid instead of the usual 3:1 ratio, and this accelerates evaporation. Sometimes, on very rainy, wet days, slides can look good on phase contrast microscopy but do not G-band well, lacking contrast. Sometimes this can be cured by putting the unstained slides in the hot oven for another round of drying (aging). The temperatures which affect slide-making results are that of the ambient air, that of the slides and water with which they are coated, and that of the surface on which they are dried (hot plate versus forearm versus benchtop). By varying one or more of these temperatures, we control the speed with which evaporation takes place. We have, for example, switched from room temperature fixative and slide rinse water to cold fixative and water in very dry conditions in order to counteract the rapid evaporation. Drying *in situ* coverslips on wet paper towels with ice around them has been a common technique in many laboratories. Drying slides in a very cold or very warm room can disrupt proper cell spreading and chromosome morphology, and make banding suboptimal. It is important to have control over the temperature of the laboratory. Hansen [87] recommends changing the temperature of the water that the slides are coated with before dropping cells on the slides to help control spreading. She uses water from 20 °C to 48 °C and prefers 41 °C to 47 °C for most slides.

2.4.7 Fixative ratio

The alcohol in the fixative is a cell-hardening agent, and the acetic acid is a softening agent. By changing the ratio of these two components, the cell membrane can be strengthened or softened to get less or more spreading, respectively. Thus, some laboratories might choose to use 2:1 methanol–acetic acid instead of the usual 3:1 ratio for bone marrow cultures that are difficult to spread, or for *in situ* harvested amniocytes. Alternatively, they might put amniocytes into 6:1 methanol–acetic acid on days when scattering is a problem. We recommend a conservative approach of keeping with the standard 3:1 fixative unless all else fails, because this yields the optimum banding quality and preserves cells best. We may vary the ratio of the fixative used to flood the slide after the cells are dropped on in order to accelerate drying, on occasion. Multiple fixations can strengthen cell membranes, improve chromosome morphology, and rid the sample of debris such as lysed red cells. Overnight fixation in the refrigerator or freezer can also strengthen cells and make them tolerate slide-making better. Tumors and difficult bone marrow specimens such as ALL marrows often yield better spreading and sharper chromosomes after incubation in the freezer.

2.4.8 Quality and freshness of fixative

Methanol–acetic acid does two things to the cell: as the pH of the cell is lowered, most acidic groups like -COO- become -COOH, permanently denaturing them; then the fixative dehydrates the cell and replaces the water with methanol, altering proteins and DNA [18]. Very dry cells are required for good spreading. Since both methanol and acetic acid are hygroscopic (absorb water from the air), the two components can be contaminated by water in the reagent bottles. The mixture also absorbs water upon standing.

Water contamination in fixative will decrease the spreading and quality of the slide [17]. Also, bad lots of alcohol and acetic acid do occur occasionally. Some years ago, the manufacturer of the acetic acid we had used for years changed the plastic liner material on the bottle. The acid became contaminated with this material, becoming useless for cytogenetic purposes. It is good practice to test new lots on a small harvest before risking a large one.

2.4.9 Height from which cells are dropped

Although a study by Lambson et al. [88] showed no correlation between chromosome spreading and the height from which cells are dropped (between 0.1 and 0.31 meters), increased height is sometimes used to increase spreading. Gibas and Jackson [89] specify the use of increased heights for dropping leukemic bone marrow cells. Our laboratory uses this as a last resort on large-ploidy, tightly compacted cells from solid tumors that will not spread any other way.

2.4.10 Wicking effects

When the slide is held up in the air during cell dropping, the coating of water and fixative are accumulated at the edges of the glass slide. Water can move back onto the cell spreading area and affect drying. Placing the slide on a paper towel with the long edge parallel to the bench, and quickly draining the excess fluid can solve this problem. The slide can be brought back to horizontal and/or the opposite long edge can similarly be drained on the towel. This also seems to encourage cell spreading due to the wicking effect of the slide draining. The goal is a consistent drying surface that leads to a constant rate of drying that in turn yields the most consistent quality spreading across the slide.

2.4.11 Air flow

Cell drying speed can be increased or decreased by changing the airflow over the slide. This can be accomplished by blowing on slides, by waving them in the air, or by using a fan or, as is sometimes used in the *in situ* method, an aquarium air pump. The air pump has the added advantage of a humidifying capability, when the air is passed through a bottle of water before releasing it over the slides. Most effective slide-drying chambers make use of a small fan that can be adjusted to produce various movement rates. Be aware of air movement in the slide-making area: people whisking past or air conditioning vents may be a source of drying problems. One caveat when blowing on slides to dry them: cells from the mouth can be deposited on the slide, and cause interpretation problems with interphase FISH studies.

2.4.12 Dilution factor

A common mistake in slide-making is applying too many cells on the slide so that there are lots of metaphases from which to choose. Crowded cells do not spread and band as well as optimally diluted cells because of physical crowding and an altered drying kinetic, as well as other factors, such as extracellular RNA and protein. Cell crowding also slows drying time. If spreading is a problem, consider further diluting the specimen.

2.4.13 Slide cleaning and labeling

Slides may be used with success from the manufacturer's package, depending on how clean they are [90]; however, cleaning slides before use facilitates uniformity of spreading and increases metaphase quality and banding quality (any oil on the slide will ruin spreading). Our laboratory cleans slides by placing them in 95% ethanol just before use, wiping them with a tissue three or four times in one direction, and then dipping them back in the alcohol before coating them with water for slide dropping. Some other laboratories prefer to use detergents or combinations of detergents and alcohol cleaning. If labeling manually, be sure to use a number 3 pencil or harder. It is unproductive to clean slides and then allow pencil lead debris to dislodge from the label during staining and stick to the metaphases. Indelible marking pens are also used successfully and avoid this problem. Many laboratories have pre-made labels from the LIS for labeling slides; however, caution must be taken as with any pre-labeling system not to use them on the wrong patient. When converting to pre-printed labels, check them to make sure both the glue and printer ink are resistant to aging techniques, staining and de-oiling procedures.

2.4.14 Slide type

It is remarkable how difficult slide-making can become when changing the type or brand of slides, or when the manufacturer changes something in slide production. Find a brand that holds a uniform layer of water, without streaks or beading up, with no pits or manufacturer's "draw lines" in the glass, and it should produce decent slides. As glass slides age, they oxidize, starting around the edges. Spreading that differs from edge to center is a sign of a slide that is oxidized. Manufacturers are starting to make available to the cytogenetics community slides that are stored under nitrogen inside of foil packages to circumvent the oxidation problem.

2.4.15 Cell and culture type

In our hands, there are certain cell types that lend themselves to slide-making better than others. All uncultured, "direct" harvests (from bone marrow, CVS, or solid tumors) present more difficulty in the making of good slides than their cultured counterparts. Blood specimens tend to be the easiest from which to make good slides, and bone marrows tend to be more difficult, with amniotic fluid specimens in between. Epithelial cultures are more difficult than fibroblast cultures (e.g., certain amniotic fluid or tumor cultures) because they do not seem to swell as large in the hypotonic solutions.

2.4.16 Culturing and harvesting techniques

Culturing variables (e.g., micro versus macro blood cultures, number of bone marrow cells used for inoculum, primary versus secondary or subcultured fibroblast cultures) and harvesting techniques (e.g., type, amount, duration, and temperature of hypotonic solution and use of ethidium bromide) can affect the quality and spreading of the cells and may require some slide-making adjustments. Primary amniotic fluid cultures contain more dead epithelial cells than subcultures do, and these cells may interfere with drying and contribute to scattering and other drying problems. Blood or bone marrow cultures inoculated with too many cells per milliliter of medium will never look as good as they would have had the culture not been crowded.

2.4.17 In situ cell drying

Once residual fixative is thoroughly removed from around the coverslips or off of the chamberslide, the manipulations are performed to assist chromosome spreading according to the ambient conditions, if commercial or other slide-drying chambers are unavailable. If conditions are perfect, few manipulations are necessary. In dry weather, humidity may be supplied as for suspension slide-making (wet paper towels under the dishes, ice to slow the drying, breathing over slides/coverslips, steaming over hot water or humidifier) as well as using moist air created by bubbling air through a flask of water and then gently onto the coverslip/slide.

If no metaphases are seen, the most likely explanation, given a healthy culture, is metaphase breakage, and the next coverslip should be dried faster. If cells are present but chromosomes are encapsulated in cytoplasmic membranes, the next coverslip should be given more humidity and a longer drying time. Coverslips with heavy growth will never give as good spreading in the center of the colonies, as the interphase cells will physically prevent them from relaxing; picking coverslips at the correct cell density is almost as important as manipulating cells during drying.

2.4.18 Slide-making for FISH studies

Several references state that slides for *in situ* hybridization will hybridize best if they appear gray rather than black on phase microscopy. While this is true, the darker or more cytoplasmic cells may be worked with. First, we flood the slide with fixative and allow it to dry; this step will lighten most cells and make them flatter and more accessible to the probe. The other pre-hybridization steps to deal with cytoplasmic or three-dimensional cells are to soak them longer in 2x SSC before performing the hybridization, and/or to pretreat them with a pepsin step (see Chapter 16, section 16.9.4, Protease Pretreatment for Standard Slide Preparations). Three-dimensional cells may yield a large signal depth of field, requiring a lot of focusing up and down during analysis to detect all signals. During hybridization, difficult specimens may be denatured at higher temperatures and for longer times to be sure that the DNA is fully single stranded. Otherwise, almost all slide-making conditions have yielded good FISH results, in our hands.

2.4.19 Slide aging

When G-bands were first integrated into the clinical laboratory scheme, one troubling aspect of the new method was that only slides that had been allowed to sit for 3 or 4 days were suitable for banding. Until they were aged, chromosomes resisted banding procedures, so turnaround time was lengthened. After experimenting, it was discovered by many laboratorians that heating the chromosome preparations on the slides in an oven or on a hot plate for 12–24 hours at 40–60°C or 20 minutes at 90–95°C would obviate the problem, and slides could be G-banded immediately. This process has never been entirely understood, but it seems likely that driving off water is involved. The major change in chromosomes caused by aging may be the oxidation of the protein sulfhydryl groups [91]. Aging of slides is reported to degrade the chromosomal DNA [92], and this may explain why some banding methods and FISH methodologies are so affected by artificial aging by slide heating. All G-band methods, and many other methods as well, are preceded by artificial aging via heating or sometimes microwaving slides. The aging process is very important, as it gives better contrast and crispness even to Q-banded chromosomes, which do not really require pre-aging. Because Q-bands do not require aging, sequential Q-banding to FISH has been useful in our laboratory. It is also important to be consistent in aging (or not aging) slides for other methods, such as C-banding and G-11, to control treatment times. In our experience, slides for FISH can be aged or not. Signals tend to be much sharper and more discrete for aged preparations; however, overaging can make signals dim or may cause failure of the hybridization. Slides that are to be saved for several weeks or months for FISH or other applications may be kept in a desiccated, oxygen-deficient environment (e.g., under nitrogen gas in a Ziploc bag or in liquid nitrogen) at -20°C until needed, in order to keep them from aging naturally. Slides that have been baked for more than 10 minutes in a 90–95°C must be re-hydrated before use for FISH (see Chapter 16, section 16.9.4, Aging and baking slides). It is possible to ruin slides for G-banding by leaving them in a hot oven for too long, (e.g., 90°C for 2 hours). Because Q-bands do not require aging, sequential Q-bands to FISH are useful in our laboratory.

2.5 Chromosome staining

There are three stains capable of differentiating all chromosomes: G-, R-, and Q-banding (Figure 2.2). The analytical stain of choice in the United States is G-banding, because it is simple and gives a lot of detailed information. Some parts of Europe use R-banding for a primary stain, and Q-banding has been preferred by some cytogeneticists for certain types of study (e.g., hematologic malignancies, and CVS direct preparations, on which it is difficult to obtain good G-bands) because of its independence from pretreatment variables and the usefulness of the bright Y chromosome for determining sex. However,

Q-banding is becoming less important for these applications as laboratories improve bone marrow methods using G-bands, and as CVS direct preparations are being replaced in some laboratories with interphase FISH for aneuploidy on uncultured chorionic villi cells. The Q-band variants are also very useful for ascertaining chromosome origin (see Chapter 6, section 6.2.1, QFQ Clinical significance, and Chapter 10, section 10.3, Germ cell tumors – UPD and imprinting). This was once especially helpful in determining whether engraftment had taken place in post-bone marrow transplant specimens (now interphase FISH is used instead, since much larger cell numbers can be scored), in looking for uniparental disomy of chromosomes 14 or 15, and to demonstrate complete isodisomy in teratomas, among other things.

Other stains that may be used in certain cases include AgNOR (silver) for staining acrocentric stalks with active nucleolar organizing genes; C-banding to stain the genetically inert heterochromatin; G-11 to stain a subgroup of heterochromatin and differentiate between, for example, chromosomes 19 and 20 or X and Y; C-dot stain to distinguish centromeric areas; endonuclease stain (Alu-banding, etc.) to allow specific patterns of heterochromatin; and FISH with various probes to discover the origin, makeup, and order of chromosomal and subchromosomal units (see Chapter 6, Chromosome stains; Chapter 16, Fluorescence *in situ* hybridization). After staining has been accomplished, slides are examined and metaphase cells are photographed or electronically imaged (Chapter 15, Computer imaging), chromosomes are analyzed (Chapter 7, Human chromosomes: Identification and variations; Chapter 9, Constitutional chromosome abnormalities; Chapter 11, section 11.7.2, Cytogenetic methodology; Chapter 12, section 12.2.16, Chromosome analysis), and reported (Chapter 8, ISCN: The universal language of cytogenetics; Chapter 23, Laboratory information system).

2.6 Chromosome microscopy/analysis

Once chromosome preparations have been banded, usually with G-banding in most laboratories, chromosomes are analyzed under a microscope for both numerical and structural aberrations. The slide is scanned in a methodical, field-by-field manner, either by traveling up and down or right to left on the slide using a low power lens (10–16 \times), searching for metaphase cells. Once found, each cell is examined under high power (60 \times or 100 \times lens, usually oil immersion type). After determining whether the cell is of reasonable quality, it is counted, and at least partially analyzed, looking for sex chromosomes and checking other chromosomes of interest, depending upon the reason for referral. For example, for a referral of CML, the 9s and 22s would be checked routinely for the translocation between 9 and 22 long arms that characterize the disease (see Chapter 11, section 11.4.1, Chronic myelogenous leukemia). For a referral of Down syndrome, the focus would be on detecting an extra copy of chromosome 21 (see Chapter 9, section 9.1, Numerical abnormalities). The stage coordinates for each cell are recorded on the analysis sheet along with the chromosome count and any abnormalities seen, as well as the sex chromosome makeup and any other pertinent findings. Often, a notation of the position of certain chromosomes is recorded for each cell, so that it can be reidentified at another time or by another technologist. For example, the chromosome closest to the 6 and 12 o'clock positions or 3 and 9 o'clock positions may be recorded; this is usually unique for all the cells of one study. Some cells are completely analyzed under the microscope to ensure that each chromosome matches its pair (homologue), and also to determine if all the bands are present and in the correct order. Experienced technologists can tell all chromosomes apart by size, shape, and banding patterns. Chromosomal banding patterns are best learned by repeated karyotyping, using a good example of chromosomes at various lengths (see Chapter 7, Human chromosomes: identification and variations).

A suitable cell is usually reasonably well spread and well banded, although quality may be compromised in various disease states; however, in cancer analyses, it may be the poor cells that will demonstrate clonal abnormalities. Once a suitable cell is found, it is counted by one of several methods:

1. Locating the cell under the high-power oil lens of a microscope and counting in a systematic fashion, by mentally dividing the cell into halves, quadrants, or other subdivisions, and counting one subdivision at a time, continuing until all subdivisions are completed. Counting may be done in ones or twos. Usually, cells counted by this method are recounted to confirm the original chromosome number.
2. Printing a photograph or captured image of the cell from an imaging system, and then marking the print with a pen or marking crayon to facilitate accuracy.
3. Electronically counting the image using an imaging system software feature; this marks all chromosomes by mouse-click and keeps a tally.
4. Preparing a karyogram from a captured image, either by manually cutting out each chromosome from a print or by using imaging software electronically “cut” the karyogram or to mark each chromosome on a spread as it is analyzed on the screen. Usually, cells counted by methods 1–3 are recounted to confirm the original chromosome number.

5. To determine the cytogenetic makeup of each patient's sample, multiple cells, usually twenty per case, are counted (e.g., most routine cytogenetic referrals involving a constitutional abnormality) and/or completely analyzed (e.g., all neoplastic cases). Often cells from more than one culture are analyzed for reasons of quality control, because sometimes the abnormal cells are confined to a certain culture or cultures. If a cell has more or less than 46 chromosomes, the cell should be analyzed to determine what is extra or missing, and this information, along with other data, such as the microscope stage coordinates, is recorded on the analysis sheet or into the LIS/imaging system, if the laboratory is paperless.

The best representative cells, normal or abnormal, are photographed or imaged (see Chapter 14, section 14.5, Capturing the microscopic image and Chapter 15, Computer imaging) and some or all are completely analyzed (e.g., comparing band presence, position and size on every homologous pair). This may be accomplished by making a hard copy of some or all of the cells, and analyzing the rest by eye under the microscope or from a computer image of a spread (a cell which has not been cut out or karyotyped) or on a print. To analyze by eye, chromosomes are all identified by first finding the homologues of each pair in some methodical sequence and then comparing their bands for differences. Some technologists prefer to go through the cell in numerical order from chromosome 1–22 and X and Y, and some find the smallest chromosomes first or the acrocentric chromosomes first, and then go on to the others. Within these five photographed images collectively, coverage must be represented at least once for every chromosome pair, for example, no overlapped or unclear regions. Some laboratory protocols call for two different technologists to check each cell or to perform different parts of the study as a quality check. It is recommended that any question of abnormalities be either flagged for director review when prepared on an analytical software system, or recorded on the analysis sheet and initialed by the director or designee after verification.

Most guidelines require documentation of two karyograms from each abnormal stemline clone, and one for abnormal sidelines and normal cells; for normal cases, at minimum, a total of two or three per case are prepared.

Once analysis is complete, a karyotype will be devised which will explain the normal or abnormal findings in specific nomenclature format (see Chapter 8, ISCN: The universal language of cytogenetics). Most guidelines also require a minimum of two karyograms (pictorial representation via the systematic arrangement of chromosome pairs in numerical order) per nonmosaic case. Specimens found either constitutionally mosaic or showing neoplastic clonal evolution may require one additional karyogram to represent each additional cell line.

2.6.1 Chromosome abnormalities

Each chromosome is composed of two chromatids joined together at the primary constriction, which is usually called the centromere. The sections of the chromosome on either side of the centromere are called arms. Human chromosomes are classified morphologically based on the relative position of the centromere. In any metaphase spread of human chromosomes, some chromosomes have their centromere in the middle, some have it at one end, and some have it somewhere between these two points. The terminology of chromosomes reflects these differences. Chromosomes are described as metacentric (centromere in the middle), submetacentric (centromere a little away from the middle so that one arm is definitely longer than the other), acrocentric (centromere close to one end), or telocentric (centromere right at the end) (see Figure 2.18 and Chapter 7, section 7.2, Description of human chromosome shapes). There are no truly telocentric chromosomes in the normal human karyotype, although some tumor rearrangements may appear telocentric. In the international system for human chromosome nomenclature, short arms are designated as p arms (for petit), and long arms are called q arms [32].

Every metaphase chromosome must have a centromere to attach to the spindle fiber apparatus during cell division. Chromosomes that do not have centromeres tend to be lost from the daughter cells because they lag behind in anaphase and are excluded from the reformed nucleus in telophase. The exception to the rule is the formation of small “double minute” (dmin) chromosomes found in some tumors. These dmins are commonly the site of gene amplification, and may have a mechanism for inclusion in daughter cells. Some chromosomes also have a secondary constriction, called a stalk (stk), which is a variable, thin portion that does not stain very darkly with Giemsa stain. Stalks frequently appear on the short arms of acrocentric chromosomes, connecting the satellites (small knobs of chromatin usually found at the ends of the short arms of the acrocentrics) to the centromere. These secondary constrictions on the acrocentric (“D” and “G”, see Figure 2.18) chromosomes are sites of ribosomal RNA synthesis in the interphase nucleus and are termed the nucleolus organizing regions (NORs). Secondary constrictions are sometimes seen on other chromosomes, such as chromosome 9, just below the centromere. On photographs, secondary constrictions may be confused with gaps because they are so thin and pale; they can be seen through the microscope, however, and may thus be differentiated from true gaps. Gaps in chromosomes may be due to chromosome breakage, as in Fanconi anemia, or fragile sites, such as fragile X syndrome [93]. The normal human chromosome number is 46. This is termed the diploid or $2n$ complement, which results from the fertilization of a haploid egg, with 23 chromosomes (the human ‘ n ’

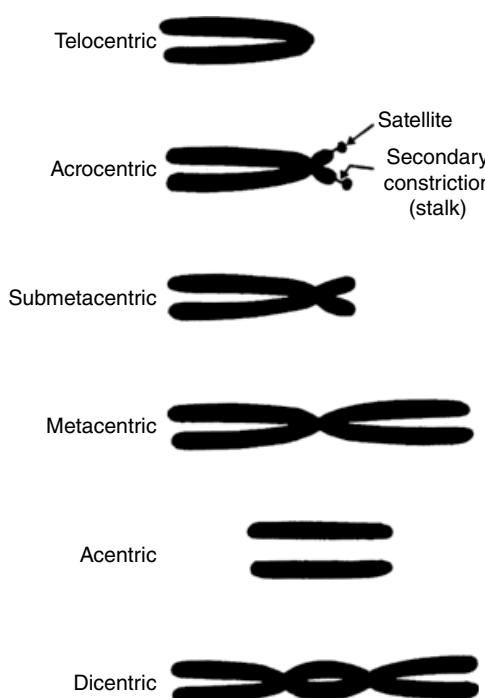


Figure 2.18 Chromosome shapes. The size and shape of a chromosome is an integral part of identifying a chromosome, along with its banding pattern (not shown).

number), or by a haploid sperm, both of which are termed gametes (see Chapter 1, section 1.2.4, Meiosis). This number can vary due to a number of errors in cell division, that is, during meiosis, fertilization, or mitosis. If the error happens meiotically, it can result in aneuploidy (cells other than diploid), or if the error is due to a mistake of fertilization (e.g., extra sets of chromosomes from either a second sperm or the polar body of the egg), it can lead to ploidy errors, such as triploidy – 69 chromosomes – or tetraploidy – 92 chromosomes. If the error arises post zygotically during mitotic division, it may lead to mosaicism, or the presence of more than one clone in the zygote (see later). Such findings would be called constitutional, meaning present in the subject's basic hereditary makeup. Alternatively, one cell in the bone marrow of an adult could undergo nondisjunction of chromosome 8, leading to a clone with trisomy 8 and hematologic disease (see Chapter 11). This finding would be called an acquired abnormality, since it is not innate, and represents a new change in the genetic makeup of that clone of cells. Acquired abnormalities are present only in the neoplastic cells, but not in the normal, nondiseased cells, so often these studies require extreme attention to find the abnormal cells.

During the cell count, an experienced technologist can also detect many structural chromosome rearrangements. A chromosome that is missing material is said to be deleted for that portion. If a segment is repeated, it is termed a duplication. A chromosome may also exhibit an exchange of material with another chromosome (called a translocation), which can either be balanced or unbalanced. In a balanced translocation no chromosomal segments are lost or gained. An unbalanced translocation involves loss or gain of one or more chromosomal segments resulting in partial monosomies or trisomies, respectively. These may imply, in a non-neoplastic chromosome study, a parental balanced translocation, inversion, or other rearrangement from which the unbalanced chromosome is derived by meiotic mis-segregation. An acquired unbalanced translocation in a malignancy is, on the other hand, usually caused by a mitotic event in the malignant cells. Sometimes a karyotype must be performed on a stimulated blood culture from a cancer patient in order to determine the origin of an abnormality seen in the cancer, if all the cells examined in the study show the same abnormal karyotype.

Sometimes a balanced translocation in an individual may form unusual meiotic configurations in preparing for disjunction in the gametes. A phenomenon called 3:1 meiotic disjunction can occur, with unequal sharing of the translocation chromosomes as the cell divides. This can result in an offspring with aneuploidy and unbalanced translocations, and can also lead to a state called uniparental disomy, in which both homologues of a pair of chromosomes come from the same parent, and there is no contribution from the other parent. For example, a translocation involving chromosomes 1 and 15 in one parent could

nondisjoin so that the subsequent daughter cell acquires both normal and translocated chromosomes 15. When the two gametes merge, one of the now three chromosomes 15 will become lost as a result of anaphase lag (loss of a chromatid at anaphase because it is not attached to the spindle and so does not get drawn to the pole), a phenomena known as trisomy rescue (see Chapter 10, section 10.2.2, Whole chromosome uniparental disomies). If the lost chromosome was from the second partner, the embryo will acquire two chromosomes 15 from the same parent, and thus have uniparental disomy for chromosome 15. Depending upon which parent the two chromosomes 15 came from, the offspring will have either Prader–Willi or Angelman syndrome (see Chapter 10, section 10.2.1, Chromosomal syndromes).

Another type of translocation commonly seen in constitutional studies is the fusion of two acrocentric chromosomes leading to a bi-armed chromosome termed a Robertsonian translocation, as a result of the discovery of such translocations in grasshoppers by Dr. W.R.B. Robertson in 1916. More than one per thousand liveborn children carry such a translocation, and it can be either balanced or unbalanced. Some Down syndrome children have Robertsonian rearrangements of the extra chromosome 21 with involvement of one of the other acrocentric chromosomes (note: the chromosome number would be 46, even though the genetic material is unbalanced). These may be inherited from one of the parents who can carry the balanced form of the Robertsonian translocation. Genetic counselors would give a much higher recurrence rate for parents of a translocation Down patient than for parents of a Down syndrome child with 47 chromosomes and an extra 21 because half of the gametes will contain the translocation and of these, due to improper disjunction, some may carry two normal chromosomes 21 as well as the translocation copy.

Some translocations occur at clusters within specific sequences, as with the familial translocation between chromosome 11 short arm and chromosome 22 long arm at bands 11q23.3 and 22q11.2. This is the most commonly occurring translocation in constitutional abnormalities, and the breakpoints occur at long AT-rich palindromic sequences (sequences with mirror image bases that hairpin back on themselves). It is not known why palindromic sequences are prone to translocation.

Other types of imbalance include duplications and deletions caused by unusual alignment of chromosomes when there is an inversion present. An inversion may involve the centromere of the chromosome (pericentric inversion) or not (paracentric). During meiosis, in order for the chromosome to pair its entire length gene for gene, the inverted chromosome has to make a loop configuration inside an inchworm shape taken by the homologue (Chapter 9, Figures 9.17 and 9.18). This may result in unbalanced gametes (see Chapter 9, section 9.2.4, Inversions), with particular duplications and deletions (or deficiencies). Carriers of inversions have an increased risk for miscarriages and unbalanced offspring because of this meiotic error.

Cytogenetic imbalance can also arise from extra chromosomes not identifiable by any definitive banding patterns. These are called marker chromosomes. Sometimes a marker can be characterized with FISH and special stain methods to be composed of multiple chromosomes mixed together, or they may be a small part of one chromosome with insufficient banding to be recognizable. Chromosomes may form ring structures, and these may be formed from a single chromosome or from multiple chromosomes. They may be constitutional or acquired in cancer. They may represent a single homologue in a euploid karyotype or they may be supernumerary. Rings often appear in different forms in different cells from the patient due to sister chromatid exchange (see Chapter 6, section 6.3, Sister Chromatid exchanges, and Chapter 13, Chromosome Instability Syndromes), becoming double in size, a figure 8, or may even open up and appear as a nonringed chromosome during this process.

Aneuploidy may also arise from technical artifact from the preparation (often termed “random chromosome loss”). The small chromosomes are commonly lost because of a small tear in the cytoplasmic membrane through which they may escape, either during harvest or slide-making. One of these escaped chromosomes may also drift into a neighboring metaphase during slide drying. Rules for distinguishing between technical artifact and true mosaicism, as defined by the International Standing Committee for Chromosome Nomenclature in the ISCN book [94], is to take these random gains and losses into account, with true mosaicism requiring the presence of two or more cells with an extra specific chromosome in order to be called significant (“clonal”), and three or more cells with a missing chromosome to be considered clonal.

2.6.2 Mosaicism

Mosaicism usually arises after fertilization, through inaccurate segregation of chromosomes at mitosis. When this occurs, different cell lineages (hence, karyotypes) that have originated from a single zygote will be present in different cells or tissues within that individual, such as the finding of some cells in a specimen with 45 chromosomes and only one X chromosome, and the remaining cells with 46 chromosomes and two X chromosomes, as seen in mosaic Turner syndrome individuals. Other examples of documented mosaic syndromes include Pallister–Killian syndrome, trisomy 20 mosaicism, and confined placental mosaicism (see Chapter 5, section 5.6.2, Confined placental mosaicism and Uniparental disomy). A much less common type of mosaicism, called chimerism, is the result of two embryos (zygotes) fusing into one, so that there is a mixture of two cell lineages that are completely unrelated (e.g., half male and half female, or half trisomic for chromosome 13 and half for chromosome 18).

If two or more chromosomally distinct cell lines are found, it becomes reportable if at least two chromosomes are observed with the same structural abnormality or additional chromosome, or at least three cells are observed with the loss of the same chromosome [94]. In vitro culturing can lead to a mitotic error (cultural artifact); therefore, finding the identical abnormality in an independent culture(s) becomes critical to interpreting mosaicism (see Chapter 4 for a discussion of primary cultures). When mosaicism is suspected but is either not found, or is found but does not meet reportable standards, another specimen source, for example, skin, buccal mucosa or another tissue, or the use of FISH, may help confirm or rule out its presence.

Two mechanisms have been postulated to account for errors of segregation, as shown in Figure 2.19. Nondisjunction is the failure of the two chromatids to separate at the centromere at metaphase, causing both chromatids to go to the same daughter cell, leading to one hyperdiploid (more than the correct diploid number) cell and one hypodiploid (less than the correct diploid number) cell. Anaphase lag, the second mechanism for errors of segregation, is the loss of a chromatid at anaphase due to the lack of its attachment to the spindle, and so it does not get drawn to the pole. This causes one hypodiploid and one normal daughter cell.

The level of mosaicism among the various tissues of an organism depends on the stage of development at which the error in division occurred (Figure 2.20). If misdivision occurs at the first cell division after fertilization, it is possible for all tissues in the body to be affected, with mosaic lines of 50% each, assuming equal survival and mitotic activity. If the misdivision occurs after the three cell types (ectoderm, mesoderm, and endoderm) have been established, the abnormal cells could be localized to one cell type (e.g., skin fibroblasts only); if it occurs later still, abnormalities could appear in only one organ of the body (e.g., bladder). The phenotypic effect could be dependent on the percentage and location of abnormal cells present in the body. Thus, it is important to be aware that analysis of a different tissue source in prenatal studies may be necessary (e.g., peri-umbilical blood, cultured urine) in order to find certain mosaic states, or that sometimes extra-fetal tissues, such as placenta or fetal membranes, will show an abnormal line (confined placental mosaicism, which can lead to growth retardation), while the fetus is karyotypically normal (see Chapter 5, section 5.6.2, Confined placental mosaicism and uniparental disomy). See Figure 2.21.

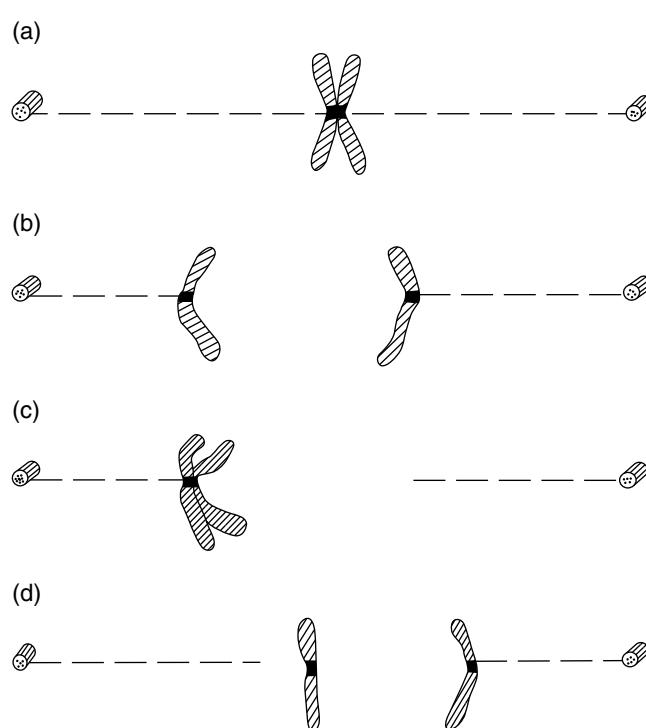


Figure 2.19 Normal and abnormal chromatid separation at mitosis. (a) Chromosomes at metaphase plate between centrioles. (b) Normal disjunction at anaphase – one chromosome at each centriole. (c) Nondisjunction at anaphase – two chromatids travel to one centriole, resulting in a cell with an extra chromosome and a cell with a missing chromosome. (d) Anaphase lag – one chromatid fails to attach to the spindle and is usually excluded from the nuclei and lost from both daughter cells.

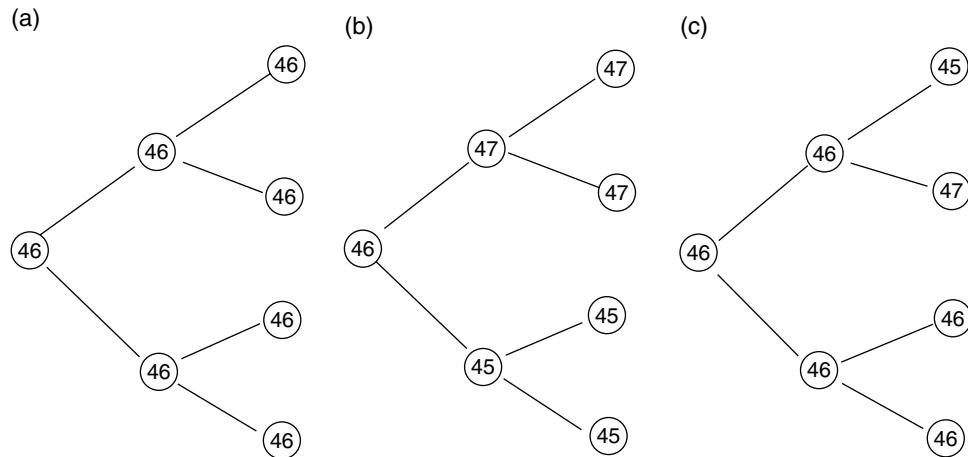


Figure 2.20 The development of mosaicism in the early zygote. (a) Normal cell division. (b) Unbalanced segregation at the first mitotic event caused by nondisjunction. This results in cell lines with 45, 46, and 47 chromosomes, and is often present in all tissue types examined. (c) Unbalanced segregation at the second mitotic event caused by nondisjunction. This also results in cell lines with 45, 46, and 47 chromosomes; however, if tissues have begun to form into separate germ layers, one or more of the abnormal cell lines could be confined to a single tissue type, such as skin, which is derived from the particular germ cell layer involved.

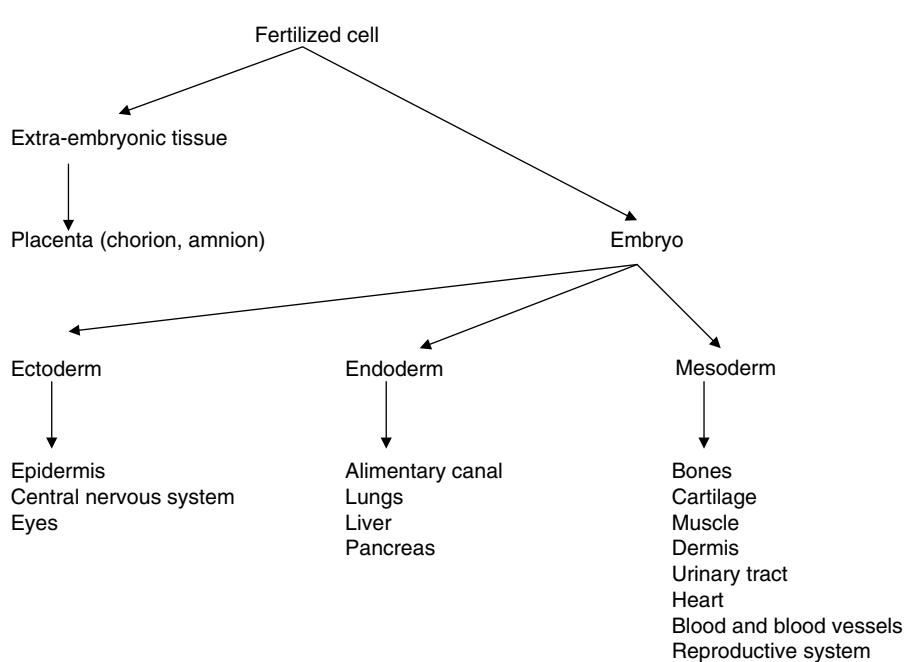


Figure 2.21 Genealogy of the organs of the body. In checking for mosaicism, tissue from different groups should be examined. Note, for example, that blood is mesodermal in origin, and skin is ectodermal.

Guidelines from the Association of Clinical Cytogenetics in the United Kingdom [95] describe a three-level definition for interpreting mosaicism, by Hsu and Benn [96], when finding at least one abnormal cell in prenatal studies:

Level I, when a single aberrant metaphase is found within only one, otherwise normal colony, or in only one suspension culture harvest.

Level II, when an abnormality is confined to only one culture vessel, whether it includes one entire colony or aberrant chromosomes are noted among normal cells from one monolayer flask.

Level III, or true mosaicism, when the abnormality has been documented in two or more independent culture vessels.

See Chapter 5, section 5.4.8, Mosaicism, for the actions to be taken and factors to consider when resolving a potential prenatal mosaic situation.

There is no way to totally eliminate the possibility of mosaicism in the patient under study, because not all cell types are present in the sample being studied. The sample is representative of the cells of the body from which it came, and not representative of other tissues from the individual. However, it is possible to count enough cells to eliminate certain levels of mosaicism within that tissue source. Hook's tables [97] apply to lymphocyte studies, and the studies of Clausen et al. [98] and Cheung et al. [99] are calculated for amniotic fluids. In each study, the statistical analysis is based on the assumption that the sample obtained is truly random (though it probably is not) and that all cells grow at the same rate and behave the same in the *in vitro* culture flask (which is probably not true either). These tables are very useful as a guideline for assessing the confidence levels for mosaicism, because they show the percentage of mosaicism excluded and the confidence levels that may be accepted if a specific number of cells have identical counts (note that "counts" may denote analysis as well, to look for structural mosaicism). The recommended count of 20 identical cells on a patient sample means that mosaicism of greater than 14% may be excluded with a confidence level of 95%. Ruling out mosaicism of greater than 10% for the same confidence level requires counting 30 identical cells on a sample. Increasing counts to 50 identical cells will only improve the detection rate to 6%, with the same confidence level; thus the small improvement may not justify the additional 20 cells counted. On the other hand, if there is enough clinical evidence to warrant the investigation for low-level mosaicism, it may be necessary to examine a greater number of cells or to obtain a sample from a different tissue source, especially when the clinical diagnosis strongly suggests a syndrome that is not found by studying 20 metaphases from a lymphocyte culture. Additional cells may also be warranted for a cancer workup or for the investigation of fragile sites or breakage syndromes; however, even a single, abnormal neoplastic cell may be reported if that aberration was previously reported in a prior specimen from that patient [94].

Because long-term incubation can increase the risk for cultural artifact, finding an abnormal cell in prenatal material requires its own criteria for differentiating true mosaicism from sporadic artifactual changes (pseudomosaicism). For example, each cell lineage should be observed in more than one independent primary culture; therefore, every colony in all primary cultures, except the one that had the abnormality, would be checked until a second colony can validate the finding. If it was only observed in that one original culture, but the finding correlates with clinical findings, it may be necessary to perform alternative testing (e.g., FISH, array, or follow-up amniocentesis). Abnormal findings in prenatal tissue could also stem from extra-fetal tissues, such as placenta or membranes (confined placental mosaicism), while the fetus is normal. In particular, an extra copy of chromosome 2 or chromosome 7 has been reported as an artifact of prenatal cultures and usually have no clinical significance (see Chapter 5, section 5.6.2, Confined placental mosaicism and uniparental disomy). See Figure 2.21. These situations have been discussed extensively in the literature [100–112]. (See also Chapter 5, Prenatal chromosome diagnosis). If no further cells (or colonies, in the case of *in situ* culture analysis) from an independent culture are found identical to the one abnormal cell (or colony) already noted, then the aberrant cell can usually be assumed to be artifactual, and mosaicism of greater than 10% can be excluded at a 95% confidence level. If other cells with the same aberration are found in a second, independent culture source, low-level mosaicism may be present and should be reported, particularly if the karyotype of the minor line represents a clinically described condition and clinical findings are consistent with that cytogenetic finding. It is important to stay current with new guidelines for interpreting mosaicism that are published from time to time.

Another method for detecting mosaicism is to utilize interphase fluorescence *in situ* hybridization (FISH), if probes are available for the interphase detection of the abnormality. FISH can be used to extend the number of cells scored for a given abnormality and to investigate alternative tissues with different germ cell layer origins, such as epithelial tissue in skin or buccal smears. It is important to first know how the patient's metaphase cells behave with the probe used, since alpha satellite DNA probes may have variant patterns in some individuals, with extra signals or even missing signals in the normal cells (see Chapter 16). Microarray CGH analysis also provides analysis for delineation of structural abnormalities, comparison of inherited abnormalities between parent and child, or investigation of possible cryptic unbalances to complete the report (see Chapter 18).

2.6.3 Chromosome breakage

Cultured chromosomes may naturally exhibit gaps and breaks in chromatids as a result of cultural artifact. The lesions vary from a staining gap where the break took place to complete displacement of the chromosome fragment far removed from the chromosome. These gaps and breaks can lead to chromosome rearrangements, including translocations, inversions, and ring chromosome formations. There are autosomal recessive conditions that predispose individuals to chromosome breaks and

radial formations (Chapter 13, Figure 13.2) and consequently to cancer. Examples include Bloom syndrome, Fanconi anemia, and Ataxia telangiectasia (see Chapter 13, Chromosome instability syndromes).

Some regions of the genome that have repetitive DNA sequences may exhibit chromosome gaps and breaks in specific breakpoints in the karyotype known as fragile sites. An example of a fragile site is the fragile Xq27.3 that causes the phenotype for Martin Bell (fragile X) syndrome. The test for this condition was a cytogenetic breakage study until the advent of a DNA test for the repeat DNA that is now the standard of care for diagnosing the fragile X both in carriers and in affected individuals. It is important to document breaks and gaps in chromosome studies because there may be a clinical cause.

2.6.4 Karyotyping a cell

The noun karyotype originally meant just an arranged layout of the chromosomes of a single cell, but with time the meaning expanded to include the written description of the cut-out cell because the two were both considered descriptions of the same thing. However, in the 2005 *International System for Cytogenetic Nomenclature* (ISCN), in order to distinguish between the two ideas, the chromosome layout became called a karyogram, and the written description as well as the chromosomal makeup of the individual became known as the karyotype. The verb for making the karyogram is not described by the ISCN, and since “karyogramming” does not resonate, we will continue to use the verb “to karyotype” as the act of arranging chromosomes for analytical comparison. Here we will discuss the principles used in the making of a pictorial karyogram. See Chapter 8 for the use of ISCN nomenclature to describe the karyotype in writing.

The karyogram is an orderly arrangement of chromosomes (46 in human, 44 autosomes arranged in pairs, one from each parent, and two sex chromosomes) according to international conventions [112]. The chromosomes may be cut from a photographic print and arranged on a preprinted form with areas marked for each chromosome and its group or they can be digitally arranged via cytogenetic analytical software. If manually cut, chromosomes may be fastened to the form by gluing, taping, or using photographic dry mounting tissue and heat. In most clinical laboratories, however, manual methods have been replaced with digital imaging software systems that can automatically arrange chromosome images onto a karyogram format, with guidance from the operator using mouse and keyboard commands. Computer-assisted karyotyping has helped to enhance the quality and reduce the time required to prepare a karyogram.

Chromosomes were originally categorized by size and morphology into seven groups, A-G, by the 1961 Denver Conference [113] (Figure 2.18). With the advent of banding, the 22 pairs of autosomes (nonsex chromosomes), numbered 1–22, became individually identifiable. The sex chromosomes were named X and Y. Although they were not given numbers, they were assigned to groups based on their size; the X chromosome to the C group, and the Y chromosome to the G group. When chromosomes are not banded (see Chapter 6, section 6.1.5, Conventional (solid) staining methods), they can be assigned to groups but cannot all be identified individually. When they are banded by the G-, Q-, or R-banding methods, each chromosome can be positively identified by number as well as by group. The distinguishing characteristics of each chromosome when G-banded are given in Chapter 7, Human chromosomes: identification and variations.

Chromosomes are always arranged on the karyogram with the short arms uppermost. Groups are arranged in alphabetical order. The sex chromosomes may be placed together at the end or separated to their groups; either way is acceptable.

The largest chromosomes are in the A group (two each of numbers 1, 2, and 3). Chromosomes 1 and 3 are metacentric chromosomes, 1 being larger than 3, and the number 2 chromosomes are slightly submetacentric.

The B group consists of two pairs of chromosomes that are large and strikingly submetacentric; the short arms about one-fourth the length of the long arms. Without banding treatment they cannot be identified individually. They are numbered 4 and 5.

The C group contains the most chromosomes and is the most difficult group to karyotype in both banded and nonbanded spreads. The chromosomes are medium-sized and are all submetacentric; they are numbered from 6 to 12. The X chromosome also belongs to this group. In the nonbanded state, these chromosomes cannot be individually identified, so they are arranged roughly in order of decreasing size: the 6s are the largest, and the 7s and the X are the next largest. Chromosomes X and 7 are about the same size, but the X is more metacentric than the 7. The 11s are the most metacentric, and the 9s are variable size depending on the length of the heterochromatic region just below the centromere. The 8s, 10s, and 12s have the shorter short arms, and the 12s are the most submetacentric.

The D group consists of three pairs of medium-sized acrocentric chromosomes, numbered 13, 14, and 15. In the nonbanded spread, all Ds look alike. Tiny short arms with satellites are often visible and vary greatly in size and intensity of staining.

The E group consists of three pairs of chromosomes that are about the same length as the D group chromosomes but have clearly defined short arms. The 16s are metacentric, and the 17s and 18s are submetacentric. The 18s have the shortest short arms in this group.

The F group contains two pairs of small metacentric chromosomes, 19 and 20, which cannot be individually distinguished without banding treatment.

The G group contains two pairs of autosomes, numbers and 22, and the Y sex chromosome. The 21s and 22s are small acrocentrics that frequently have short arms with satellites, similar to the Ds. The Y chromosome can be very similar to the other Gs, although it does not have satellites, and may have more defined short arms. It can also vary in size and is sometimes larger than the 18, although its short arms are always small.

The normal female has two X chromosomes and no Y chromosome, so that the nonbanded spread has 16 C group chromosomes and only four G group chromosomes. The normal male, with only one X chromosome, has only 15 C group chromosomes but has five G group chromosomes because of the presence of the Y.

A note about satellites: other chromosomes (autosomes, the Y chromosome) can acquire satellites as a normal variant (see Chapter 9). In addition, the short arms of the acrocentric chromosomes can acquire the Yqh region without an effect on the phenotype, and can be seen in females as well as males.

2.6.5 Banded karyograms

Karyotyping banded chromosomes follows the same principles described for the nonbanded chromosomes but uses the additional information provided by the banding pattern to identify each chromosome by number. Because the band number depends upon the elongation of the chromosome, band descriptions vary for a given chromosome and are difficult to verbalize. Chapter 7, Human Chromosomes: Identification and Variations, shows G-banded chromosomes at various lengths.

2.6.6 Haploid band number and band levels

Band level is a term representing the total number of bands countable in a haploid set of autosomes plus the sex chromosomes. The band level that can be obtained by the various banding procedures depends on the degree of mitotic chromosome condensation achieved by the cell when it was harvested. It is desirable to aim for the highest level of band resolution as possible. Landmark bands that were the first demonstrated on metaphase chromosomes represented a band level of about 250 bands per haploid set. At this stage in late metaphase, all chromosomes can be identified individually but even quite large abnormalities are invisible. The Paris Conference schematic [114] represents chromosomes at the 400-band level; this is obtained from chromosomes at a slightly earlier stage of metaphase.

A band level of 550 (see Figure 2.12) provides sufficiently high resolution for detection of many small rearrangements without being prohibitively difficult to analyze. This level corresponds to prophase or prometaphase, when many small, distinct bands can be demonstrated that will later condense to form the well-known landmark bands (see Table 2.7). Blood and amniotic fluid samples are capable of obtaining this length, but bone marrows are usually shorter. Band levels of up to 850–900 bands per haploid set are obtainable using stimulated blood cultures, and amniotic fluid cells are capable of occasional high band lengths as well. These very high band levels are used to define very small abnormalities. However, for detecting certain abnormalities such as pericentric inversions, may be more apparent in shorter cells, so cells of various lengths should be examined.

There are a number of methods to determine the band length of a given cell [45–50]. One method, developed at the Cytogenetics Laboratory of Vancouver General Hospital [49], uses the dark G-bands from five different chromosomes, counting all bands except the centromere on the X and 10, 11p, 12q, and 1p31–p32. The total obtained is related to the haploid band levels, from 350 to 850. The number of bands counted represents approximately 7% of the total haploid count at band levels of 500 and above, and slightly less at lower band levels. The sum of the bands on these segments on the ISCN schematics is as follows: 18 bands at the 350-band level, 21 bands at 400, 40 bands at 550, and 60 bands at 850. In counting

Table 2.7 Comparison of bands on four chromosomes at two different band levels

Chromosome	Approximately 400	550–600 Band level
7q	2 dark bands	Each of the two major dark bands splits into 2 dark and 1 light
10q	3 dark bands (upper or proximal is darkest)	Proximal dark band splits to 2 dark and 1 light; also 1 dark band appears in the next distal light band, 10q22
11p	2 dark bands, very close together	The 2 dark bands separate and a thin dark band appears, close to terminal
Xp	1 large dark band	The 1 dark band splits into 2 dark bands with 1 light band between them; distal dark band is the largest

p=short arm; q=long arm.

the actual bands on the chromosomes of a spread, the total obtained might be intermediate to the four guidelines, in which case an extrapolation can be made. Alternatively, certain bands can be used as a reference to assess the entire cell, since they split into additional bands at specific band levels [50]. Many laboratory guidelines require band assessment, and often band level of the best quality cell is reported on the final report. The College of American Pathologists (CAP) requires the reporting of band resolution on the final report for non-neoplastic studies.

Many patient samples will show variations in regions of certain chromosomes, such as increased or decreased heterochromatic regions, variation in sizes of short arms, stalks, and satellites of acrocentric chromosomes, and so on. These variables, or heteromorphisms, which usually carry no phenotypic effects, are covered in detail in Chapter 7, Human chromosomes: identification and variations.

2.6.7 The complete cytogenetic study

There are guidelines for the components of the complete cytogenetic study, which are followed by most laboratories. These include the guidelines of the American College of Medical Genetics (ACMG) [115], College of American Pathologists (CAP) [116], and European Cytogenetic Association (ECA) and other country-directed guidelines [117]; in addition, there may be state or country-specific standards also available. These guidelines are updated regularly and can be printed from the websites of the organizations mentioned above (see Chapter 25, section 25.2.7, Credentialing and guidelines). Guidelines will specify how many metaphase cells are to be examined, photographed or imaged, analyzed or karyotyped, and how many interphase cells for FISH are to be examined and imaged.

For most constitutional studies, counting 20 cells and finding them identical is sufficient to provide the cytogenetic diagnosis. It may, however, be necessary to examine a greater number of cells (see 2.6.2, Mosaicism) or to obtain further cells from a sample of a different tissue source if the clinical diagnosis strongly suggests a syndrome that may not be detected by studying 20 metaphases from a lymphocyte culture (e.g., Hypomelanosis of Ito, Pallister–Killian syndrome). Cancer predisposition studies for the presence of fragile sites and breakage may also require analysis of an even greater number of cells for meaningful statistics.

For many samples, the quality of the spreads chosen for analysis should be the best that can be found on the slide. The major exception to this rule is in cancer cytogenetics, in which the abnormal cells are sometimes of poorer quality than the normal cells, and may be nondiploid (e.g., near-tetraploid or near-haploid). For this reason, some laboratories routinely document the quality of a study during analysis: for example, *excellent* for spreads with sharp, clear bands and no overlaps; *good* for spreads in which all chromosomes can be identified, though some overlaps are present; and *fair* for spreads that can be analyzed but are unsuitable for capturing and karyotyping.

Exceptions to the rule of reporting prenatal mosaicism in multiple colonies include the appearance of a colony with an extra chromosome 2 or chromosome 7, both of which have been reported as an artifact of prenatal cultures and are usually considered to have no clinical significance. However, even a single aberrant cell in a bone marrow sample could represent a neoplastic line and may need to be reported, if relevant to the clinical picture [94]. The laboratory director may choose to report a single aberrant cell if it seems relevant, considering the clinical findings or patient history.

2.6.8 Karyograms, karyotypes, and the final report

After a case has been analyzed microscopically, images are made of representative metaphase cells either with a photographic or digital system. The number of images to be made will be established by the laboratory director, but guidelines generally include minimal recommendations. Prints of chromosome spreads that are not karyotyped enable technologists to check their analyses. Some laboratories, particularly those in cancer work, photograph as many as 30 consecutive spreads for later analysis.

All photographic materials, both negatives and prints, must be clearly identified with the patient's name and the case number, and careful records must be kept of the photographic work, either in a special file or in each patient's file, following the protocols established for each laboratory. An identification system for storing digital images is usually programmed into the computer imaging system, or can be added during analysis.

The final report should be prepared as soon as the study is completed, and copies sent to the relevant referral physicians, hospital records departments, or laboratory/hospital information computer systems. The report should include (per ACMG/CAP guidelines) the patient name and birth date, identification number such as medical record number and test encounter number laboratory accession number, referring physician, test requested and performed, clinical indication for the study and ICD10 code if known, source of the specimen (amniotic fluid, blood, etc.), date of service, date received, culture type and number of vessels examined, number of cells and colonies examined, number of analysis and karyograms prepared, stains used,

quality, band level, karyotype, cytogenetic results, interpretation, recommendations, preliminary and final report dates, and the Director's signature. The results are expressed using ISCN (the current version), as well as in a verbal interpretation, which can correlate the findings with the clinical presentation and can comment on recommendations for genetic counseling or further cytogenetic or other studies of the patient or the family of the patient.

2.6.9 Sources of error in analysis and reporting

It is extremely important to avoid errors in genetic studies, because prognostic, diagnostic, and outcome information, treatment decisions, and often the life and death of a patient may depend upon accuracy. Because almost all stages of a cytogenetic study involve subjectivity and individual expertise on the part of the cytogeneticist, it is important to be aware of some potential sources of errors.

Mistakes can occur at various stages, from specimen collection to the actual interpretation of the karyotype. Cases can be mixed up during culturing, harvesting, slide-making, slide or record sheet selection at the microscope, printing of the film and karyotyping. Each case must be properly labeled throughout the cytogenetic procedure – preferably by the patient's name and an identification number (e.g., accession number, bar code, medical record number, etc.). The CAP guidelines require two separate, unique identifiers on each sample. It is also helpful to work in an atmosphere that is free from distractions and to handle each case separately when setting up cultures, making slides, performing microscopy, printing, adding probes to slides, and karyotyping. Extreme care should be taken to avoid cross-contamination of specimens during harvesting and slide-making. It may sometimes be necessary to repeat work using other cultures or new samples to clarify discrepant results.

Poor growth of cultures and poor banding also present the greatest sources of potential error to the technologist. Cultures that yield few metaphases of poor quality, such as compact spreads and cells with many overlapping chromosomes or background debris, may result in incorrect microscopic analysis because the technologist may fail to detect mosaicism, translocations, ring or marker chromosomes, or other very small chromosome abnormalities. If the technologist is not satisfied, new slides should be prepared, a repeat culture performed, or a repeat specimen requested. It is still as important as ever to obtain the highest quality banding resolution possible, because even though microarray technologies are improving and increasingly available, they do not cover the entire genome, do not detect balanced rearrangements, and will not be offered to all patients. Poor quality banding may also be a source of error by preventing the detection of translocations, duplications, deletions, or other structural abnormalities. If the technologist has any doubt, new slides should be prepared, a harvest repeated on a culture, or a repeat specimen requested.

The banding pattern of each chromosome should be closely examined in each study, and every chromosome pair should be compared band for band at least once to ensure that no differences exist between homologous chromosomes. If an unusual pattern is observed, all means necessary, including supplementary banding or molecular procedures, should be employed to rule out or confirm any abnormality. Preparation of more than one karyotype helps to ensure that all chromosomes can be visualized without crossovers or other clarity obstruction. If there are still regions of some chromosomes that are unclear, examine more cells on the slides on the microscope or on prints. Some unusual findings may turn out to be due to variations in the slide-making and staining, limitations of the image reproduction system, or to the location of the chromosome within the metaphase: chromosomes tend to stretch on the outer edge of the metaphase, overlapping chromosomes can confuse banding patterns, and extraneous stain can be mistaken for chromosome segments.

Some abnormalities, termed "cryptic," are not readily detectable by karyotype analysis. For example, if the light telomere bands from two different nonhomologous chromosomes exchange, neither will appear much different than the normal state. Subtelomere specific probes have been designed to investigate such rearrangements (see Chapter 16, section 16.3.1, Subtelomeric-specific probes). Another example is seen in chronic myelogenous leukemia, where the diagnostic translocation between the long arms of chromosomes 9 and 22 may be obscured by involvement of a third chromosome or may present as a small insertional rearrangement that does not include a translocation of the entire distal segments of the 9 and 22. FISH for the translocation and/or polymerase chain reaction (PCR) DNA testing should clarify these rare cases because the cryptic rearrangement still results in a fusion between the *BCR* and *ABL1* genes.

Finally, obtaining another opinion is valuable. Consultation with other technologists or laboratories may prove invaluable in many cases.

2.7 Laboratory procedure manual

All of the methods used in the cytogenetics laboratory should be updated regularly and be made available to all laboratory personnel working with them. They may be in a loose-leaf binder or on a computer-based system. The computer-based system, if on a common drive of an institutional intranet, is easiest to keep up to date for everyone. If a paper based methods

system is utilized, when newer, updated versions are created, all copies of the old methods must be archived with date that the method was discontinued so that all personnel will be using the same guidelines for every method. Personnel must review all methods annually, or whenever changes are made, to be sure that they are correct and that the correct methods are being used. It is a good practice to have all personnel review all of the methods at least annually to be sure that current and correct methods are being used. The laboratory director and department chair, if appropriate, are also responsible to review the methods semi-annually or when any change is made.

The essential parts of a laboratory protocol are:

Title: The title should have a name that would represent the procedure uniquely, along with a filing identification for easy retrieval. The name of the writer(s) of the procedure, as well as laboratory director and institution, could also be included in this initial section.

Purpose: What the method does, what it is used for, or how it relates to patient care.

Principle: How the method works. This is important for troubleshooting.

Materials/Supplies: What needs to be present in the laboratory in order to undertake the method; ordering information, such as vendor, size or volume of stock reagent along with its catalog #; how to formulate and store the reagent solutions; whether any specific safety conditions are required in their handling; and what the expiration times are after a stock reagent has been defrosted or opened, and for solutions that have been prepared from that reagent.

Method: This should include any safety warnings specific to the use of any reagent, as well as what steps are used to complete the procedure. **Tips:** Sometimes procedures are not always straightforward and there may be specific variables or limitations, which when they occur, may have troubleshooting tips to handle these occurrences. These can be added either within the procedure or after the Method section. **References** and **Additional readings:** Any literature references would be placed at the end of the Method section.

Approval signoffs: Before any procedure can be put into production, approval signoffs by laboratory director and/or department chair must be documented, which would include the date, their name(s) clearly typed or printed, followed with their signature(s). Signatures must be verifiable. This process is repeated for each change and for routine annual reviews. All laboratory personnel responsible for performing any procedure must also document that they reviewed all procedures and any subsequent changes to a procedure, at initial implementation and annually thereafter, even if no changes have been implemented.

The laboratory should also keep a record of policies. These can include the probes used for various panels, turnaround times, guidelines for an acceptable band length for prometaphase studies, etc. These are also reviewed yearly or semi-annually by the technologists and the laboratory director and/or department chair.

This chapter, while detailed, is a simplified glimpse into the workings of a cytogenetics laboratory as it existed when this book was written. Cytogenetics is a continuously changing field, and it is important to continue learning from the literature and networking with others at meetings and whenever we can. There are few schools teaching this necessary laboratory science, and so it is our duty to pass our experience and knowledge on to others, and their duty to do the same.

References

1. Gardner EJ. *History of Biology*, 2nd ed. Minneapolis: Burgess, 1965.
2. Hsu TC. *Human and Mammalian Cytogenetics: An Historical Perspective*. New York: Springer-Verlag, 1979.
3. Painter TS. Studies in mammalian spermatogenesis. II. The spermatogenesis of man. *J Exp Zool* 1923; 37:291–336.
4. Tjio JH, Levan A. The chromosome number of man. *Hereditas* 1956;42:1–6.
5. Hsu, TC. Mammalian chromosomes in vitro I.The karyotype of man. *J Hered* 1952; 43:172.
6. Hughes A. Some effects of abnormal tonicity on dividing cells in chick tissue cultures. *Q J Microscopical Sci* 1952; 93:207–220.
7. Nowell PC. Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. *Cancer Res* 1960; 20:462–464.
8. Moorehead PS, Nowell PC, Mellman WJ, Battips DM, Hungerford DA. Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp Cell Res* 1960; 20:613–616.
9. Caspersson T, Farber S, Foley GE, Kudynowski J, Modest EJ, Simonsson E, Wagh U, Zech L. Chemical differentiation along metaphase chromosomes. *Exp Cell Res* 1968; 49:212–222.

10. Caspersson T, Zech L, Johanson C. Differential banding of alkylating fluorochromes in human chromosomes. *Exp Cell Res* 1970; 60:315–319.
11. Yunis J. High resolution of human chromosomes. *Science* 1976; 191:1268–1270.
12. Ledbetter DH, Riccardi VM, Airhart SD, Strobel RJ, Keenan BS, Crawford JD. Deletions of chromosome 15 as a cause of the Prader-Willi syndrome. *N Engl J Med* 1981; 304:325–329.
13. Fryns JP, Kleczkowska A, Decock P, Van den Berghe H. Angelman's syndrome and 15q 11–13 deletions. *J Med Genet* 1989; 26:538.
14. Greenberg F, Elder FFB, Haffner P, Northrup H, Ledbetter D. Cytogenetic findings in a prospective series of patients with DiGeorge anomaly. *Am J Hum Genet* 1988; 43:605–611.
15. Hall J. Catch 22. *J Med Genet* 1993; 30:801–802.
16. Dobyns WB, Stratton RF, Parke JT, Greenberg F, Nussbaum RL, Ledbetter DH. Miller-Dieker syndrome and monosomy 17p. *J Pediatrics* 1983; 102:552–558.
17. Lawce H. How Colcemid Works. *JAGT* 2002; 28(1):5–9.
18. Knight L. The effect of Colcemid. *Karyogram* 1980; 6(3):31.
19. Holmquist GP, Motara MA. The magic of cytogenetic technology. In: Obe G, Basler A, eds. *Cytogenetics*. Berlin: Springer-Verlag, 1987; 30–47.
20. Taylor EW. The mechanism of colchicine inhibition of mitosis. *J Cell Biol* 1965; 25(I):145–160.
21. Waters K. Colcemid effect. *Appl Cytogenet* 1995:75.
22. McGill M, Pathak S, Hsu TC. Effects of ethidium bromide on mitosis and chromosomes: a possible material basis for chromosome stickiness. *Chromosoma* 1974; 47:157–167.
23. Cox DM, Niewczas-Late V, Riffell MI, Hamerton JL. Chromosomal mosaicism in diagnostic amniotic fluid cell cultures. *Pediatr Res* 1974; 8:679–683.
24. Peakman DC, Moreton MF, Robinson A. Prenatal diagnosis: techniques used to help in ruling out maternal cell contamination. *J Med Genet* 1977; 14:37–39.
25. Spurbeck JL, Carlson RO, Allen JE, Dewald GW. Culturing and robotic harvesting of bone marrow, lymph nodes, peripheral blood, fibroblasts, and solid tumors with in situ techniques. *Cancer Genet Cytogenet* 1988; 32:58–66.
26. Yunis J, Ramsey N. Retinoblastoma and sub-band deletion of chromosome 13. *Am J Dis Child* 1978; 132:161–163.
27. Curry C, Lanman J, Magenis RE, Brown M, Bergner E, Shapiro L. X-linked chondrodystrophy punctata with ichthyosis and chromosomal localization to Xp. *Am J Hum Genet* 1982; 34:122.
28. Buehler E. The tricho-rhino-phalangeal syndrome(s): chromosome 8 long arm deletions: is there a shortest region of overlap between reported cases? *Am J Med Genet* 1984; 19:113–119.
29. Allanson J, Bixenman H. A new generation of chromosomal syndromes. *Karyogram* 1985; 11:71–76.
30. Yunis J. Comparative analysis of high-resolution chromosome techniques for leukemic bone marrows. *Cancer Genet Cytogenet* 1982; 7:43–50.
31. Yunis J, Bloomfield C, Ensrud K. All patients with acute non-lymphocytic leukemias may have a chromosome defect. *N Engl J Med* 1981; 305:135–139.
32. Hagemeijer A, Smit E, Bootsma D. Improved identification of chromosomes of leukemic cells in methotrexate-treated cultures. *Cytogenet Cell Genet* 1979; 23:208–212.
33. Yunis J. New chromosome techniques in the study of human neoplasia. *Hum Pathol* 1981; 12:540–549.
34. Brown M. How to improve your peripheral blood chromosome preparations. *Karyogram* 1980; 6(6):81–86.
35. Dutrillaux B, Viegas-Pequinot E. High resolution R- and G-banding in the same preparations. *Hum Genet* 1981; 57:93–95.
36. Sha J, Verma R, Rodriguez J, Dosik H. Human chromosomes in prenatal diagnosis: a one-step high resolution technique. *Prenat Diag* 1983; 3:253–256.
37. Wheater R, Roberts S. An improved lymphocyte culture technique; deoxycytidine release of a thymidine block and use of a constant humidity chamber for slide making. *J Med Genet* 1987; 24:113–115.
38. Hsu T, Pathak S, Shafer D. Induction of chromosome cross-banding by treating cells with chemical agents before fixation. *Exp Cell Res* 1973; 79:484–487.

39. Yu R, Aronson M, Nichols W. High resolution bands in human fibroblast chromosomes induced by actinomycin D. *Cytogenet Cell Genet* 1981; 31:111–114.
40. Yunis J. Mid prophase human chromosomes. The attainment of 2000 bands. *Hum Genet* 1981; 56:293–298.
41. Eichenbaum S, Krumins E. A simple and reliable method of chromosome banding for prenatal diagnosis using a bromodeoxyuridine pulse. *Prenat Diagn* 1983; 3:291–296.
42. Ikeuchi T. Inhibitory effect of ethidium bromide on mitotic chromosome condensation and its application to high-resolution chromosome banding. *Cytogenet Cell Genet* 1984; 38:56–61.
43. Wang R, Hsu T, Ramkissoon D. Improvements of a procedure for increasing mitotic cells suitable for high resolution banding. *Mammal Chrom Newsletter* 1986; 27:1–4.
44. Yunis J, Ball D, Sawyer J. G-banding patterns of high-resolution human chromosomes 6–22, X and Y. *Hum Genet* 1979; 49:291–306.
45. Wellborn JL, Wellborn R. Quantitative determination of banding resolution for G-banded chromosomes. *Appl Cytogenet* 1993; 19:57–59.
46. Stallard R, Johnson W. Non subjective method for estimating the resolution of banded chromosomes. 34th Meeting abstracts. *Am J Hum Genet* 1983; 155A.
47. Huret JL, Leonard C, Aurias A. Proposal for scoring the quality of the banding of chromosomes. *Hum Genet* 1987; 75:373–377.
48. Kau YS, Kao GA, Walters CS. Banding resolution of amniotic cell chromosome preparations for prenatal diagnosis. *Am J Clin Pathol* 1990; 93:765–770.
49. Josifek K, Haessig C, Pantzar T. Evaluation of chromosome banding resolution: a simple guide for laboratory quality assessment. *Appl Cytogenet* 1991; 17:101–105.
50. Zabawski J, Wiktor A, Sikora M, Van Dyke D. Use reference bands to accurately estimate ISCN band levels 400, 550, and 850. *JAGT* 2005; 31(1):9–13.
51. Drouin R, Holmquist GP, Richer C-L. High resolution replication bands compared with morphologic G-bands. In: Harris G, Hirschhorn K, eds. *Advances in Human Genetics*, vol. 22. New York: Plenum Press, 1994 (22); 47–115.
52. Webber L, Garson M. Fluorodeoxyuridine synchronization of bone marrow cultures. *Cancer Genet Cytogenet* 1983; 8:123–132.
53. Gibas L, Slobodan G, Barr M, Jackson L. A simple technique for obtaining high quality chromosome preparations from chorionic villus samples using FdU synchronization. *Prenat Diagn* 1987; 7:323–327.
54. Schollmeyer E, Schafer D, Frisch B, Schlerermacher E. High resolution analysis and differential condensation in RBA-banded human chromosomes. *Hum Genet* 1981; 59:187–193.
55. Sutherland GR, Baker E, Fratini A. Excess thymidine induces folate sensitive fragile sites. *Am J Med Genet* 1985; 22:433–443.
56. Griffiths MJ, Strachan MC. A single lymphocyte culture for fragile X induction and prometaphase chromosome analysis. *J Med Genet* 1991; 28:837–839.
57. Boucher B, Norman C. Cold synchronization for the study of peripheral blood and bone marrow chromosomes in leukemias and other hematologic disease states. *Hum Genet* 1980; 51:207–211.
58. Yu M, Yu C, Yu C, Maidman J, Warburton D. Improved methods of direct and cultured chromosome preparations from chorionic villus samples. *Am J Hum Genet* 1986; 38:576–581.
59. Stubblefield E. Synchronization methods for mammalian cell cultures. *Meth Cell Phys* 1968; 3:25–44.
60. Barnes I, Maltby E. Prometaphase chromosome analysis as a routine diagnostic technique. *Clin Genet* 1986; 29:37–83.
61. Lew S. Simple synchrony for lymphocyte cultures. *Karyogram* 1986; 12:17–19.
62. Camargo M, Cervenka J. Pattern of chromosomal replication in synchronized lymphocytes. I. Evaluation and application of methotrexate block. *Hum Genet* 1980; 54:47–53.
63. Richardson VB, Littlefield LG, Sayer AM, Peterson PT. Cell-cycle-stage specificity of the methotrexate block as resolved by x-ray induced chromosome damage. *Cytogenet Cell Genet* 1994; 66:126–128.
64. Yunis J, Sawyer J, Ball D. The characterization of high resolution G-banded chromosomes of man. *Chromosoma* 1978; 67:293–307.

65. Morris C, Fitzgerald D. An evaluation of high resolution chromosome banding of hematologic cells by methotrexate synchronization and thymidine release. *Cancer Genet Cytogenet* 1985; 14:275–284.
66. Biegel JA, Leslie DS, Bigner DD, Bigner SH. Hydroxyurea synchronization increases mitotic yield in human glioma cells. *Acta Neuropathol (Berl)* 1987; 73:309–312.
67. Arrighi F, Hsu TC. Experimental alteration of metaphase chromosome morphology. *Exp Cell Res* 1965; 39:305–308.
68. Zakharov A, Egolina N. Differential spiralization along mammalian mitotic chromosomes. 1. BudR-revealed differentiation in Chinese hamster chromosomes. *Chromosoma* 1972; 38:341–365.
69. Viegas-Pequinot E, Dutrillaux B. Une methode simple pour obtenir des prophas et des prometaphases. *Ann Genet (Paris)* 1978; 21:122–125.
70. Marcus M, Nielsen K, Goitein R, Gropp A. Pattern of condensation of mouse and Chinese hamster chromosomes in G2 and mitosis of 33258 Hoechst-treated cells. *Exp Cell Res* 1979; 122:191–201.
71. Prantera G, Pimpinelli S, Rocchi A. Effects of distamycin A on human leukocytes in vitro. *Cytogenet Cell Genet* 1979; 23:103–107.
72. Rocchi A, DiCastro M, Prantera G. Effects of DAPI on human leukocytes in vitro. *Cytogenet Cell Genet* 1979; 23:250–254.
73. Dewald G, Dines D. Chromosome analysis of pleural effusions. *Karyogram* 1983; 9(4):49–53.
74. Latos-Bielenska A, Hameister H. Higher resolution banding techniques in the clinical routine. *Clin Genet* 1988; 33:325–330.
75. Muravenko OV, Amosova AV, Samatadze TE, Popov KV, Poletaev AI, Zelenin AV. 9-Aminoacridine: an efficient reagent to improve human and plant chromosome banding patterns and to standardize chromosome image analysis. *Cytometry A* 2003; 51(1):52–57.
76. Hoo J, Jamro H, Schmutz S, Lin C. Preparation of high resolution chromosomes from amniotic fluid cells. *Prenat Diagn* 1983; 3:265–267.
77. Belling J. On counting chromosomes in pollen mother cells. *American Naturalist* 1921; 55:573–574.
78. Conger AD, Fairchild LM. A quick freeze method for making smear slides. *Stain Technol* 1953; 28:281–283.
79. Rothfels K, Siminovich L. An air drying technique for flattening mammalian cells grown in vitro. *Stain Technol* 1958; 33:73.
80. Spurbeck JL, Zinmeister AR, Meyer KJ, Jalal SM. Dynamics of chromosome spreading. *Am J Hum Genet* 1996; 61:387–393.
81. Hliscs R, Muhlig P, Claussen U. The spreading of metaphases is a slow process which leads to a stretching of chromosomes. *Cytogenet Cell Genet* 1997; 76(3–4):167–171.
82. Verma R, Babu A. *Human Chromosomes: Principles and Techniques*, 2nd ed. New York: McGraw-Hill, 1995;13.
83. Claussen U, Michel S, Muhlig P, Westermann M, Grummt UW, Kromeier-Hauschild K, Liehr T. Demystifying chromosome preparation and the implications for the concept of chromosome condensation during mitosis. *Cytogenet Genome Res* 2002; 98(2–3):136–146.
84. Claussen U, Mazur A, Rubtsov N. Chromosomes are highly elastic and can be stretched. *Cytogenet Cell Genet* 1995; 66:120–125.
85. Bangs CD, Donlon TA. Chromosome preparations from cultured peripheral blood cells. In: Dracopli NC, Haines JL, Korf BR, Moir, D, Morton, C, Seidman, C, Seidman, J, Smith, D., eds. *Current Protocols in Human Genetics*. New York: John Wiley and Sons, 1994; 4.0.1–4.1.19.
86. Lundsteen C, Lind A. A test of a climate room for preparation of chromosome slides. *Clin Genet* 1985; 28:260–262.
87. Hansen S. Slide preparation. *Karyogram* 1980; 6(5):66–67.
88. Lambson B, Mendelow B, Bernstein R. Metaphase spreading in chromosome preparations is minimally affected by mechanical disruption. *Karyogram* 1986; 12:30–32.
89. Gibas L, Jackson L. A new hypotonic solution for cytogenetic analysis of leukemia bone marrow cells. *Karyogram* 1985; 11:91–92.
90. Bull RM, Hoyt LA, Waters KM. Slide cleaning. *Appl Cytogenet* 1995; 21(1):9–10.
91. Evans HJ. Some facts and fancies relating to chromosome structure in man. *Advances in Human Genetics* 1977; 8:347–438.

92. Mezzanotte R, Vanni R, Flore O, Ferrucci L, Sumners AT. Ageing of fixed cytological preparations produces degradation of chromosomal DNA. *Cytogenet Cell Genet* 1988;48:60–62.
93. Dewald GW, Buckley DD, Spurbeck JL, Jalal SN. Cytogenetic guidelines for fra(X) studies tested in routine practice. *Am J Med Genet* 1992;44:816–821.
94. ISCN (2013): *An International System for Human Cytogenetic Nomenclature*. Shaffer LG, McGowan-Jordan J, Schmid M (eds). Basel: S Karger, 2013.
95. Prenatal Diagnosis Best Practice Guidelines 2009; V1.00. Association for Clinical Cytogenetics. www.cytogenetics.org.uk
96. Hsu LYF, Benn PA. Revised guidelines for the diagnosis of mosaicism in amniocytes. *Prenat Diagn* 1999;19:1081–82.
97. Hook EB. Exclusion of chromosomal mosaicism: tables of 90%, 95%, and 99% confidence limits, and comments on use. *Am J Hum Genet* 1977;29:94–97.
98. Claussen U, Schafer H, Trampisch HJ. Exclusion of chromosomal mosaicism in prenatal diagnosis. *Hum Genet* 1984; 67:23–28.
99. Cheung SW, Spitznagel E, Featherstone T, Crane JP. Exclusion of chromosomal mosaicism in amniotic fluid cultures: efficacy of in situ versus flask techniques. *Prenat Diagn* 1990; 10:41–57.
100. Peakman DC. Chromosomal mosaicism in amniotic fluid cell cultures. *Am J Hum Genet* 1979; 31:149–155.
101. Simpson JL, Martin AO, Verp MS, Elias S, Patel VA. Hypermodal cells in amniotic fluid cultures: frequency, interpretation and clinical significance. *Am J Obstet Gynecol* 1982; 143:250–258.
102. Bui TH, Iselius L, Lindsten J. European collaborative study on prenatal diagnosis: mosaicism, pseudomosaicism and single abnormal cells in amniotic fluid cell cultures. *Prenat Diagn* 1984; 4:145–162.
103. Hsu LYF, Peris TE. United States survey on chromosome mosaicism and pseudomosaicism in prenatal diagnosis. *Prenat Diagn* 1984; 4:97–130.
104. Park JP, Moeschler JB, Rawnsley E, Berg SZ, Wurster-Hill DH. Trisomy 20 mosaicism confirmed in a phenotypically normal liveborn. *Prenat Diagn* 1984; 4:97–130.
105. Worton DG, Stern R. A Canadian collaborative study of mosaicism in amniotic fluid cell cultures. *Prenat Diagn* 1984; 4:131–144.
106. Hsu LYF, Kaffe S, Perlis TE. Trisomy 20 mosaicism in prenatal diagnosis – a review and update. *Prenat Diagn* 1987; 7:581–596.
107. Richkind KE, Apostol RA, Puck SM. Prenatal detection of trisomy 5 mosaicism with normal outcome. *Prenat Diagn* 1987; 7:143.
108. Zadeh TM, Peters J, Sandlin C. Prenatal diagnosis of mosaic trisomy 9. *Prenat Diagn* 1987; 7:67–70.
109. Freiberg AS, Blumberg B, Lawce H, Mann J. XX/XY chimerism encountered during prenatal diagnosis. *Prenat Diagn* 1988;8:423–426.
110. von Koskull H, Ritvanen A, Ammala P, Gahmberg N, Salonen R. Trisomy 12 mosaicism in amniocytes and dysmorphic child despite normal fetal chromosomes in fetal blood sample. *Prenat Diagn* 1989; 9:433–437.
111. Wilson MG, Lin MS, Fujimoto A, Herbert W, Kaplan FM. Chromosome mosaicism in 6,000 amniocenteses. *Am J Med Genet* 1989; 32:506–513.
112. Mendelian inheritance in man on line. Johns Hopkins University. <http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM>
113. Denver Conference (1960): A proposed standard system of nomenclature of human mitotic chromosomes. *Lancet* 1:1063–1065.
114. Bergsma D, ed. An international system for human cytogenetic nomenclature. Basel: S. Karger for the National Foundation-March of Dimes. *Birth Defects* 1978;14(8).
115. <http://www.acmg.net/>
116. <http://www.cap.org/>
117. Hastings RJ, Cavani S, Bricarelli FD, Patsalis PC, Kristoffersson U, ECA PWG Coordinators. Cytogenetic guidelines and quality assurance: a common European framework for quality assessment for constitutional and acquired cytogenetic investigations. *European Journal of Human Genetics* 2007; 15:525–527. doi:10.1038/sj.ejhg.5201809; published online 14 March 2007 <http://www.nature.com/ejhg/journal/v15/n5/full/5201809a.htm>

Contributed protocols

IMPORTANT: No protocol included in this manual should be used for clinical testing unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements for all chemicals used in the laboratory.

Protocol 2.1 Slide-making

Contributed by Oregon Health & Science Center, Knight Diagnostic Laboratories, Clinical Cytogenetics Laboratory

I. Principle

Good chromosome banding is dependent on appropriate slide preparation. The purpose of this procedure is to obtain an adequate number of metaphases/slide with appropriate morphology. Variables affecting preparation include relative humidity, room temperature, drying time, and number of cells/slide. Chromosomes that are too flat do not produce crisp banding; chromosomes that are too raised appear too dark and have a halo appearance under phase microscopy. These dark cells frequently result in uneven and poor banding. The perfect phase contrast image is one that is crisp and even with no visible cytoplasm.

Safety warning

All tissue specimens should be handled as biohazardous, using Universal Precautions. Use the laminar flow hood for all steps up to harvest spin. Wear a laboratory coat and protective gloves for all steps through slide-making. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with Sanimaster 4 (made fresh weekly) or 70% ethanol. Cover cuts with protective bandages even when gloves are worn. Dispose of Pasteur pipettes in sharps container. Wash hands thoroughly after removing gloves.

II. Materials

1. Chromosome cell suspension
Dilute pelleted suspension with fresh fixative to achieve a cell dilution that is thin enough to allow proper chromosome spreading but thick enough to find mitotic figures easily (cloudy but not milky-appearing suspension).
2. Float glass slides with painted end for labeling
3. Pasteur pipette and bulbs
4. Phase microscope with 16 \times lens
5. Blotting material, such as KimWipes
6. Hygrometer and thermometer for determining ambient humidity and temperature
7. Tray with wet paper towels for dry conditions

III. Method

The following is an example of a slide-making method for a clean, average sample on a day with 40% relative humidity and a temperature of 72 °F (22 °C).

1. Remove glass slides from the box and place them in a Coplin jar containing 95% ethanol.
2. Remove one of the slides from the ethanol and clean the upper surface of the slide with a KimWipe, using two or three forceful strokes to clean off manufacturing residues.
3. Dip the slide back in the ethanol and then into a beaker of clean, distilled water. Dip it in the water 8–10 times, or swirl it until the water coating is even and the signs of the mixing of alcohol and water disappear.
4. Hold the slide horizontally and make sure the water coating is even. Then place the long edge of the slide on an absorbent towel to drain excess water.
5. Gently aspirate some of the fixed cell suspension into a Pasteur pipette and, holding the slide at a 20–30° angle on the towel and starting at the nonlabel end, drop 2–4 drops of cell suspension from about 1/4 in. from the slide surface. The drops should not stream down the slide but should make round patterns. Drain the slide on paper towel.

6. Drop 4–6 drops of fixative across the slide, overlapping the drops.
7. Drain the slide on a paper towel or with a KimWipe.
8. Label the slide with patient information, slide number, and date.
9. When the slide has dried until rainbow colors appear on the surface, warm the slide on the back of the hand or the thigh for a few seconds.

IV. Notes

Technique variations for different environments:

1. For conditions in which the humidity is very high (45% or greater), try drying the slide continuously on hand, leg (thigh), or warming plate (temperature 35–40 °C).
2. For conditions in which the humidity is very low (30% or less), place slide on a stack of water-soaked paper towels and leave until completely dry.
3. Slide-making under other conditions may require combinations of drying environments that can only be discovered by experimentation. For example, use a very thin layer of water on very humid days or thicker on very dry ones. Drain slide after each drop of suspension is added on humid days and wipe back of slide several times on very humid days before warming. Do not wipe back of slide before putting on wet paper towels on very dry days.
4. Check on a phase microscope. If cells are gray or chromosomes are scattered, warm longer. If cells are very dark and show visible cytoplasm, reduce or eliminate warming.

Protocol 2.2 Slide-making

Anonymous contributor

I. Principle

Even though slide-making is the final step in the harvesting procedure, a technologist must have the ability to troubleshoot and customize a procedure to yield optimal results.

II. Materials

1. Methanol
Store in an enclosed and secure flammable cabinet, separate from acids, other reactive substances and human contact. Never lift bottle by cap. Transport in a rubber safety carrier. Label all receptacles containing this reagent. Use proper fume ventilation and PPE when handling reagent. Disposal must be in accordance with federal, state and local environmental control for flammable materials.
2. Glacial acetic acid
Store in a cool, dry, well-ventilated place away from incompatible materials. Never lift bottle by cap. Label all receptacles containing reagent. Transport in a rubber safety carrier. Label all receptacles containing reagent. Disposal must be in accordance with federal, state and local environmental control regulations. Use proper fume ventilation and PPE when handling reagent.
3. Modified Carnoy's fixative
Mix 1 part acetic acid to 3 parts methanol. Keep at 20 °C. Prepare fresh daily, as needed. *Warning:* use proper fume ventilation and PPE when handling reagent. If spreading is a problem in dry climates or regions, see Note 1.
4. Wheaton staining tray for 20 slides, Science Products #900200, Fisher Scientific #08-812.
5. Microscope slides
6. Sterile, distilled water

Cleaning slides

Slides are first visually inspected; any slide with even a questionable flaw should be discarded in the sharps container. With the scratchy label side all facing the same direction, slides are placed diagonally in a glass slide container, with both slide front and back freely exposed to the cleansing reagent. Fill the dish with fixative (see Note 2) so that the slides are fully immersed, and allow to sit for 15 minutes. Fixative is carefully decanted, and slides are rinsed twice in sterile distilled water.

- Fresh sterile, distilled water is added so that the slides are completely immersed, and the container is stored in the refrigerator. Expiration: 3 months. NOTE: Fixative must be discarded in container collector for Flammable Material pickup.
7. Warming plate at 40 °C and 60 °C
 8. Coverslips 24 × 50 mm
 9. Sealant (use under fume hood)
 10. Tweezers for lifting clean slides out of staining tray
 11. Paper Towels (for drainage)

III. Method

Slide-making is performed in a humidified, temperature-controlled environment (see Note 3). Never leave more than one centrifuge tube uncovered at a time. Do not drop more than one case at a time.

1. Cells should have had at least three 10 mL fixative changes and should have had its final centrifugation (1000 RPM for 10 minutes) so that the cells are in a pellet, ready for slide-making.
2. Depending upon size and condition of cell pellet, withdraw fixative up to an equal volume to the cell pellet. Resuspend pellet gently but ensuring that all material has been released in suspension.
3. Test slide surface by removing a slide from the water holder and running fresh fix down its surface. If the fixative does not flow straight and smoothly, for example, it buckles in pockets, discard slide and try another. If the problem persists, try another slide container and/or change fixative reagents. If the problem still persists, try a different slide lot or manufacturer (see Note 4).
4. If FISH is required and a more concentrated slide is desired, make the FISH slide first and then dilute pellet for routine cytogenetic analysis.
5. Labels for slides should have at least two patient identifiers, including patient name and lab accession number, vessel #, slide #, tech initials and date made.

Slide-making

6. Slide-making method 1: Lay wet slide onto paper towel and run a couple drops of fixative on the water surface. Resuspend cell pellet, withdraw 0.5 mL of cell suspension in Pasteur pipette and gently release two drops equidistant onto horizontal slide. Allow slide to remain flat for 1 minute. It should not become dry. Gently roll slide to its horizontal edge, drain excess, dry back, wave once and place onto 40 °C hotplate until dry. Check under phase microscopy to see if cells are spread sufficiently and metaphase concentration is of ideal density. Add more fixative if cells are too dense and try again. If the fixative dries too quickly (before a minute), increase humidity or reduce time it lays on paper towel before transferring to the hotplate. Label and initial.
7. Slide-making method 2: Remove slide from storage container without draining water from surface. Add a couple drops fixative to water and release two drops of cell suspension equidistant on slide while slowly tilting slide to its horizontal edge. Hold horizontal for one minute. Slide should not be dry yet. Dry back, blow gently on surface and place on 40 °C hotplate until dry. Check under phase microscopy for spreading and morphology. Label and initial.
8. Slide-making method 3: (a) place two drops fixative on slide, tilt slide at a 45° angle and gently run 2 or 3 drops down the center of the slide. (b) Release cell suspension from a higher distance. Lift slide to a vertical position; hold again for one minute, allowing the cells to drain slowly down the slide surface. Blow on slide, dry back, and place on 40 °C hotplate until dry. Check under phase microscopy. Label and initial.

Slide quality evaluation

9. If the distance between nuclei is too dilute (you see only sparse nuclei under a microscope field), bring pellet to a 10 mL volume with fixative and centrifuge at 800–1000 RPM for 10 minutes. Remove supernatant, resuspend in less fixative and re-drop.
10. When complete, resuspend pellet in 5 mL fresh fixative, close tightly and store in refrigerator for 6 months (see Note 5).
11. Artificially age slides for G banding on a 60 °C hotplate overnight. For emergency results, bake slides in a 96 °C for 45 minutes.

IV. Notes

1. Getting chromosomes to spread properly in very dry or very humid conditions can be difficult. Replacing the 1:3 ratio of acetic acid to methanol with 1:2 or 1:1 acetic acid–methanol solution, may help. The validation process must ensure that all subsequent steps have not been detrimentally affected by the ratio change.

2. FIXATIVE is a caustic reagent and requires advance planning in its usage – from storage and preparing, to use and discarding, in order to avoid any potential health hazard risks. Its fumes should be controlled via a fume hood. The staining dish filled with fixative should remain closed and undisturbed while slides are immersed. The dish should also be properly labeled of its contents and danger. A bottle containing either fixative or its reagents should never be carried by holding only the top of the jar; large reagent containers should be carried inside a bottle carrier specially designed for high-risk transport [for example, we use Rubber Bottle Carrier (red), 1 gal/5 pts, Lab Safety Supply #3328R]. Once complete, the fixative should be easily discarded with minimal handling and with no risk of spillage. As accidents may occur, precautions must be taken that, if spillage does occur, that it is contained and not within physical harm to the user. Spill directions should be visually displayed and/or easily referenced. In the event of a spill that does not harm the user, follow your institution's guidelines for toxic chemical spills. In the event of spillage onto the user, remove any potentially affected clothing immediately and immerse in the safety shower for 15 minutes. Seek medical attention immediately, whether you feel a burning sensation after the wash or not.
3. Fluctuation in temperature and humidity can affect the spreading of chromosomes during slide or coverslip drying. Hypotonic time may need adjusting if slide-making results are consistently less than optimal. Optimal harvest parameters in our laboratory are:

Humidity	Temperature	Hypotonic time
40–60%	75–88°F (24–31 °C)	25 min (unstimulated); 10 min (stimulated)

4. Slide-making depends heavily on the ability for metaphase chromosomes, held together within a fragile membrane, to flow unimpeded on the slide surface until it can rest and spread, without bursting too early (chromosome “soup”) or holding too tightly (chromosome “fudge”). Any obstruction that increases or decreases friction, or any change in environmental conditions (seasonal changes, humidity, temperature and airflow) that will increase or decrease fixative evaporation time, could affect slide quality. Culture and harvest parameters can also affect metaphase spreading.
5. Fumes from the fixative solution are corrosive and may eventually affect a refrigerator's electrical parts. Covering the tube with parafilm may help, but it is still not a total solution. Fixative is also flammable; therefore, care must be taken with historically held cell pellets. Storing these pellets in the freezer may help retard DNA degradation.

Protocol 2.3 Making wet slides for chromosome analysis

Adapted from a personal communication with Laura Adomaitis.

I. Principle

Making slides depends not just on the quality of the harvest, but it also depends on the atmospheric conditions of the day and on the quality of all reagents and materials used. The cytogenetics technologist must be resourceful enough to be able to recognize certain recurring problems and through an understanding of the process, be able to handle the unforeseen.

Safety precautions

Fixative is caustic and flammable and thus should be used and stored with proper fume ventilation, with full protection from spillage (see Note 1, Handling fixative).

II. Materials

General Supplies

1. Sterile bottled water, 1000 mL
2. Glass Pasteur pipettes, 5¼ in.
3. Sterile plastic Pasteur pipettes, 5¼ in.
4. Microscope slides
5. Microscope coverslips
6. CytoSeal
7. Glass slide tray/holder

8. Humidifier/temperature readout and controlled environment
9. Hot plates set to 40°C (for drying), 60°C (for aging overnight), and/or 90–96°C if same-day aging is needed for STAT cases
10. Phase microscope
11. Chemical harvest fume hood
12. Access to eye wash and shower
13. Vinyl labcoat and gloves

Reagents

1. 1:3 Fixative: Add 1 part glacial acetic acid to 3 parts absolute methanol. This solution should be made fresh periodically throughout the day. CAUTION: FLAMMABLE, CORROSIVE. Use proper PPE and proper ventilation to reduce caustic vapors.

III. Procedure

Preparing clean slides

1. Inspect each slide to ensure there are no scratches, streaks or chips. Discard defective slides in sharps container.
2. Stack slides into glass tray inserts.
3. Inspect glass dish to ensure there are no cracks. Glass dish should be able to hold 100 mL of liquid. Place dish within a protective tray (to control accidental spillage) under the chemical fume hood.
4. Add 25 mL of glacial acetic acid to 75 mL of methanol and carefully pour into glass dish. (Adjust volume if glass dish does not hold 100 mL) (see Note 2, Handling spillage).
5. Slowly immerse one slide tray insert into the glass dish of fixative. Cover and allow tray to sit for 5 minutes. Leave metal holder attached to tray. Meanwhile, fill a second glass dish with 100 mL of sterile, bottled water and place alongside the fixative dish.
6. After 5 minutes, carefully lift the slide tray insert above the fixative, allow fixative to drain into the dish, and place the tray of slides into the second glass dish with sterile bottled water.
7. Take this second dish to the sink, pour off water while running copious water, and add enough fresh, sterile bottled water to immerse the upper edge of the slides. Label dish cover with contents, date of cleaning and initials of preparer, and place in 4°C until ready to use. Slides can be prepared in advance and stored at 4°C for 2 weeks.
8. Continue the process with the second slide tray. Slowly immerse the next slide tray insert into the fixative dish under the fume hood and repeat the process until all slide trays have been cleaned and stored.

“Dropping” slides

NOTE: Work with only one patient at a time and keep only one centrifuge tube uncovered at a time. Change pipettes between centrifuge tubes, even on the same patient.

1. Confirm that humidity is 60–80% and temperature is 70–85°F (21–29°C) (the lower the temperature, the higher the humidity should be) in the area where slides are being made.
2. Depending on the size of the fixed cell pellet, supernatant is aspirated to 0.5 mL level or in equal volume with the size of the cell pellet. Cell suspension should be slightly opaque (see Note 3, When cell clumps are visible).
3. Confirm that name and accession # on tube matches slide label to be affixed. Number labels with Tube ID and unique slide identifier #. For example, 72T1 and 72T2 would be the first two slides made from culture 72T.
4. Remove one cold, wet (water) slide with forceps and run a few drops fixative along its surface (see Note 4, If fixative does not run down the slide evenly). Wipe back. Drop cell suspension onto wet slides in one of several methods below. **Do not rush the drying step.** Allow the fixative to complete its motion. You will see Newton's rings (rainbow) appear along the fixative surface. A ruffled edge indicates that the fixative “bubble” has imploded and the cells are planting. Place the slide on a 40°C hotplate until surface is dry.
5. Label slide and check under the phase microscope. If the concentration and spreading is optimal, make one more slide. If, however, the quality or quantity can be improved, change what needs to be changed (see Note 5: Troubleshooting) and re-drop.

Slide dropping positions

Horizontal method

Place slide on countertop. Run fixative over its surface. Drop 2 equidistant cell suspensions onto the fix/water mixture. After 30 seconds, drain the residual fixative onto a paper towel and wipe the back. Wave the slide up and down once and watch for Newton's rings (see Note 6, The vanishing rainbow). As soon as ruffled edges appear, place slide onto the 40°C hotplate.

Tilted horizontal method

Tilt slide on its long side at a 30° angle. Run fixative gently across surface. Gently place 2 drops at the top edge of slide and tilt the slide as the cells run down the short width of the slide. Blow gently and watch surface. When Newton's rings and edge ruffles appear, place on hotplate to dry. If, however, the cells are tight, wave the slide at a slow pace for 30 seconds. If they are still tight, tap gently on counter twice, wave, repeat tap and wave until slide is dry.

Tilted vertical method

Remove slides with water still retained on surface of slide. Place a couple of fixative drops to the surface and tilt slide lengthwise at a 30° to 60° angle (see Figure 2.22). Add the cell suspension while tilted, allowing cells to run down the surface, from label to lower edge. Tap moderately on counter edge, about 1 second between taps, until slide is dry. Place on 40°C hotplate until completely dry.

IV. Notes

1. Handling fixative: Acetic acid can cause severe burns. Although methanol may neutralize some of its toxicity, it is still a reagent that should be used with a fume hood and any contact should be immediately treated. Do not carry any bottle containing fixative by the lid, as accidents have been reported because the top was not securely closed. Thermo Fisher Scientific SDS for acetic acid provides the following first aid measures for acetic acid exposure [1]:

Eye contact: Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required.



Figure 2.22 Tilted vertical slide method. Tilt wet, cold slide at a 30–60° angle, rinse with fixative, and allow two drops of cell suspension to run from label to lower edge. Tap moderately on counter edge, about 1 second between taps, until slide is dry.

Skin contact: Wash off immediately with plenty of water for at least 15 minutes. Immediate medical attention is required.

Inhalation: Move to fresh air. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance; induce artificial respiration with a respiratory medical device. Immediate medical attention is required.

Ingestion: Do not induce vomiting. Call a physician or Poison Control Center immediately.

2. Handling spillage: In the event of a spill while under the fume hood and within the protective tray, suction spillage into glass decanter and discard in liquid chemical waste receptacle. If the spill occurs in any other uncontrolled manner, dial 'O' for Operator and call for a "Code 100" to alert the assistance of the Chemical Emergency Cleanup team. See First Aid Measures for immediate attention.
3. When cell clumps are visible: Allow larger clumps to settle (or remove them to a labeled tube), or take the cell-fixative suspension from above the particles, without remixing) in order to have a cleaner slide preparation.
4. An uneven fixative runoff: Many factors can obstruct the metaphase rhythm and expansion during the slide-making process. Air movement caused by the fume hood fan, centrifuge vibration on the countertop, nearby construction rumbling the foundation, can all affect the critical moment when the cell collapses onto the glass surface. Therefore, conditions need to be ideal when making slides. Sometimes, however, the cause may have nothing to do with the air or countertop. In the early 2000s, a trusted slide manufacturer decided to improve their slides with a chemical that made their appearance whiter and for a lab, gave a much cleaner look. But the chemical created submicroscopic nicks into the glass surface that caused havoc to the cytogenetics community. If the fixative does not run smoothly down the slide, the cells may also find difficulty resting flat on its surface. Discard or re-clean the slide, and if the problem persists, find a new lot or different manufacturer, preferably one that the cytogenetics community currently uses with proven success, and compare results.
5. Troubleshooting
 - a. Too crowded
 - Dilute and re-drop.
 - b. Lots of nuclei but very few metaphases
 - Too much blood set up in culture; not enough buffy coat; too long in hypotonic or wrong concentration; mitogen expired; Colcemid® not added; harvested at improper time range; pregnant women have a low yield; children or patients on medications may also have a low yield.
 - c. Too sparse
 - Re-spin and make more concentrated.
 - d. Too tight
 - Check humidity; slides are drying too fast; re-fix and try again; use warm slides (e.g., drop cell suspension while slide is on 40°C hotplate); blow on slide while still wet; shorten wait time before placing on hotplate; try another slide method; dilute; re-fix and aggressively pipet before dropping; layer 1 : 1 or 2 : 1 (methanol : acetic acid) fixative on slide before dropping the cell suspension (this should slow down the drying process).
 - e. All I got was chromosome soup
 - Check that humidity isn't too high or temperature too low; fixative is drying too slowly; too long in hypotonic; dry flat; try a different method; do not blow on slide or wave.
 - f. Short, dark, and stubby
 - Slides drying too quickly; check humidity/temperature. Could be a harvest problem: take off more supernatant before adding hypotonic; leave more supernatant over pellet before first fixative; slow down the fixation process; reduce time or quantity in Colcemid®.
 - g. Chromosomes constantly too skinny
 - Try using blood/bone marrow hypotonic with a ratio of 1 : 2 sodium citrate/KCl.
 - h. Intruding cytoplasm
 - Re-fix and mix well. Could be a harvest problem: not enough time in hypotonic; slow down fixation process.
 - i. Uneven homologues, especially on the periphery
 - Slow down slide-making; hold back placing slide on hotplate until the edges show ridges. Could be a harvest problem: slow down first fixative procedure.
 - j. Mushy chromosomes
 - Check fixative reagents; fix pellet a couple more times; increase slide aging; decrease trypsin time during banding; decrease stain concentration.
 - k. Full of holes
 - Insufficient aging; insufficient drying after banding before covering
 - Hot plate temperature too high (should be 60°C for overnight or 96°C for the "quick-bake" aging process in 30–45 minutes).

The vanishing rainbow

Although Robert Hooke first described this rainbow phenomenon in his 1664 book *Micrognathia*, Isaac Newton in 1717 was the first who studied its formation. The refraction of light at varying interferences between the convex fixative drop resting on the flat glass surface creates this rainbow effect. As the convex fixative bubble “flattens,” the cells become pressed onto the glass slide surface. This provides a visual indication for when the fixative meniscus collapses. http://en.wikipedia.org/wiki/Newton's_rings

Reference

1. Thermo Fisher Scientific Material Safety Data Sheet (MSDS) for Acetic Acid, CAS# 64-19-7. Revised July 30, 2010. 2nd rev.

Protocol 2.4 Slide-making

Adapted from the third edition of the *AGT Cytogenetics Laboratory Manual*, contributed by Brigham and Women’s Hospital, Boston, MA, p. 51.

I. Principle

Slide-making is best done after the cells have been in fixative at 4 °C overnight. However, good results can be achieved from material that is freshly harvested.

II. Procedure

1. Aspirate the remaining supernatant from the last centrifuge procedure. (If tubes have been sitting overnight, mix and then centrifuge the tubes for 10 minutes at 1200 RPM.) Add sufficient fresh 3:1 fixative (methanol-acetic acid) to make the cell suspension slightly turbid. Note: The amount of fixative added will vary depending on the pellet size.
2. Using a diamond-tipped pencil, follow your institution’s requirements for labeling slides, for example, case number and second patient identifier, tube letter, and slide number. Depending on atmospheric conditions and the characteristics of each sample, obtaining the best results may necessitate a variety of methods for preparing the slides before dropping the cells. Slides can be dipped in cold distilled water; steamed over a hot water bath; steamed with breath; dipped in methanol and allowed to dry; or soaked in 6:1 fixative.
3. Gently resuspend the cell pellet, using a Pasteur pipette. Drop 3–6 drops of cell suspension at one end of the slide and allow the drops to spread across the slide surface. Place the slide on a slide warmer at 75 °C to dry.
4. Check the slide for the presence/absence of metaphase spreads and for the degree of spreading using a phase contrast objective. Use this information to adjust or modify your slide-making conditions. Record the quality of the preparation in the Harvest Quality Assurance book.
5. Slides can be stained with fluorescent stains immediately. For Giemsa staining, it is best to allow the slides to dry on the slide warmer at 75 °C for approximately 2 hours and 15 minutes or bake for 1 hour at 90 °C.
6. Unstained slides are stored on metal trays by case number in slide files.

Protocol 2.5 Slide preparation

Adapted from a protocol from the third edition of the *AGT Cytogenetics Laboratory Manual*, that was contributed by Stanford University, Palo Alto, CA, pp. 61–63.

I. Principle

Chromosome slide preparations are made by dropping harvested cell suspensions on a wet slide, flooding with fixative, and air-drying. Properly prepared slides will yield mitotic figures that are well spread and of a contrast, when observed with phase microscopy, that will allow adequate G banding and subsequent chromosome analysis to be performed. Many different methods exist for slide preparation, and the following is presented as one protocol which, when consistently applied, will

satisfy the above requirements. It is not intended to constrain individual technicians methodologically, only qualitatively. Any variation that produces results consistent with the following quality control guidelines is acceptable.

Specimen

Harvested cell suspension from any chromosome analysis protocol. Slide preparations are best made on the same day of harvest, mainly as a matter of convenience. Slide preparation can be delayed for one or more days without problem and may in some cases benefit (e.g., difficult bone marrow) harvests. Metaphase spreading on harvests left longer than several days may be problematic.

II. Materials and equipment

1. KimWipes
2. Microscope slides, frosted (VWR #48312-079)
3. Paper towels
4. Absolute methanol, (JT Baker #9070-1)
5. Glacial acetic acid (JT Baker #9507-1)
6. Pasteur pipette, 5-3/4 (VWR #14673-010)
7. Oven, 90°C
8. Phase contrast microscope

Reagent preparation

9. Methanol-acetic acid fixative: One part glacial acetic acid to three parts absolute methanol. Make fresh.

Quality control

Quality control is an ongoing process every time slides are made. Each slide should be examined under phase microscopy to determine if a sufficient number of metaphases show adequate spreading and contrast. Never make more than two or three slides without monitoring their quality. For specifics of manipulating the process to optimize spreading and contrast, see Troubleshooting.

Unacceptable results: Preparation in which a preponderance of metaphases are of such poor spreading or contrast as to preclude successful G-banding and analysis.

III. Procedure

1. Store slides in a Coplin jar of absolute methanol. Remove slide from methanol and polish with a folded KimWipe. Dip the slide back in methanol and swirl it in a beaker of distilled water until the methanol is dissolved and a uniform, thin film of water covers the slide.
2. Holding the ground glass end between thumb and finger, vertically blot the long side of the slide on a paper towel to remove excess water. Keeping the edge in contact with the paper towel, lower the slide until it forms a 30° angle with the benchtop, with uniform water film up.
3. From a horizontally held Pasteur pipette, 1–2 inches above the slide, place 3 drops of cell suspension evenly spaced onto the slide, moving successively toward the frosted end. The droplets should strike the slide one-third of the width from the top of the horizontally positioned slide and should burst and spread evenly as they strike the slide.
4. Tilt the slide back to vertical and drain it a moment. Lower it again to a 30° angle and, dropwise with a Pasteur pipette, flood the slide with fresh 3:1 methanol–acetic acid fixative, starting at the upper corner and moving toward the ground glass end. This will evenly displace any remaining water and allow the slide to dry uniformly.
5. Again drain the slide for a moment, wipe off the back, and then air-dry the slide in such a manner as to provide good chromosome spreading and morphology (see Troubleshooting).
6. Label slide according to laboratory requirements, which may include laboratory number, second patient identifier, culture letter, unique sequential number, and date.
7. Artificially age the slide in an oven at 90°C for 30 minutes.

IV. Troubleshooting

Procedural consistency is essential when preparing chromosome slides. This will subsequently allow consistent and systematic G-banding. To achieve consistency, each slide must be monitored with phase contrast microscopy. Slides are examined for good cell concentration, chromosome spreading, morphology and mitotic index.

1. Good chromosome preparations should be spread adequately for easy counting and analysis. Overspreading should be avoided as this will cause artifactual hypomodal chromosome counts and difficulty in photomicroscopy.
2. Chromosomes should appear sharp and dark, without “light halos” surrounding them. Likewise, they should not be fuzzy, pale or “grayed out.”
3. The most critical and variable step is air-drying the slides. This must be worked out for each session, then adhered to and monitored for each slide. In general, poorly spread chromosomes with halos are drying too fast and may be slowed by placing them to dry on a wet paper towel or at a vertical or steep angle, breathing lightly on the slide after fix flooding or by chilling the fix. Also in general, pale, gray, or overspread chromosomes are drying too slowly and may be corrected by lowering the drying angle, briefly warming the slide, or waving the slide before setting it down to dry.
4. Drying is very sensitive to humidity and temperature, and so will vary from day to day. A certain amount of imagination and experimentation is helpful. Remember, though, that simplicity of technique is desirable and will lead to more consistent results and subsequently to easier and better trypsin G-banding.
5. Heating of the slide is also important. This is artificial aging and should be done within the limits stated in the procedure. Overheating or underheating, whether by varying temperature or time, will lead to under- or oversensitivity to trypsin pretreatment during G-banding.

References

1. Personal communication with Mike Brown's technique, Clinical Cytogenetics Lab, Oregon Health Sciences University, Portland, Oregon
2. Hack M, Lawce H, eds. *The Association of Cytogenetic Technologists Cytogenetics Laboratory Manual*. San Francisco: Association of Cytogenetic Technologists, 1980.

Protocol 2.6 Slide preparation procedure

Adapted from a protocol from the third edition of the *AGT Cytogenetics Laboratory Manual*, that was contributed by the University Medical Center, Ohio State University, Columbus, Ohio, pp 73–74.

I. Principle

A homogeneous cell suspension is dropped on detergent or acid alcohol-cleaned slides from a height and dilution such that the metaphase spreads as well as the chromosomes themselves will be spread apart from one another without breaking the cell membranes. A ruptured cell membrane will result in chromosomes scattering on the slide surface, producing metaphases that are both difficult and inappropriate to analyze. After dropping the cell suspension, the slides are placed briefly on a hotplate and then into a hot oven to aid the drying process. The slides are banded according to the technique deemed necessary for the type of analysis. Slide preparation is the single most important step in achieving good results. Poorly prepared slides will result in poor banding and tedious analysis.

II. Materials

1. Fixative
 - a. Methanol, analytical reagent grade
 - b. Glacial acetic acid, analytical reagent grade

Preparation of working solution

1. Measure with a 100 mL graduated cylinder, 75 mL (3 parts methanol) to 25 mL (1 part) glacial acetic acid.
2. Pour into 100 mL bottle and cap lightly. Label as “Fix” with warning: CORROSIVE/FLAMMABLE. Prepare fixative immediately before it is needed. Always keep bottle capped when not in use. Discard daily any unused portion into safety canister, according to your institution’s Chemical Management Guidelines. Mark date, type and concentration of compound, quantity discarded and initials on tag attached to safety can.

Quality control

1. Microscopically check first slide dropped, assessing quality and quantity of metaphase spreads present.
2. Adjust dropping technique accordingly for successive slides dropped.
3. Check microscopically each successive slide dropped from that culture. Readjust dropping technique accordingly.
4. Check first slide on each remaining culture tube to be dropped, and each successive slide thereafter, for chromosome spreading and background cytoplasm.
5. Tolerance limits: Aim for well spread chromosomes with no cytoplasm.

III. Procedure

1. Using a Pasteur pipette, place three drops of fixed cell suspension evenly over clean slide immersed in 37°C distilled water.
2. Slides can be shaken and/or blown on gently to facilitate chromosome spreading.
3. Place on wet paper towels for 5–30 seconds if humidity 40%.
4. Place on 65°C slide warmer for 5–30 seconds, as necessary.
5. Check slide using phase contrast microscope for quality of cell suspension, number of metaphases, quality of metaphases, and spreading. Document on specimen setup sheet the quantity and quality of metaphase spreads present in each culture tube.

IV. Troubleshooting

1. Slide too thick with nuclei and/or mitotic figures
 - a. Dilute with more fixative.
2. Slides too thin
 - a. Spin and remove some of the fixative.
3. Poorly spread and/or cytoplasmic background
 - a. Increase height from which cells are dropped from pipette onto slide.
 - b. Use additional washes in fixative.
 - c. Increase concentration of glacial acetic acid in fixative.
 - d. Substitute 50% glacial acetic acid in fixative.
 - e. Use steam and less time on slide warmer.
 - f. Use combination of above methods.
4. Scattered chromosomes and/or broken spreads
 - a. Decrease height from which cells are dropped from pipette onto slide.
 - b. Use more time on slide warmer.
 - c. Use colder water and/or frozen slides.
 - d. Use combination of above methods.
5. If none of the above rectifies the problem, refrigerate and drop the following day.

CHAPTER 3

Peripheral blood cytogenetic methods

Helen J. Lawce¹ and Michael G. Brown²

¹Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA

²(retired), Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA

3.1 Using peripheral blood for cytogenetic analysis

Peripheral blood is the most commonly utilized tissue for determination of constitutional karyotypes because it usually yields the longest and best-banded chromosomes (Figure 3.1) and can be obtained easily. Peripheral blood is obtained in a relatively noninvasive manner; is inexpensive to culture; requires only short culture durations; can be recultured from the original sample; and can be mailed successfully. Peripheral blood lymphocytes are not dividing in normal, healthy adults; they must be stimulated to divide by exposure to mitogens, such as phytohemagglutinin or pokeweed antigen.

Clinical uses of stimulated peripheral blood cultures include determination of the constitutional karyotype of patients requiring genetic diagnosis or genetic counseling; determination of the constitutional karyotype of families of individuals with chromosome abnormalities to determine carrier status of a familial translocation; comparison of lymphocyte karyotypes with those of other tissues (skin, amniotic fluid, or chorionic villus sampling) for confirmation of structural and numerical abnormalities or mosaicism; determination of the constitutional karyotype of patients with hematologic malignancies or solid tumors for comparison with chromosome abnormalities in their neoplastic cells (see Chapter 11, section 11.7.2, Cytogenetic methodology); assessment of chromosome damage in individuals exposed to environmental hazards; aid in the diagnosis of chromosome breakage syndromes such as Bloom syndrome, Fanconi anemia (Chapter 13, section 13.2, Fanconi anemia), and ataxia telangiectasia; and to provide material for interphase and metaphase FISH (fluorescence *in situ* hybridization) studies. Most acquired abnormalities in hematologic malignancies are studied with bone marrow or unstimulated blood cultures; however, certain hematologic malignancies, such as chronic lymphocytic leukemia, are best studied with stimulated blood cultures, using various mitogens (e.g., pokeweed mitogen or IL-4) for various culture durations (see Chapter 11, Protocols 11.3–4). This chapter will deal mainly with culture of non-neoplastic blood cultures.

Clinical uses of unstimulated blood cultures include determination of the acquired karyotypes of spontaneously dividing cells from hematologic malignancies (see Chapter 11, section 11.7.2, Cytogenetic methodology), and constitutional karyotypes from directly harvested or 24-hour cultures of neonatal blood samples (e.g., umbilical cord blood from newborns or periumbilical blood samples (PUBS) obtained prenatally). This is possible because of the presence of immature cells (blasts) in these samples that are capable of dividing and yielding mitotic cells that can be analyzed for STAT chromosome results.

Although blood cultures have many advantages, they are not the best choice for every situation. Evaluation of cases in which mosaicism could be present could miss some of the different cell lineages when only blood is studied, because some cell lines may be lost over time (especially in rapidly dividing tissues) or may not manifest in blood tissue at all (e.g., Pallister–Killian syndrome and mosaic trisomy 20). There are three germ layers in the embryonic blastoderm: **endoderm**, the innermost layer that gives rise to the epithelium of the digestive tract, the respiratory organs, bladder, vagina, and urethra (from which many amniotic cells may originate); **mesoderm**, the middle layer, which gives rise to all connective tissues, including muscular, skeletal, circulatory, lymphatic, and urogenital, plus body cavity linings; and **ectoderm**, the outer layer, which forms skin, nervous system, and so forth. Blood is from mesodermal germ cell layers and will not exhibit cell lines that are confined to ectoderm or endoderm, nor will it confirm abnormalities seen in some extra-fetal cell lineages such as certain confined placental mosaicism (CPM). In spontaneous abortuses, it is extremely important to study extra-fetal material in addition to blood, skin,

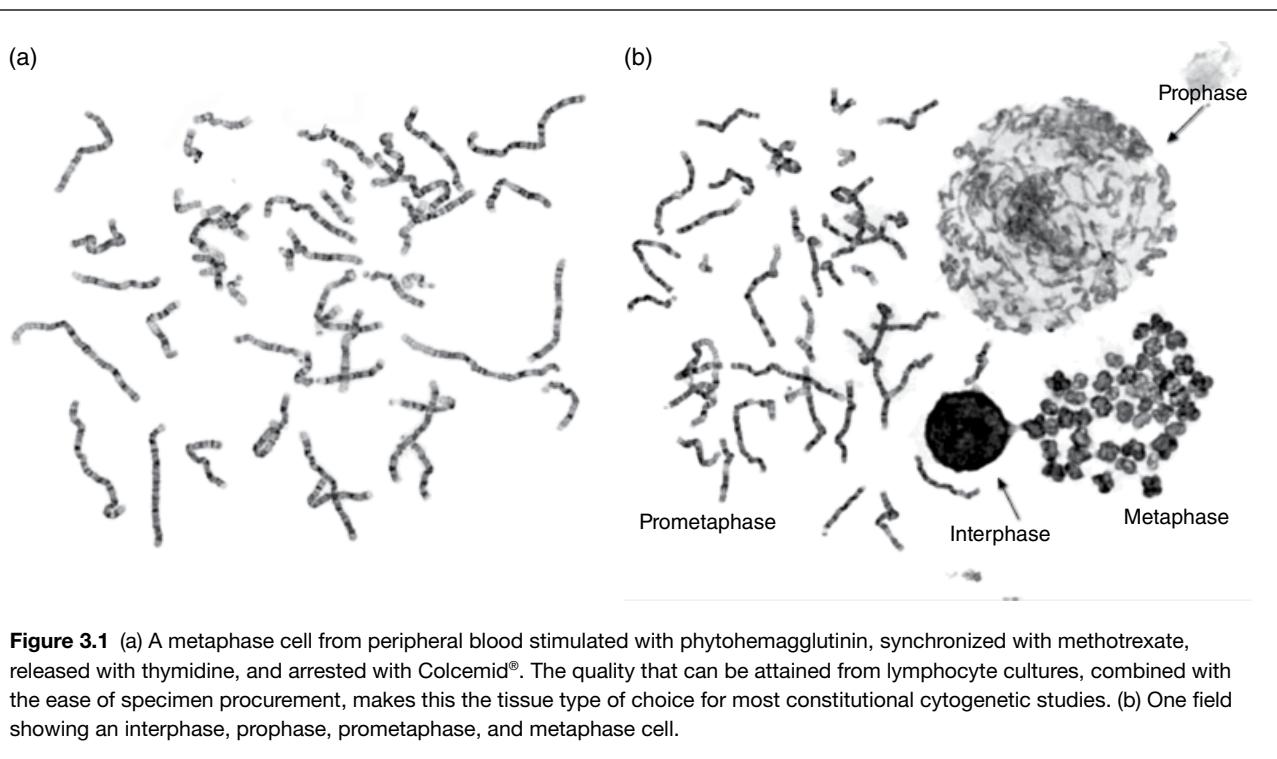


Figure 3.1 (a) A metaphase cell from peripheral blood stimulated with phytohemagglutinin, synchronized with methotrexate, released with thymidine, and arrested with Colcemid®. The quality that can be attained from lymphocyte cultures, combined with the ease of specimen procurement, makes this the tissue type of choice for most constitutional cytogenetic studies. (b) One field showing an interphase, prophase, prometaphase, and metaphase cell.

etc., to look for confined placental mosaicism (see Chapter 5, section 5.6.2, Confined placental mosaicism and uniparental disomy). In suspected mosaics, other tissue (skin, gonad) should be examined cytogenetically, if possible, in addition to blood cultures.

Research uses of stimulated blood cultures include creating lymphoblast cell lines; performing microdissection and PCR (polymerase chain reaction); gene mapping; comparative genomic hybridization (CGH); chromosome organization research; animal karyotyping; and chromosome evolutionary studies.

3.2 Special uses of peripheral blood cultures

3.2.1 Chromosome instability syndromes

Peripheral blood cultures are used to study the genetic syndromes with DNA repair, replication, or recombination defects. Examples of these include Fanconi anemia [1–8], ataxia telangiectasia [9,10], and Bloom syndrome [9]. The phenotypic pattern includes growth restrictions, immune deficiencies, and predisposition to malignancy. Fanconi anemia is an autosomal recessive disorder characterized by progressive pancytopenia, diverse congenital malformations, and a predisposition to malignancies [3–5]. Clinical signs vary and can include some or all of the following: cafe-au-lait spots, absent thumbs, microcephaly, radio-ulnar synostosis or aplasia, hypotelorism, short stature, renal and genital anomalies, heart defects, hearing loss, intellectual disabilities, low birth weight, and aplastic anemia [2]. Owing to the variable expression of the disorder, diagnosis by clinical manifestations alone is unreliable. Fanconi anemia cells exhibit a higher percentage of chromosome breaks and radial formations than cells from normal individuals when cultured with DNA-damaging clastogenic agents, such as diepoxybutane (DEB) and mitomycin C (MMC) [2]. Breakage analysis combined with the clinical picture provides an accurate diagnosis of patients with Fanconi anemia. Treatment for Fanconi anemia patients is different from that for other anemias, so it is very important to obtain the correct diagnosis. For example, treating a Fanconi anemia patient with radiotherapy, chemotherapy, or immune suppression before bone marrow transplant would have devastating results because of their chromosome instability. Current therapy strategies include bone marrow transplant, androgen therapy, and hematopoietic growth factors.

Cytogenetic breakage analysis is most commonly performed on peripheral lymphocytes. Cultures are set up in the usual manner and cultured in the clastogenic agent for at least 48 hours. Metaphases (50–100) of nonbanded Giemsa slides are scored for breaks and radial formations. Control cultures are performed also to determine effectiveness of the clastogenic agent and to determine reference ranges [2]. Prenatal testing has been performed [6] on perumbilical blood samples and on chorionic villi and amniotic fluid cells for couples with a previously affected child. Normal baseline breakage frequencies are being developed on these latter cell types, as well as normal and affected fibroblasts. Various cell types have different sensitivities to clastogenic agents, so the dosage has to be carefully established.

The molecular pathogenesis for Fanconi anemia is unknown, but 13 genes (complementation groups A, B, C, D, etc.) have been found [7,8] (Chapter 13, section 13.2, Fanconi Anemia). Cloning and characterization of all the complementation groups is work currently in progress. Patients will benefit by their classification into the various complementation groups and the establishment of a correlation between their genotypes and phenotypes. Understanding more about the genes responsible for the disorder will enable new drug treatments and gene therapy.

Ataxia telangiectasia is an autosomal recessive condition that includes symptoms, such as progressive cerebellar ataxia, disorders of skin, gonad and endocrine systems, immunodeficiency, and oculocutaneous telangiectases. In many patients, spontaneous chromosomal breakage (of both chromatids at homologous sites) is found in lymphocytes and less in fibroblasts. This breakage may fluctuate in frequency during the patient's lifetime. Ataxia telangiectasia patients often exhibit spontaneous chromosomal rearrangements, often involving chromosomes 7 and 14. Lymphocytes from these patients grow slowly and respond poorly to mitogens. B- and T-cell mitogens are often used in the evaluation of AT.

Bloom syndrome patients exhibit long pointed facies, rash on cheeks and nose, and immunodeficiencies. The hallmark of Bloom syndrome is spontaneous symmetrical quadriradial figures in lymphocytes and fibroblasts. A tendency to undergo homologous interchanges may be manifested as an increase in sister chromatid exchanges (see Chapter 6, section 6.3.3, Sister chromatid exchanges). All of these genetic conditions are heterogeneous, and symptoms may overlap with other diseases and conditions. Thus, a method to diagnose the chromosome instabilities is valuable.

The instability syndromes may demonstrate an elevated spontaneous breakage rate in peripheral blood cultures, but each can be cultured in the presence of specific clastogens to quantitatively test for the condition. For ataxia telangiectasia, cells are cultured as usual for 72 hours, and are exposed to either G2 X-irradiation or bleomycin for the final 3 hours. For Fanconi anemia, either DEB or MMC is added to the blood cultures to induce increased breakage but more specifically to form multiple chromosomal rearrangements known as radial forms (triradials, quadriradials, etc.) (see Chapter 13, section 13.2, Fanconi anemia). For Bloom syndrome, BrdU is added to blood cultures for two complete cell cycles after which sister chromatid exchanges are monitored. For all of these, multiple positive and negative controls are necessary to establish comparative parameters. Also, because these diseases are relatively rare and interpretation may be complex, only experienced laboratories should perform tests, or samples should be split with an experienced laboratory until proficiency is attained.

3.2.2 Fragile sites

Fragile sites are chromosome breaks or gaps that occur at a high frequency in a particular breakpoint in some individuals, often expressed under specific culture conditions. Some of the fragile sites are inherited, and of these, the fragile X at Xq27.3 has clinical significance as it is commonly associated with a group of patients who, in many cases, demonstrate familial intellectual disabilities. Other fragile sites occur in the autosomes in response to folate deficiency or other chemical treatments and do not seem to be clinically significant at this time. One such autosomal fragile site, fragile 16q22, manifests spontaneously in culture yet seems clinically inert.

Historically, the fragile Xq27.3, although occasionally seen in cells grown in regular cultures, usually required folate deficiency to manifest itself. Often the use of several culture parameters was performed to diagnose the condition. The usual methods for inducing expression of the fragile X were folate deprivation, either by culturing the cells in medium with low folic acid (Medium 199, for example) or by using antifolates such as trimethoprim, which is an antibiotic, or with addition of thymidylate synthetase inhibitors, such as FUdR or methotrexate. Excess thymidine would also induce the fragile X site expression. There are now DNA tests for fragile X that are more reliable than the chromosomal fragile X tests, especially for detecting fragile X carrier status. FMR1 repeat and methylation studies are now the test method of choice for fragile X syndrome. However, it is highly recommended that standard cytogenetic studies using G-banded chromosomes be a part of the fragile X workup, because a significant number of cases referred for fragile X testing turn out to have numerical or structural chromosome aberrations, instead of the fragile X syndrome.

Another caveat is that some putative fragile sites may be interstitial NOR insertions. We have seen two cases of interstitial insertions of NOR-positive stalks into chromosome 20 long arm, and other autosomes have also been seen with stalks inserted in one of the arms (Stupca, personal communication). Positive silver staining in the area will confirm the finding (see Chapter 6, section 6.2.8, Silver staining for nucleolus organizing regions).

3.3 Peripheral blood constituents

Peripheral blood is formed by hematopoiesis in the bone marrow, and the lymph nodes contribute lymphocytes and monocytes, as well. When the marrow fails, other tissues may become hematopoietic, such as spleen.

Peripheral blood contains the following components: erythrocytes (red blood cells), leukocytes (white blood cells), platelets, serum proteins, lipids, fibrinogen, glucose, water, gases, salts, antibodies and other protective substances, hormones, and excretory products such as urea, uric acid, and creatinine. If whole blood is allowed to clot, the remaining liquid is called serum. If an anticoagulant (such as heparin) is added to whole blood and the blood is allowed to settle or is centrifuged, the clear fluid that

occupies the upper half of the centrifuge tube is called plasma. The red cells settle to the bottom of the tube, and between the plasma and the red cells is a buff-colored or whitish layer called the buffy coat, which contains primarily white cells. When the plasma and coat are withdrawn from the red cells, a plasma-leukocyte suspension (PLS) is obtained.

The mononuclear leukocytes, called lymphocytes, are the cells used for routine cytogenetic studies. In normal circulating peripheral blood, about 20–40% of the white blood cells are lymphocytes; the remaining cells are neutrophils (segmented), 40–60%; eosinophils, 1–3%; basophils, 0–1%; and monocytes, 4–8%. Fewer than 2% of lymphocytes are large lymphocytes. Most are small lymphocytes, of which 55–75% are T cells (thymus-dependent cells responsible for cellular immunity), and 15–30% are B cells (bursa-dependent cells responsible for humoral immunity or antibody production). The T and B cells are not distinguishable from each other when observed using a light microscope, but they do differ in their response to mitogens. In the presence of mitogens, small lymphocytes undergo a process known as transformation and de-differentiate into a short term lymphoblastic cell, which is a large cell possessing staining properties of the nucleus that are similar to those seen in blasts, and now, most importantly, have the ability to divide when stimulated (Figure 3.2). Peripheral

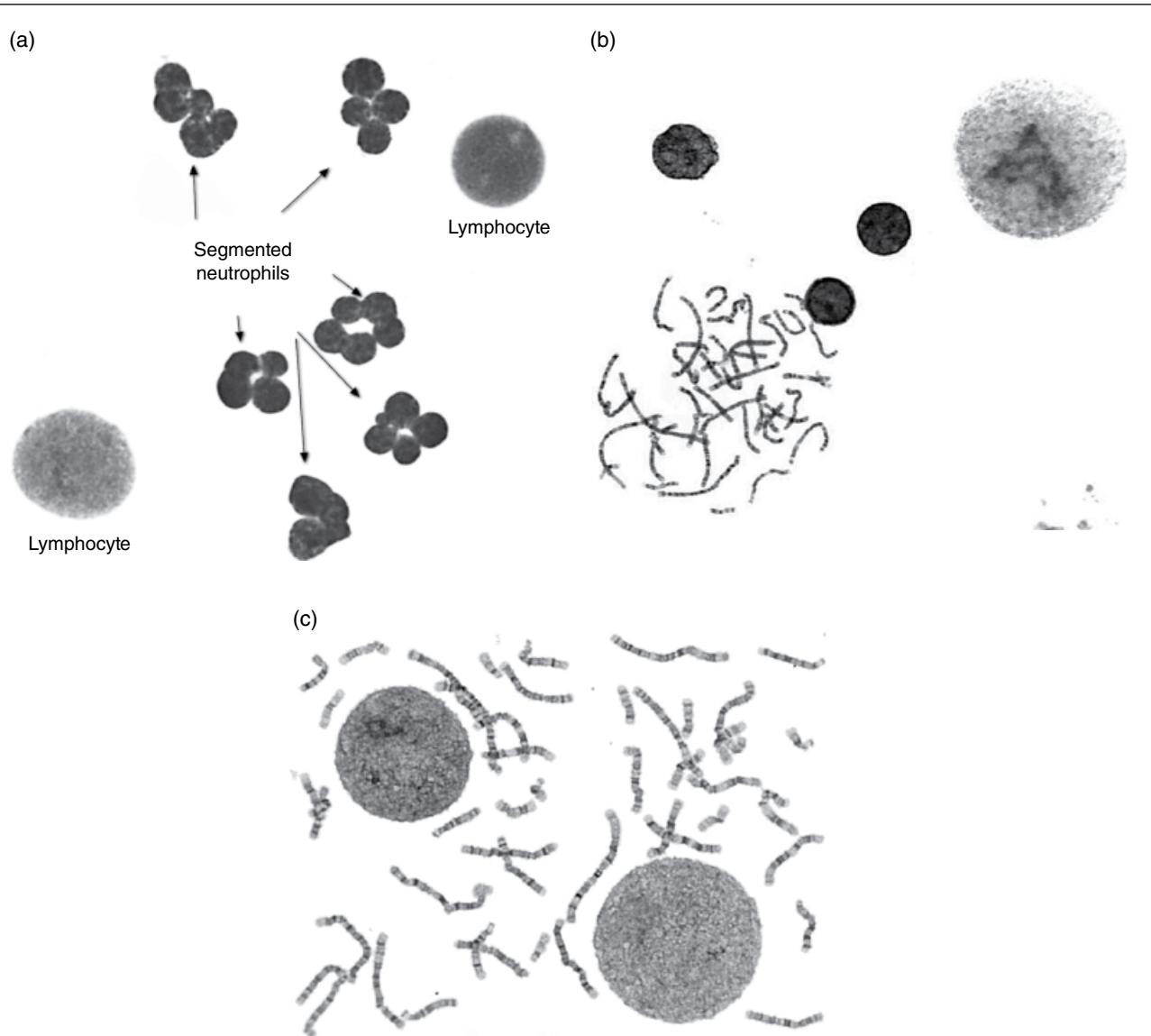


Figure 3.2 Interphase and metaphase nuclei in peripheral blood cultures. (a) Lymphocyte cultures after 24 hours. Notice the multilobed polymorphonuclear cells (segmented neutrophils) from the myeloid series that do not respond to mitogens. (b) Lymphocyte cultures after 48 hours. Note the presence of transformed (large) and nontransformed (small, dense) nuclei. (c) Lymphocytes from a 72-hour culture. Most polymorphonuclear cells are absent, and the lymphocyte nuclei are large, with open, decondensed chromatin. It is the action of the mitogens that transforms the nuclei.

blood does not normally divide in culture without external stimulating agents (mitogens). Mitogens include plant lectins (carbohydrate-binding proteins that can agglutinate red cells), such as pokeweed mitogens and phytohemagglutinin (PHA), viruses such as Epstein–Barr (EBV), and proteins from bacterial cells. Certain mitogens like concanavalin A (ConA) or phytohemagglutinin stimulate primarily the T-cell population (thymus-dependent lymphocytes), and others (e.g., pokeweed mitogens and Epstein–Barr virus) affect primarily the B cells (bone marrow-dependent lymphocytes). Some, such as pokeweed, stimulate both to some degree. Certain diseases (e.g., ataxia–telangiectasia, athymic DiGeorge syndrome, and lymphomas) may respond better to a B-cell mitogen. Occasionally, use of a combination (cocktail) of B- and T-cell mitogens may be useful, as in study of animal chromosomes, which can be difficult to grow (see Chapter 24, Protocol 24.3 Whole blood microculture).

3.4 Specimen handling [12,16–18]

3.4.1 Stimulants

The most commonly used mitogen for cytogenetic studies is phytohemagglutinin. When the T-cell lymphocytes are exposed to this plant antigen (an extract from the red kidney bean), they respond as they would in the body to a nonself (foreign) substance. This response causes the mature T cells to dedifferentiate into a T-lymphoblastic cell. It is in this immature blast form that the T cell has the capacity to synthesize DNA in preparation to undergo mitosis. During the first 24-hour exposure to PHA, the T cells undergo this transformation, and as the culture duration increases, they undergo several rounds of cell division. Each round is approximately 18 to 22 hours, and results in a wave of mitotic cells every day in culture. Removal of PHA from cultures after day one will not alter mitotic stimulation, although this is rarely done in the clinical cytogenetics laboratory. Peak mitotic activity is reached around 68–70 hours in culture and is considered to be the optimum harvesting point for chromosome studies [11] (Figure 3.2). However, mitotic figures are seen using other culture times: in newborns, because there may be many circulating blasts present, especially in a cord blood sample, a few metaphases may be seen as early as 24 hours, a larger number seen in a 48-hour culture, with the 72-hour culture being optimum; in adults, especially pregnant women, although 72-hour cultures are quite satisfactory, a 96-hour culture may produce a higher number of mitotic figures. Therefore, the most appropriate culture duration may depend on a number of factors including: the patient's age and response to PHA, the temperature of the laboratory's incubator and the culturing conditions and harvesting methods used by each laboratory. Every laboratory should coordinate the setup time and harvest time, along with any culturing and harvesting procedures, to optimize the recovery of the best number and quality of metaphases that will ensure the highest quality cytogenetics analysis.

Most of the mitoses seen in 48-hour cultures represent cells that have undergone one division; most in the 72-hour cultures have completed two divisions. Lymphocyte cultures degenerate after four to five cell divisions and cannot be further stimulated. Peripheral blood cultures from prenatal and newborn infants usually yield sufficient mitoses at 48 hours, and this shorter culture can be useful for getting quick results on STAT specimens.

Phytohemagglutinin is available as PHA-M, a mucoprotein, and as PHA-P, a more purified and potent protein from which the polysaccharide moiety has been removed [12,13]. The PHA-M form is most commonly used in cytogenetics laboratories, as the dosage is much less difficult to determine. Note that PHA is a large molecule and will not pass through a 0.2- μm filter. It should be added to the culture medium after filter sterilization.

In the past, batches of commercially produced PHA were quite variable, because they were tested for their agglutinating ability rather than their mitogenic ability [14,15]. PHA that has been tested in tissue culture for its ability to initiate division is available from major tissue culture companies. Most laboratories use 1–2% PHA per volume as a final concentration.

The concentration of PHA in the culture medium is important. Too much PHA is toxic, and not enough yields poor mitotic response. It may be useful to add PHA to a bottle of medium (volume 100–500 mL) rather than to each 5–10-mL culture to ensure a more precise and uniform dosage. This bottle of complete medium should be used within 2–4 weeks because reconstituted PHA loses approximately 30% of its mitogenic effect over a 1-month period. PHA is normally frozen at receipt and aliquoted into a few doses, and refrozen until use.

As stated previously, lymphocytes cannot divide more than a few times before they die. However, immortalized lymphocyte cultures can be created using EBV, which transforms B-cell lymphocytes. These cultures may be kept for months or years, facilitating research and ensuring a continuing source of material for study. One possible clinical use of transformed lymphocytes in the clinical laboratory is for storage of a supply of abnormal cell lines for use in FISH studies as a positive control. One must always remember, however, that these now immortalized cells may not respond exactly as normal cells, and that over time in culture, cytogenetic changes can occur that may need to be evaluated from time to time.

3.4.2 Anticoagulants

The anticoagulant of choice is usually sodium heparin. Powdered heparin without preservatives is preferred and may be made reconstituted in sterile saline when needed and at any concentration desired. Liquid heparin usually contains alcohol as a bacteriostatic agent but is often used successfully. However, heparin that contains phenol is highly toxic and should not be used [13]. The optimal concentration of heparin is 10–25 IU/mL blood. However, small samples can withstand large amounts of heparin without apparent toxicity as long as the heparin is part of an isotonic solution. Clotted blood may be salvaged by mincing the clot with scissors or a scalpel to release cells from the fibrin clot or using collagenase to enzymatically dissolve the fibrin in the clot, and then inoculating the dissociated cells into blood culture medium containing PHA. Heparin is not needed at this step as the cells from the clot were derived from a sample that was completely clotted and no longer contains clotting factors.

Lithium heparin, EDTA, citrate, and others may interfere with cell culturing. However, blood received in the wrong anticoagulant can often be salvaged. Setting up the blood promptly may minimize effects of a wrong anticoagulant, and it is important to heparinize the blood or the culture medium to prevent clotting. Other anticoagulants (e.g., acid citrate dextrose (ACD), EDTA, etc.) act by chelating calcium. Since calcium is present in the medium, clotting will occur if cultures are not anti-coagulated with sodium heparin.

3.4.3 Culturing

Samples should have all the required patient information, and a unique identifier should be assigned. Cultures should be labeled with two identifiers, for example, laboratory accession number and patient name.

Blood cultures are performed by inoculating whole blood or separated white blood cells into culture medium that has been supplemented with fetal bovine serum, L-glutamine, and sometimes antibiotics. High concentrations of animal serum may be detrimental to growth, and 10% serum is optimal for blood culturing.

Strict sterile technique must be followed during the entire culturing procedure. Any contamination introduced will be toxic to the blood cells and will result in an insufficient number of mitotic cells for harvest and analysis.

Cultures may be set up using the macro technique, in which plasma-leukocyte-suspension (PLS) is inoculated into medium without the red cells, or the micro technique, in which sodium heparinized whole blood is used. For the macro method, the buffy coat may be settled out by gentle centrifugation (800 RPM for 5–10 minutes) or gravity sedimentation (room temperature for 30 minutes to 1 hour) [19]. The PLS is drawn off and mixed, and a volume of 0.1–0.4 mL of the white cell suspension is added to a 5–10 mL volume of complete medium (with PHA) to produce a cell culture with $1-2 \times 10^6$ leukocytes per milliliter of medium. A variation of this method is to draw cells directly from the buffy coat layer into a sterile pipette without removing all of the plasma and to use these cells to initiate the culture.

For the micro method, NaHep (sodium heparin) whole blood is inverted several times to mix it thoroughly, and 0.2–0.5 mL is inoculated into 5–10 mL of complete medium with PHA. Red blood cells (RBCs) do not interfere with the ability of the stimulated lymphocytes to undergo mitosis, but they do metabolize essential nutrients, especially glucose. In the micro method, workers overcome this problem by adding a relatively small volume of whole blood to a relatively large volume of prepared medium. Mature granulocytes degenerate quickly and are not transformed [13]. Several cultures may be set up to ensure good results, and at least two cultures should be initiated unless there is insufficient sample for more. The inoculum of blood to be added to a culture may be specified by volume (0.25–1.0 mL or numbers of drops) or by final cell concentration ($1-2 \times 10^6$ /mL medium) [13,18,20,21]. Watt and Stephen [22] recommend using 0.8 mL of blood for normal adults, 0.6 mL for infants and children, and 1.0 mL for women who are pregnant or immediately post partum to establish 10-mL cultures. These volumes take into account not only the average white count for each category, but also the percentages of lymphocytes usually present. With blood from newborns or from patients with a high hematocrit (e.g., elevated RBCs), less blood should be used in setting up the culture, because there may be too many red cells per milliliter of blood, interfering with cell response to PHA and ultimately cell growth. Concentration of lymphocytes may not always be strictly critical, but it may be important if the patient is ill or if the condition of the specimen is less than ideal.

Some laboratories adjust the cell concentration in each patient's culture. A number of protocols call for cell determination by hemocytometer, electronic cell counter, or by reference to a recent white count and differential. Some protocols also call for the use of a cell separation medium to recover only leukocytes from blood [23,24].

Blood can be cultured 4 or more days after it is collected, which allows the specimen to be mailed to the laboratory or cultures to be reset if the first attempt fails due to some technical error. Fresh specimens grow best and the mitotic yield declines as the specimen ages. Care must be taken to avoid extremes in temperatures. Mailed specimens packed in Styrofoam containers are usually insulated sufficiently. Specimens should be stored at room temperature or refrigerated at temperatures above 4 °C, but never frozen.

Very small blood samples do not store well in a large container because they tend to dry out. If a small container cannot be used, add sterile medium to provide moisture. Vacutainers not completely filled should have the vacuum released by removing the stopper or by inserting a sterile hypodermic needle into the top to equilibrate the pressure.

In order to culture blood samples for 72 hours and avoid weekend harvests, it is best to arrange for samples to be obtained on Monday, Tuesday, or Friday. When this is not practical, blood received on Wednesday may be kept for one day and cultured for 96 hours, kept for 2 days and cultured for the usual 72 hours, or put into culture immediately and cultured for 48 hours. Blood received on Thursday may be kept for one day and cultured for 72 hours or be set up immediately and cultured for 96 hours. Whole blood may be kept at room temperature or stored in the refrigerator above 4°C. Alternatively, blood can be set up as usual, except for omitting the PHA, and can be stored in the refrigerator overnight. Timing of the culture should begin when the PHA is added and the culture is placed in the incubator.

3.5 Cell culture equipment and supplies

Cell culture requires a constant temperature growth chamber, a sterile work area, and a centrifuge. The growth chamber may be as simple as a water bath or as complex as the many kinds of incubators available. These may be atmospheric incubators (which use tightly closed flasks with the gas atmosphere controlled inside the flask, or a nongas-mediated buffer system such as HEPES) or CO₂ incubators (humidified or dry) in which the atmosphere of the incubator is controlled and growth vessels may be left open to gas exchange.

3.5.1 Materials for cell culture

A wide variety of sterile glassware and plasticware is available for tissue culture. For peripheral blood cultures, tubes and centrifuge tubes are often used, as well as flasks, bottles, and chamber slides (flasks in which microscope slides are built into the bottom). Plasticware that has been treated to allow cell adhesion, which is necessary for long-term culture, may not be suitable for short-term cultures. Polypropylene tubes are better than polystyrene for culture if the harvest is performed in the same tube, because they resist the effects of fixative breakdown products (i.e., methyl acetate, which over time dissolves polystyrene plastic tubes).

3.5.2 Solutions for tissue culture

Media

Culture medium (plural: media) comes in many varieties. A basic medium contains the minimum essential components, but enriched and modified media may contain extra amino acids, vitamins, and other additives, such as fatty acids, pyruvates, ATP, cholesterol, thymine, uracil, ribose, and guanine. A complete medium (ready to use) is the chemically defined medium plus any necessary supplements, such as serum (at 10–30% concentration), L-glutamine, and antibiotics. L-glutamine is usually added to complete medium because it is an unstable amino acid that in storage becomes D-glutamine, a form cells cannot use. The time required to change L-glutamine to D-glutamine depends on the temperature and pH. At 4°C, 80% of the L-glutamine remains after 3 weeks [25]. At room or incubator temperature, the amount diminishes more rapidly. For this reason, most media require additions of L-glutamine when they are prepared and once every 3 weeks thereafter. In studies conducted by Sigma, L-glutamine was most stable at pH 7.3 and deteriorated at high and low pHs [25]. L-glutamine can be stored at -10°C for up to one year. An Invitrogen product called GlutaMAX is very stable at room temperature and at 37°C for long periods, and also has the advantage that it does not produce as much ammonia as standard forms of L-glutamine.

Media can be purchased in several forms. Powdered medium is the least expensive form, but it requires dilution with water and sterilization. Most formulas must be filtered, but some special formulas can be autoclaved [26]. The main disadvantage to powdered medium is that it is difficult to obtain a high enough quality of water with which to dilute it. Sterile liquid medium can be purchased in 1× concentration (for use) or in 10×, 50×, and 100× concentrations. Working strength medium is the most convenient and the most expensive. Concentrates must be diluted with sterile water according to directions given by the supplier. Lyophilized (freeze-dried) media are also available and must be reconstituted before use. The advantage of lyophilized medium over liquid medium is a longer shelf life. Our laboratory purchases 1× medium so that the supplier controls the water for endotoxins.

Liquid culture medium can be stored at refrigerator temperature for up to 1 year. Complete medium (with serum) has a shelf life of 4 weeks at 4°C. The most commonly used media for blood culturing are Eagle's MEM (minimum essential

medium), MEM alpha, DME (Dulbecco's modified Eagle's), BME Medium 199, RPMI 1640 and 1603, Ham's F-10, and McCoy's 5A. Iscove's modified Dulbecco's MEM is a synthetic medium designed for lymphocyte culture without serum [22]. Special studies, such as heritable fragile sites or synchronized cultures for prometaphase studies, may require specific media. Of these, the most popular for most culture conditions is RPMI 1640.

Buffers

Cells can survive in medium with pH 6.6–7.8, but optimal growth is obtained at levels between 7.0 and 7.4. The use of a properly buffered medium is very important. Since cells are constantly producing acidic products, and although the buffer cannot hold the pH constant, it will slow the rate of change. To assist with this issue, most culture media utilize Phenol Red as an immediate visual indicator of the culture's pH. When the medium is yellow, it is acidic, and when pink, basic. The color representing the ideal cell culture pH range of 7.0–7.4 is a golden orange. If blood culture medium is very pink (basic pH) at the time the culture is initiated, the T cells will not react to the PHA properly, resulting in poor growth and inadequate mitotic figures. It is best to adjust the pH of the medium before inoculating blood cultures by gassing with 5% CO₂ or adding dilute HCl to achieve this golden orange color.

Sera

A complete medium contains some percentage of serum (plural: sera). Serum may be derived from bovine, human, horse, or other sources. The nature of the factors provided to cells by serum is poorly understood, but it is clear that certain types are better than others and that certain lots are more conducive to growth than others. Much depends upon the age, condition, and diet of the donor and on the way that the serum is collected and stored before use. Fetal calf (bovine) serum and autologous and homologous human serum (often AB blood group) are most commonly used for cytogenetic cultures. Fetal calf serum may contain some growth factors that are diluted or not present in neonatal or older calves, and it also contains fewer immunoglobulins. Because of its animal origin, this serum is the most likely of the tissue culture components to contain contaminants and should be subjected to quality control measures, such as sterility and growth support checks. Most suppliers will reserve a batch of serum for a short time to allow the laboratory to test and select a suitable batch [26,27].

Some laboratories prefer serum that has had the complement inactivated by treatment with irradiation or heat (56°C for 30 minutes). This denatures the complement protein and ensures that immunologic reactions against cultured cells will not occur. However, it may denature other serum proteins, as well.

Serum has a shelf life of 4 weeks at 4°C and 1 year at –20°C. Serum substitutes are now available and may be used successfully with low concentrations of serum [27]. Individual batches should be tested using the same growth support tests used for serum.

Growth factors, antibiotics, and other factors

A number of growth factors may be used in serum-free or serum-reduced media. Examples are epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and T-cell growth factor or interleukin (IL)-2) [28]. Medium conditioned by giant cell tumor cells can produce colony-stimulating activity (CSA) and stimulate human bone marrow growth in vitro [29]. Phytohemagglutinin-leukocyte-conditioned medium (PHA-LCM) may be used in bone marrow or peripheral blood cultures [30,31] (see Chapter 11). Growth factors are the subject of intensive research for theoretical reasons and for their practical use in tissue culture [32–34].

Most laboratories use antibiotics, usually penicillin–streptomycin (pen-strep) or gentamicin, in the culture medium for blood samples. The amounts commonly used are 100 U/mL of penicillin, 100 µg/mL of streptomycin, or 50 µg/mL of gentamycin final concentration in culture. Other factors important for a successful culture are temperature, pH, and CO₂ concentration. The temperature of the incubator or water bath should be set between 36°C and 37°C and should remain constant rather than fluctuating. Lower temperatures delay growth, and temperatures above 40°C usually result in cell death. The release time in synchronized cultures will vary with incubator temperature, as the cell cycle speeds up at higher temperatures (up to 37°C). Sterile supplies and good sterile technique are vital [35,36].

Certain studies require the addition of other chemicals during the culture incubation period. For example, sister chromatid exchange, lateral asymmetry, and replication banding all require the addition of BrdU (bromodeoxyuridine). Cultures to be synchronized with methotrexate for prometaphase or metaphase studies require addition of the methotrexate block and thymidine release solutions.

3.6 Harvesting peripheral blood cultures

The method of harvesting will depend on the reason for studying the chromosomes. Synchrony and chemical additives such as ethidium bromide or actinomycin D will result in more extended chromosomes. If a high mitotic index is required (e.g., to rule out mosaicism) the Colcemid® time can be increased; however, for prophase/prometaphase studies, no more than 30 minutes of Colcemid® is recommended (see Chapter 2). The hypotonic solution used for peripheral blood is usually 0.075 M KCl, but other salts may be used to affect chromatid morphology (e.g., Ohnuki's hypotonic or mixtures of sodium citrate and KCl). Hypotonic may be warm or at room temperature. The timing of the hypotonic treatment is 10–30 minutes, with some laboratories preferring two hypotonic treatments. This method is most important when harvesting whole-blood cultures. The first exposure of the cells to hypotonic solution results in the lysis of many red cells. The resulting hemolysis changes the hypotonicity of the supernatant requiring a second hypotonic treatment to optimize its effect. Fixation may be initiated by adding a few drops of 3:1 methanol–acetic acid to the hypotonic solution, or solution may be added after cells are centrifuged out of hypotonic. Multiple fixative steps should be carried out to rid the culture of the red cell ghosts and methemoglobin (brown supernatant) that is formed when red cells are lysed with fixative. It is highly recommended that the centrifuged pellet of cells in hypotonic be first suspended either by gentle pipetting or tapping the centrifuge tube before the first fixation is attempted. Following this initial fixation, several changes of fixative to completely wash the cells and clear the supernatant is advantageous to optimize chromosome spreading. See protocols at the end of this chapter for more information.

3.7 Chromosome analysis of peripheral blood

At least 20 banded metaphases should be counted and examined for sex chromosome constitution, breaks, rearrangements, dicentrics,acentrics, and other abnormal findings. A minimum of five cells should be analyzed. Band level should be adequate for the diagnosis, for example, 400 bands per haploid set (BPHS) to detect trisomies, and 550–850 BPHS to look for micro-deletions. Count guidelines should increase to at least 30 for questions of mosaicism. Our laboratory commonly examines 50–100 metaphases to look for minor cell lines, especially when one or two abnormal cells are found with the same abnormality in the first 20 metaphases. If this does not adequately answer the question, interphase FISH can be performed on the blood sample, if there is a probe available for such a study. A skin biopsy or other tissue for chromosome analysis may be requested, for examination of a different germ cell layer.

At least five metaphase cells are completely analyzed, comparing homologues band for band in detail. Two or three karyograms per cell line are prepared, and at least two additional photographs are available for examination. Panels of structurally abnormal chromosomes are also very useful and necessary to aid in proper breakpoint analysis of translocations or other rearrangements.

3.8 Storage of fixed specimens

After the harvest step, our experience is that the best high-resolution spreading and banding results when the fixed cells have been kept at room temperature. We therefore keep the cells at room temperature until the cytogenetic study is completed, and then transfer pellets to the refrigerator or freezer. If it is desirable to save fixed cell pellets for possible future FISH studies or other special stains, the cells last well if kept in a centrifuged pellet in 3:1 methanol–acetic acid, which fills the storage tube (excluding air), and stored at freezer or cold refrigerator temperatures. Slides that are already made should be stored in boxes in Ziploc bags, and kept in the freezer. For very long-term storage, the bag may be filled with nitrogen to prevent oxidation of the cells.

Acknowledgments

Thanks to Gloria Marchilli, Marilyn Arsham, and Margaret J. Barch, who originally wrote portions of this chapter in the First or Second Edition. Special thanks to Marilyn Arsham, who charted the straits of Doomgurgle, and to our soulmates, John Lawce and Sandee Brown for their constant and loving support.

References

1. Auerbach A, Rogatko A, Schroeder-Kurth TM. International Fanconi Anemia Registry: relation of clinical symptoms to diepoxybutane sensitivity. *Blood* 1989;73(2):391–396.
2. Cervenka J, Hirsch BA. Cytogenetic differentiation of Fanconi anemia, idiopathic aplastic anemia, and Fanconi anemia heterozygotes. *Am J Med Genet* 1983;15:211–223.

3. Fanconi G. Familial constitutional panmyelocytopathy. Fanconi anemia (FA). I. Clinical aspects. *Semin Hematol* 1967;4:233–240.
4. Schroeder TM, Tilgen D, Kruger J, Vogel F. Formal genetics of Fanconi's anemia. *Hum Genet* 1976;32:257–288.
5. Swift M. Fanconi's anaemia in the genetics of neoplasia. *Nature* 1971; 230:370–373.
6. Auerbach AD, Sagi M, Adler B. Fanconi anemia: prenatal diagnosis in 30 fetuses at risk. *Pediatrics* 1985;76:794–800.
7. Strathdee CA, Duncan AMV, Buchwald M. Evidence for at least four Fanconi anaemia genes including FACC on chromosome 9. *Nat Genet* 1992;1:196–198.
8. dos Santos CC, Gavish H, Buchwald M. Fanconi anemia revisited: old ideas and new advances. *Stem Cells* 1994;12:142–153.
9. von Kap-Herr C. The chromosome instability test. In: Kaplan BJ, Dale DS, eds. *The Cytogenetic Symposia*, 1994. Association of Cytogenetic Technologists: 15–1 to 15–7.
10. Hecht F, McCaw BK. Chromosome instability syndromes. In: Mulvihill JJ, Millers RW, Fraumeni JF Jr, eds. *Genetics of Human Cancer*. New York: Raven Press, 1977.
11. Michalowski A. Time course of DNA synthesis in human leukocyte cultures. *Exp Cell Res* 1963;32:609–612.
12. Difco supplementary literature. Detroit: Difco Laboratories, 1968.
13. Hamerton JL. *Human Cytogenetics; General Cytogenetics*. New York: Academic Press, 1971.
14. Hsu TC. *Human and Mammalian Cytogenetics: An Historical Perspective*. New York: Springer-Verlag, 1979.
15. Hsu TC. Mammalian Chromosomes in vitro. I. The karyotype of man. *J Heredity* 1952;43:167–172.
16. Ford EHR. *Human Chromosomes*. New York: Academic Press, 1973.
17. Priest JH. *Medical Cytogenetics and Cell Culture*. 2nd ed. Philadelphia: Lea and Febiger. 1977.
18. Zackai EH, Mellman WJ. Human peripheral blood leucocyte cultures. In: Yunis JJ. ed. *Human Chromosome Methodology*. New York: Academic Press, 1974.
19. Bartataos M, Baramki TA. *Medical Cytogenetics*. Baltimore: Williams and Wilkins. 1967.
20. Sharma AK, Sharma A. *Chromosome Techniques—Theory and Practice*. 3rd ed. London: Butterworth, 1980.
21. Verma RS, Babu A. *Human Chromosomes—Manual of Basic Techniques*. New York: Pergamon Press, 1989.
22. Watt JL, Stephen GS. Lymphocyte culture for chromosome analysis. In: Rooney DE, Czepulkowski BH, eds. *Human Cytogenetics—A Practical Approach*. Oxford: IRL Press, 1986;39–55.
23. Ali FMK. *Separation of Human Blood and Bone Marrow Cells*. Bristol, UK: IOP Publishing Ltd, 1986.
24. Pretlow TG II, Pretlow TP, eds. *Cell Separation—Methods and Selected Applications*. Vol. I. New York: Academic Press, 1982.
25. *Sigma cell culture reagent catalog*. St. Louis, MO: Sigma Chemical Company, 1990.
26. Freshney RI. *Culture of Animal Cells: A Manual of Basic Techniques*. New York: Alan R. Liss, 1983.
27. Rooney DE, Czepulkowski BH. *Human Cytogenetics: A Practical Approach*, Oxford: IRL Press, 1986.
28. McKeehan WL. Identifying the complete set of extracellular variables that influence cell multiplication in vitro. In: Patterson MK Jr, ed. *Uses and Standardization of Vertebrate Cell Cultures*. Gaithersburg, MD: Tissue Culture Association, 1984;48–57.
29. *Handbook and Catalog for the Cell Culture Scientist*. Woodland CA: J. R. Scientific, 1988.
30. Morgan S, Hecht BK, Morgan R, Hecht F. Qualitative and quantitative enhancement of bone marrow cytogenetics by addition of Giant Cell Tumor Conditioned Medium. *Karyogram* 1987; 13(4):39–40.
31. Morgan S, Poland-Johnston NK, Meloni-Balliet A, Morgan R. Methodology and experience in culturing and testing the GCT cell line for marrow cytogenetic improvement. *Karyogram* 1988; 14(1):7–9.
32. Basterga R, Foa P, Metcalf D, Polli EE, eds. *Biological Regulation of Cell Proliferation*. New York: Raven Press, 1986.
33. Burgess A, Nicola N. *Growth Factors and Stem Cells*. New York: Academic Press, 1983.
34. Ford RJ, Maizel AL, eds. *Mediators in Cell Growth and Differentiation*. New York: Raven Press, 1984.
35. Coriell GD. Contamination control. In: Patterson MK Jr, ed. *Uses and Standardization of Vertebrate Cell Cultures*. Gaithersburg, MD: Tissue Culture Association, 1984;97–103.
36. Coriell LL. Methods of prevention of bacterial, fungal, and other contaminations. In: Fogh J, ed. *Contamination in Tissue Culture*. New York: Academic Press, 1973;29–49.

Troubleshooting peripheral blood cultures

1. No growth or poor growth	Blood sample suboptimal: hemolyzed or clotted blood. No T-cell lymphocytes present (e.g., DiGeorge syn.) Few lymphocytes present. Patient on IV drugs (e.g., some newborns). Pregnant patients. Critically ill or deceased patient. Blood specimen stored too long. Culture conditions suboptimal: too much blood in culture. pH of medium too basic. No mitotic stimulant was added (see Figure 3.3). Insufficient gas exchange. Microbial contamination.	Mince clots with scalpel before culturing; request new sample. Use B-cell mitogens (pokeweed) or both T-cell and B-cell mitogens (pokeweed). Inoculate with buffy coat layer instead at whole blood. Wash cells with medium before culturing. Use buffy coat instead of whole blood. May need to request sample of tissue other than blood.
	Incubator temperature too low or too high. Medium, serum, or PHA expired. Arrest conditions suboptimal.	Request new sample. Re-set cultures with less blood (use 0.3–0.4 mL whole blood/5 mL medium). Reset cultures; gas with CO ₂ or add dilute HCl to neutralize pH. Reset cultures with proper mitogen.
	Cells lost during centrifugation. Cells not centrifuged before aspiration of supernatant. Improperly made hypotonic solution. Improperly made fixative.	Put tubes at a semi-horizontal slant; use flasks instead of tubes. Reset cultures with sterile medium; be sure draw was properly collected. Check to be sure temperature ranges from 37.0 °C to 37.5 °C and does not fluctuate widely. Reculture with fresh reagents. Check Colcemid® concentration and exposure time. Too low a concentration or too short a time may decrease mitotic index. Be sure centrifuge is set properly and working well. Reculture if necessary.
	PHA not added to medium, or accidentally filtered out by 0.2-mm pore size filtration. Insufficient hypotonic volume or treatment time. Excess blood in cultures.	Be sure the correct chemical was weighed out and water was pure. Check pH (should be 6.0–7.5). Be sure fixative ratio was not reversed. Be sure no other chemicals are contaminating the reagents, e.g., H ₂ O, plastic from bottle caps, etc.). Reculture.
	Mixing of cells in hypotonic was incomplete. Excess blood in cultures.	Use larger volumes of hypotonic solution.
	Ethidium bromide making chromosomes sticky and hard to spread. Slide-making problems.	Inoculate with less blood; use buffy coat. Rinse fixed pellets in 3 : 1 fixative or 2 : 1 fixative to clear cells. Pipet cells well.
	Drying too slowly. Cell mixing too harsh during harvest. Interval between release and harvest too long.	Reculture with less inoculum; if this is not possible, remove the upper section of the fixed pellet (fluffy) and make slides using the lower part of the pellet. Increase Colcemid® concentration.
	Block was ineffective. Mitotic arrest too long or Colcemid® concentration too high.	Slow drying of slides on dry slide-making days or speed drying of slides on humid slide-making days as a general rule; make sure slides are clean before using.
2. Suspension is thick or gelatinous	Drying too slowly. Cell mixing too harsh during harvest. Interval between release and harvest too long.	Speed up with heat, airflow, lower humidity. Avoid over-agitation of cells during harvest (e.g., harsh vortexing or pipetting). Step release time back in 10- to 20-minute increments to elongate chromosomes.
3. Chromosomes will not spread properly	Block was ineffective. Mitotic arrest too long or Colcemid® concentration too high.	Prepare fresh reagents and culture again. Keep Colcemid® time between 10 and 30 minutes maximum; keep Colcemid® concentration at 0.05 mg/mL final concentration.
4. Chromosomes are overspreading		
5. High-resolution chromosomes too short		

Contributed protocols

IMPORTANT: No protocol included in this manual should be used clinically unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements of all chemicals used in the laboratory.

Protocol 3.1 Blood culture and harvest procedure

Adapted from a procedure contributed by Oregon Health & Science University, Knight Diagnostic Laboratories Cytogenetics Laboratories

I. Principle

Blood lymphocytes are stimulated by the mitogen phytohemagglutinin (PHA) to rapidly grow and divide such that three days of culturing will yield late prophase or early metaphase cells of sufficient quantity and quality for clinical analysis. Colcemid inhibits the formation of the spindle fibers. When added to the stimulated culture, it causes mitotically active cells to be arrested in early metaphase. The synchronization of the cultures with methotrexate and thymidine allows large numbers of cells to enter early metaphase near the time of the harvest. Addition of ethidium bromide can also produce chromosomes with greater length and resolution by intercalating with the DNA, thus inhibiting chromosome condensation.

Safety warnings

A. Specimen handling

All tissue specimens should be considered hazardous. Use biohazard handling procedures and follow universal precautions. Wear a laboratory coat and protective gloves for all steps through slide-making. Cover cuts with protective bandages even when gloves are worn. Use the laminar flow hood for all steps up to harvest spin. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with fresh Sanimaster 4 (made fresh weekly). Dispose of glass Pasteur pipettes, needles and syringes in sharps container. Wash hands thoroughly after removing gloves. Do not bend, recap, break, or remove needles from disposable syringe; dispose of all needles in labeled red sharps container.

B. Methanol, absolute (Mallinckrodt #3016)

Methanol is EXTREMELY flammable; may cause flash fires. *May be fatal or cause blindness if swallowed.* Harmful if inhaled or absorbed through skin. Cannot be made nonpoisonous. In case of contact, flush skin or eyes for 15 minutes, remove to fresh air in case of inhalation. Seek medical attention if swallowed. Use gloves, lab coat, and goggles at all times. Should not be discarded down the sink, but should be collected for Safe Harbor disposal.

C. Glacial acetic acid (EM Science #AX0073-9)

Acetic acid is flammable as a liquid and a vapor. Avoid contact with skin, inhalation or ingestion. *May be fatal, if swallowed.* Causes severe eye and skin burns. In case of contact, flush skin or eyes, including under eyelids, for 15 minutes; remove to fresh air in case of inhalation. Do not induce vomiting if swallowed. Use gloves, lab coat, and goggles at all times.

D. Methotrexate, 50mg/mL (Lederle #NDC0205-5325-26)

Methotrexate (MTX) may be fatal if swallowed, or absorbed through the skin. May have reproductive effects. Wear gloves and lab coat at all times when handling.

E. Colcemid 10 µg/mL (Gibco #15210-016)

Colcemid is mutagenic, tumorigenic, embryotoxic and teratogenic with acute overexposure. Wear gloves and lab coat at all times when handling. Avoid contact with skin or inhalation, flush for 15 minutes if accidental contact.

F. Ethidium bromide (EB) (Sigma #E-1510)

Ethidium Bromide is a potential mutagen, and is irritating to the eyes, skin, and respiratory system. Flush for 15 minutes incase of accidental contact. Wear gloves and lab coat at all times.

Specimen

Lymphocytes are obtained through procurement of anti coagulated peripheral blood. Three to five milliliters of sodium-heparinized whole blood is required for optimum results. Other anticoagulants, though not recommended, may be accepted. Samples should be kept at room temperature and sent to the laboratory as soon as possible for optimum viability. Samples sent by overnight mail generally yield adequate results.

II. Materials

Supplies

1. 15 mL Corning polypropylene tube (Lab Stores #64.2005)
2. Pasteur pipette 5 ¾ » (Lab Stores #63.1614)
3. Pipette bulbs, 2 mL (Lab Stores #63.0202)
4. 5 mL sterile pipettes (Falcon #7543)
5. 10 mL sterile pipettes (Falcon #7551)
6. 1 mL sterile pipettes (Falcon #7521)
7. 25 mL sterile pipettes (Falcon #7525)
8. T-12.5 blue-capped flasks (Falcon #353018)
9. Micropipettors and sterile tips (Eppendorf)
10. Sterilizing filter unit, 115 mL, 0.2 µm (Nalgene #124-0020)

Culture medium and stock reagents

1. RPMI 1640 (Gibco #11875-051)
2. Fetal Bovine Serum (Irvine #3000)
3. Glutamine 200 mM (Gibco #250030-016)
4. Gentamicin 50 mg/mL (BioWhittaker #17-5282)
5. Incomplete Medium:
 - a. 50 mL Fetal Bovine Serum
 - b. 10 mL Glutamine
 - c. 0.5 mL Gentamicin
 - d. 439.5 mL RPMI 1640

Mix together for a total of 500 mL. Aliquot into 250 mL sterile flask or bottle and label as incomplete medium (also known as Bone Marrow Medium). Store at 4°C. Shelf life is 6 weeks.

6. Phytohemagglutinin M-Form (Gibco #10576-015)
PHA comes reconstituted and frozen. To use, thaw 10 mL vial and dispense into four 2.5 mL aliquots. Refreeze unused aliquots and thaw just before making the next batch of complete medium. Aliquot the next 10 mL vial when the last 2.5 mL aliquot is used.

Add 2.5 mL of PHA-M to 250 mL of incomplete medium (same as Bone Marrow medium). This is now **complete** medium used to grow peripheral blood T-lymphocytes. Store at 4°C. Shelf life is 4 weeks.

7. Hypotonic KCl (0.075 M)
Working Solution: Add 5.59 g KCl (Mallinckrodt #6838) to 1 liter of deionized H₂O. Aliquot into 100 mL bottles and store at 4°C. Shelf life is 4 weeks. Warm to 37°C before using.

8. Fixative (see Safety warnings)
Working Solution: Add 1 part glacial acetic acid (EM Science #AX0073-9) to 3 parts absolute methanol (Mallinckrodt #3016). Make fresh before each use.

9. Methotrexate, 50 mg/mL (see Safety Warnings above)
Stock Solution: Add 0.08 mL (80 µL) methotrexate (Lederle #NDC0205-5325-26) to 4.4 mL sterile H₂O using a TB syringe and sterile 25-gauge needle. Carefully place the used needle and syringe into a sharps container.

Working Solution: Add 0.1 mL (100 µL) of stock to 9.9 mL sterile H₂O for a concentration of 10⁻⁵ M. Store at 4°C. Shelf life: 12 months. Use 0.05 mL (50 µL)/5 mL culture to synchronize cultures.

10. Thymidine (Sigma #T-5018) (see Safety warnings)
Working solution: Add 0.025 g thymidine to 100 mL Hanks' (1×) for a final concentration of 10⁻³ M. Filter sterilize with a 0.2-µm filtration unit. Aliquot 20 mL into a T-12.5 blue-capped flask. Store at 4°C. Shelf life: 12 months. Use 0.1 mL (100 µL)/5 mL culture to release cells from methotrexate.

11. Hanks' Balanced Salt Solution (1×)
Add 1 part Hanks' 10× (Gibco #14180-020) to 9 parts sterile H₂O. Store at room temperature or 4°C. Shelf life: 12 months.
12. Colcemid 10 µg/mL (Gibco #15210-016) (see Safety warnings). Shelf life: 12 months.
13. Ethidium Bromide (Sigma #E-1510), 10 mg/mL (see Safety warnings)
EB Working Solution: Add 1 mL of EB to 9 mL of Hanks' (1×). Store at 4°C. Shelf life: 12 months.

III. Procedure

Blood setup and culture

1. Add 5 mL Complete Culture Medium to each of three 15 mL Corning polypropylene tubes. Label two of these tubes as 2A and one tube as 2AEB. Label with patient name, date, and laboratory identification information. An additional tube may be prepared and labeled as B culture depending upon study requirements. This culture will yield a better mitotic index but will not have as high-resolution chromosomes.
2. At 4:00 to 4:30 p.m., add heparinized whole blood to 5 mL Complete Culture Medium using a 1 mL pipette or Eppendorf pipette aid. Use 0.25–0.3 mL for newborns, 0.3–0.35 mL for individuals < 15 years old, and 0.35–0.40 mL for individuals > 15 years old. Cap tube and mix well.
3. Incubate cultures at 37°C for 48 to 96 hours. Incubation time is dependent upon the age of the patient, type of study and urgency of the results (48 hours for fast results and all newborns and young children, 72 hours for best results for children and young adults, and 72–96 hours for adults and women who are pregnant).
4. Add 0.05 mL of 10⁻⁵ M methotrexate to cultures 2A and 2AEB at 4:00 to 4:30 p.m. the day before harvest. Incubate cultures at 37°C overnight. Culture 2A and 2AEB are methotrexate-synchronized overnight and released for 5 hours. Culture B, if set up, is an unsynchronized culture, and is incubated until harvest time at 37°C.
5. Add 0.1 mL of 10⁻³ M thymidine to the 2A and 2AEB cultures at 8:30 a.m. the morning of the scheduled harvest. Mix well and re-incubate at 37°C until harvest.

Blood harvest procedure

1. At 1:00 p.m. on the scheduled harvest day, add 20 µL Colcemid using a micropipettor to both 2A and 2AEB cultures. Add 0.05 mL (50 µL) ethidium bromide to each 2AEB culture. Mix well and incubate for 30 min at 37°C.
2. Centrifuge each culture for 10 min at 1000 RPM.
3. Remove supernatant. Add 4 mL warm (37°C) 0.075 M KCl to each tube and resuspend cell pellet. Centrifuge for 10 min at 1000 RPM. Allow cultures to stand at room temperature for 5 minutes, if the number of cultures being harvested is less than 9 tubes.
4. Remove supernatant. Add 4 mL warm 0.075 M KCl to each tube and resuspend cell pellet. Add 5 drops of fresh fixative to each tube and mix well. Centrifuge for 10 min at 1000 RPM.
5. **Each tube must be treated individually for this step.** Remove supernatant and resuspend cell pellet. Add 2 mL fresh fixative and mix well. Add an additional 2 mL fixative and mix well. Repeat for each individual tube. Centrifuge for 10 min at 1000 RPM.
6. Remove supernatant. Add 4 mL fresh fixative to each tube. Centrifuge for 10 min at 1000 RPM.
7. After centrifugation, cell pellet may be used to make slides, or stored in a refrigerator for later use.

IV. Additional readings and references

1. Hungerford DA. Leukocytes cultured from small inocula of whole blood and the preparation of metaphase chromosomes by treatment with hypotonic KCl. *Stain Technology* 1965; 40; 333–338.
2. Francke U, Oliver N. Quantitative analysis of high resolution trypsin-Giemsa bands on human prometaphase chromosomes. *Human Genetics* 1978;45;137–165.
3. Yunis JJ. High resolution of human chromosome. *Science* 1976;191;1268–1270.

Protocol 3.2 High-resolution peripheral blood method

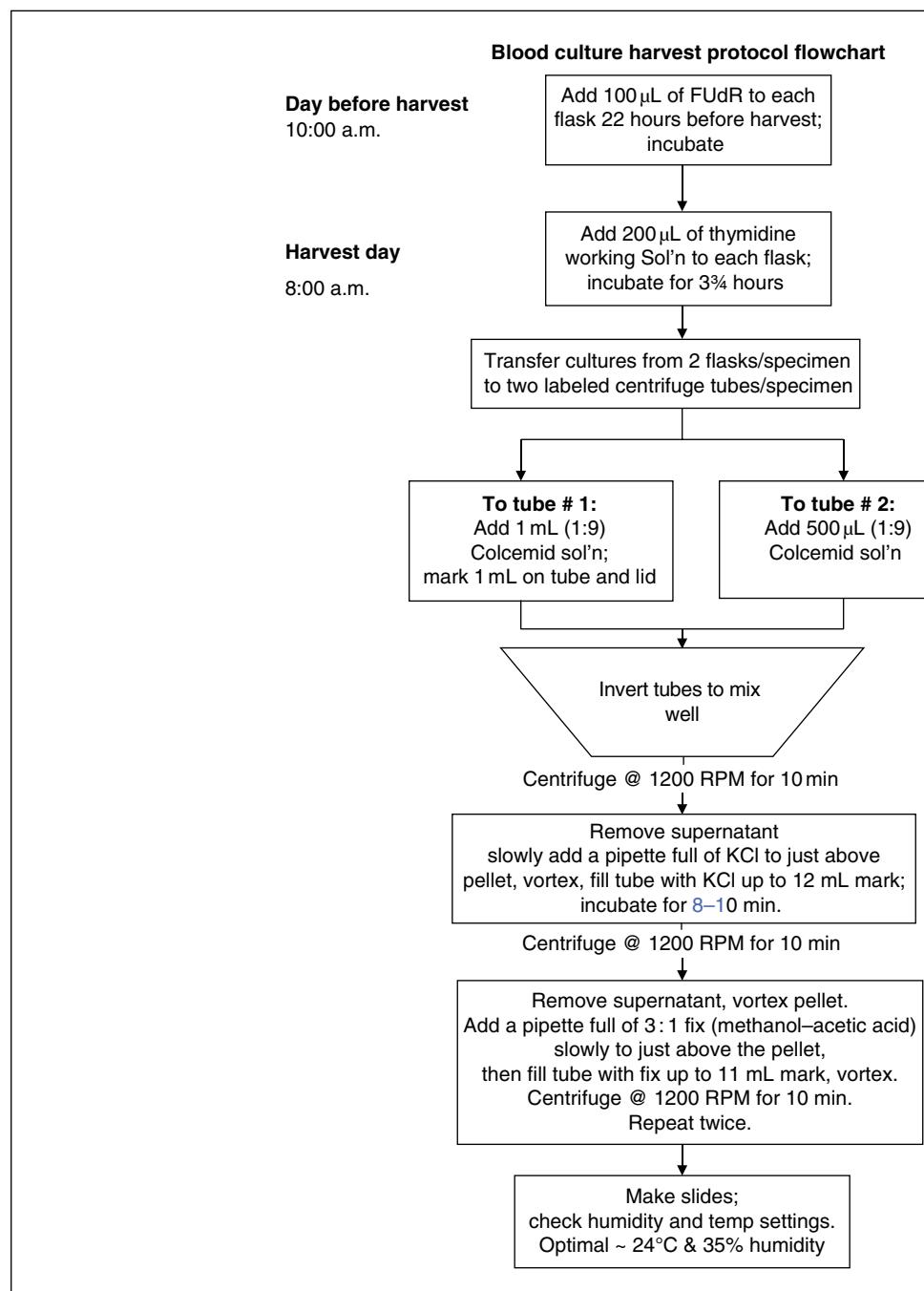
Adapted from procedures submitted by Melanie Hyde of Vancouver General Hospital, Cytogenetics Laboratory, Vancouver, B.C., Canada. Procedures were prepared by Chris Haessig, Kim Arase, Michele Currie, Birgitt Findler, Anis Habib, Deepak Dhillon, Aster Lau.

I. Principle

To provide a protocol for culturing Cytogenetic blood lymphocyte specimens, including set-up, culture, harvesting and slide-making. Blood specimens are set up in flasks and harvested after 3–4 days incubation. High-resolution chromosome analysis on blood specimens is requested when an 850-band level is required for detection of subtle rearrangements. Specimen is inoculated in T flasks containing culture medium containing a mitogen, incubated at 37°C for approximately 3–4 days, and “harvested” with solutions that will ultimately provide a satisfactory number of analyzable prophase-prometaphase mitotic cells within unobtrusive cell matrices and with minimal chromosome overlap. Slides are made and aged for staining with G-banding for analysis.

PPE: proper protective equipment

Appropriate personal coverage must be adhered at all times when handling bodily fluids. PPE includes gowns, gloves, shoes and shields, when appropriate.



II. Materials

Dulbecco's Medium, 500 mL	Invitrogen, Gibco #11885-084
Fetal Calf (Bovine) Serum (FCS), 500mL, frozen	Somagen, Irvine #3000a
Pen-Strep, 20mL, frozen	Invitrogen, Gibco #15140-148
L-Glutamine, 100 mL, frozen	Invitrogen, Gibco #250330-081
Sodium Heparin 10,000 units	Sigma #H3393
Phytohemagglutinin (PHA) M form 10mL, frozen	Invitrogen, Gibco #10576-015
Fluorodeoxyuridine (FudR), 10 mg	Sigma #F0503
Thymidine, 5 g	Sigma #T1895
Colcemid 10 mg/mL, 10 mL	Invitrogen, Gibco #15210-040
Potassium chloride (KCl), 500 mg	Fisher #P-217-500
Hanks' Balanced Salt Solution (HBSS) 1x, 100 mL	Invitrogen, Gibco #14170-120
Absolute Methanol, 4 L	Fisher #A412-4
Glacial Acetic Acid, 2.5 L liquid	BDH #ACS003-78
Sterile water, 3 L	Stores
Sterile water, 10 mL vial	Pharmacy
Flasks, Corning, 25 cm ²	Fisher #10-126-30
Culture Flasks, Cell Star, white cap, 25 cm ²	
Centrifuge Tubes, Corning, 15 mL	Fisher #05-538-51a
Tubes, Falcon, 5 mL	VWR #Ca60819-295
Pipettes, 10 mL	VWR #Ca53300-523
Transfer Pipettes, Samco, 1 mL, sterile	VWR #14670-364
Needles, 22 G	STORES
Syringes, 1 mL	STORES
Syringes, 10 mL	STORES
Slides, Microscope 25 x 75 mm	STORES
Alcohol Swabs	STORES
Isopropanol 70%, 2 L	STORES
Nu Gauze	STORES
Gloves	STORES
Red Wax Pencil	STORES
Green Wax Pencil	STORES
Black Lab Marker	VWR #52877-310
Sterile Flasks, 200 mL	
Pipette Tips 100–250 µL	
Plastic Squeeze Bottle, 200 mL for fix	

Equipment

Biohazard Hood, Nuaire	ESBE Lab Supplies Model #NU425-400
Fume Hood, Labconco Basic 47	VWR Model #22473
Incubators, Multigas	SANYO Model #MCO1755M

Refrigerator/Freezer	SANYO Model #MPR-411F
Centrifuge, Heraeus	FISHER Megafuge 1.0
Inverted Microscope, Diaphot	NIKON
Rotomix Shaker	VWR Barnstead # 58922-612
Pipet-aid, Drummond	FISHER #13-681-19
Max Memory Pipettor	VWR #53498-835
Micropipettor, P200 Gilson Pipetman	MANDEL SCI #GF23601
Timer	VWR #62344-586

Solutions

Note: Solution labels should include the following information as appropriate – e.g., description, lot#, preparation/open date, storage temp, expiration date, tech ID).

Setup and culture solutions

Fetal Calf (or Bovine) Serum (FCS or FBS) Stock Solution, 20mL (frozen)	500mL FCS (FBS) <ul style="list-style-type: none"> • Thaw FCS (FBS) • Swirl to mix • Dispense in 20-mL aliquots into 25-cm² Corning flasks • Label, date, assign a letter for lot # and record in Media/Reagent logbook • Store in freezer
Pen Strep Stock Solution, 1 mL (frozen)	20mL frozen PS <ul style="list-style-type: none"> • Thaw and swirl to mix • Dispense in 1-mL aliquots into 5-mL Falcon tubes • Label, date, assign a letter for lot # and record in Media/Reagent logbook • Store in freezer
L-Glutamine Stock Solution, 2mL (frozen)	100mL frozen L-Glutamine <ul style="list-style-type: none"> • Thaw and swirl to mix • Dispense in 2-mL aliquots into 5-mL Falcon tubes • Label, date, assign a letter for lot # and record in Media/Reagent logbook • Store in freezer
Sodium Heparin Stock Solution 4 °C	Sodium Heparin 10,000 10mL vial sterile water <ul style="list-style-type: none"> • Dispense water into Heparin using sterile 10-mL syringe/22G needle • Mix using syringe and re-dispense solution back into sterile water vial • Label, date, assign a letter for lot # and record in Media/Reagent logbook • Store in fridge
Phytohemagglutinin (PHA) Stock Solution (4 °C)	10mL frozen PHA <ul style="list-style-type: none"> • Thaw and swirl to mix • Label, date, assign a letter for lot # and record in Media/Reagent logbook • Store in fridge
Culture Medium Working Solution (4 °C)	100mL Dulbecco's 20mL FCS (FBS) 2mL L-Glutamine 1mL Pen-Strep 0.3mL Sodium Heparin <ul style="list-style-type: none"> • Dispense into a 200 mL sterile flask • Swirl to mix • Label materials added, date and initial on bottle • Store in fridge

Colcemid/HBSS Working Solution (4 °C, wrapped in foil)	1 mL Colcemid 9mL HBSS <ul style="list-style-type: none"> • Mix in sterile centrifuge tube, wrap in foil • Make up fresh the day before for each harvest <p>*NB: undiluted Colcemid wrapped in foil lasts for one month after opening</p>
--	---

Harvest solutions

Hypotonic, 0.075 M	2.8g KCl 500mL sterile water <ul style="list-style-type: none"> • Mix in clean volumetric flask and refrigerate • Dispense 12mL KCl/culture tube into a 500mL bottle, place in 37 °C incubator the day before harvest
Fixative	3 Methanol : 1 Acetic Acid <ul style="list-style-type: none"> • Swirl to mix in a plastic squeeze bottle • Make fresh throughout the day • Store in fire resistant cabinet; use with chemical fume hood • Flush unused reagent under running water for 5 minutes <p>(Editor's Note: Disposal of flammable chemicals must follow local, state and national regulatory standards. Check with your institution's guidelines for disposal of flammable material.)</p>

Fluorodeoxyuridine (FUdR) solutions

FUdR Stock # 1 (freezer, wrapped in foil)	10mg FUdR vial 10mL sterile water vial <ul style="list-style-type: none"> • Assign a letter to Stock # 1 and record in Media/Reagent logbook
FUdR Stock # 2 (freezer, wrapped in foil)	0.5mL Stock # 1 4.5mL sterile water vial <ul style="list-style-type: none"> • Mix stock # 1 with 4.5mL sterile water • Use one tube of FUdR Stock #1 to make twenty (20) tubes of 5mL aliquots of FUdR Stock #2 • Wrap in foil and label • Store in freezer
FUdR Working Solution (1 mg/L or 4 µM) (freezer, wrapped in foil)	0.1mL Stock # 2 9.9mL sterile water vial <ul style="list-style-type: none"> • Mix FUdR Stock # 2 with 9.9mL sterile water • Use one FUdR Stock #2 tube to make ~30 tubes of 10mL FUdR Working Solution • Mix each of the above solutions in a sterile centrifuge tube • Wrap all FUdR solutions in foil, label & date • Store all solutions in freezer • Thaw Working Soln as needed
For culture (0.01 mg/L or 0.04 µM)	0.1mL (100 µL) FUdR Working Solution 10mL Culture <ul style="list-style-type: none"> • Add FUdR working solution to culture flask
Thymidine Stock #1	0.2422g Thymidine 10mL sterile water vial <ul style="list-style-type: none"> • Mix thymidine with sterile water • Transfer to a sterile 10mL centrifuge tube • Wrap in foil and label • Assign a letter to Stock #1 and record in Media/Reagent logbook
Thymidine Stock #2	1mL Thymidine Stock #1 9mL sterile water <ul style="list-style-type: none"> • Mix 1mL Thymidine Stock #1 with 9mL sterile water • Wrap in foil & label • Use one Thymidine Stock #1 tube to make ten (10) tubes of 10mL Thymidine Stock #2 • Store in freezer

Thymidine Working Solution (Culture concentration = 2.4×10^{-4} g/mL)	1 mL Stock #2 9mL sterile water vial <ul style="list-style-type: none"> Mix 1 mL Thymidine Stock #2 with 9mL sterile water Use one stock #2 tube to make 10 tubes of 10mL Thymidine Working Solution Mix each of the above solutions in separate sterile centrifuge tubes Wrap all Thymidine solutions in foil, label & date Store all solutions in freezer Thaw Working Solution as needed
Culture Thymidine (2.4×10^{-6} g/mL)	0.2mL (200 μ L) Thymidine Working Solution 10mL culture <ul style="list-style-type: none"> Add 0.2 mL Thymidine Working Solution to 10mL culture medium

III. Procedure

Specimen receipt: A sodium-heparinized blood sample is cultured with mitogen PHA to induce mitotic division in T-lymphocytes. Specimens can be set up anytime prior to the scheduled incubation day and kept at room temperature or in the fridge. Specimens that are 2–4 days old should be set up the same day of arrival.

STAT Specimen: Two (2) additional cultures are set up as backup for the next incubation time.

Incubate	Add FUdR	Harvest Day
Monday →	Thursday →	Friday
Tuesday →	Thursday →	Friday
Wednesday →	Friday →	Saturday
Friday →	Monday →	Tuesday

A. Setup

- Two (2) culture flasks are set up per specimen. Two additional cultures are set up on STAT specimens as backup for the next incubation time.
- Label blood tubes with the cytogenetic acquisition number (e.g., CG16-125) and mix using Rotomix Shaker while flasks are being prepared.
- Using gloves and sterile technique under a biohazard hood, dispense 10 mL of culture medium using a sterile pipette into two (or four) 25-cm² (Cell Star) flasks.
- Add 200 μ L PHA to each flask using a micropipettor. Mark flask top with a green wax pencil to indicate PHA has been added.
- Set up only *one patient at a time* to prevent possible mixups.
- Label two (2) culture flasks per patient with patient's name and CG #.
- Double check* name and CG# of blood tube with flasks.
- Remove top of blood tube with sterile gauze.
- Using a sterile transfer pipette, add 0.3–0.4 mL of whole blood to each flask.
- Tighten caps on flasks and mix well.
- Place cultures in the appropriate baskets, as described in the above incubation table.
- At 4:00 p.m., incubate cultures at 37.5–38 °C for 3–4 days.

B. Day before harvest

- 9:00 a.m. – Add 100 μ L FUdR Working Solution using a micropipettor to each culture 22 hours before Colcemid addition.
- Mix flasks well and re-incubate at 37 °C in Harvesting Incubator.

	Timing		Add to flasks
Day before harvest	9:00 a.m.	22 hours	0.1 mL FUDR Working Sol'n
Harvest day	7:00 a.m.	3 hours 30 min	0.2 mL Thymidine Working Sol'n
		15–20 min	0.5 mL in tube 1 and 1 mL Colcemid Sol'n in tube 2
		8–12 min	12 mL KCl
Fixative		3 × washes of 3 : 1 Fix	

C. Harvest day

Colcemid treatment

The addition of the mitotic inhibitor (*Colcemid*) accumulates metaphases by preventing the formation of the spindle apparatus. Mitotic inhibitors also cause chromosome contraction. This effect is dose and time dependent.

15. At 7:00 a.m., add 200 µL of Thymidine Working Solution using a micropipettor to each culture, mix well and reincubate for 3 hours 30 minutes in Harvesting Incubator.
16. Using gloves under a biohazard hood, label two centrifuge tubes/culture with patient's name and CG # and transfer the contents from patient's flasks to the tubes. Double-check name and # to avoid mix-ups.
17. Repeat step 16 for each patient.
18. Add 0.5 mL Colcemid Solution to one culture and 1 mL to the other. Mix well and reincubate tubes at 37.5–38°C in Harvesting Incubator for 18–20 minutes.
19. Centrifuge cultures at 1200 RPM for 10 minutes.

Hypotonic treatment

Potassium chloride (KCl) lyses red cells and allows the mitotic cells to swell within the cell membrane. This treatment helps to spread the chromosomes when making slides. The following steps involving the addition of KCl must be done quickly to ensure a similar KCl time for all cultures.

20. Remove caps in order from each tube.
21. Using a clean pipette on the suction apparatus, carefully remove supernatant, leaving about 1 mL above cell button for each tube.
22. Resuspend cells using vortex to break up pellet, making sure there are no clots of blood in the suspension for the first tube.
23. Add 2–3 drops of warmed KCl (37.5–38°C) with a Pasteur pipette and vortex.
24. Add the rest of KCl from the pipette and vortex to thoroughly mix.
25. Repeat steps 22 to 24 for the rest of the tubes.
26. In order starting with the first tube, fill all tubes with KCl to a volume of 12 mL.
27. Set timer for 8–12 minutes. KCl time is dependent on technologist harvesting, temperature, and humidity.
28. Recap tubes in order and invert tubes several times to mix KCl.
29. Place tubes in Harvesting Incubator 37.5–38°C for the time remaining.

Fixation

Until this stage, cells are alive and metabolizing. Fixative preserves the cell by stopping all cell functions, and prevents further swelling.

30. After hypotonic treatment, remove tubes from harvesting incubator and centrifuge at 1200 RPM for 10 minutes.
31. Prepare fixative of 3 : 1 methanol-acetic acid, 10 mL/culture.
32. Remove caps in order from each tube.
33. Remove supernatant by suction from each tube leaving 1 mL above cell button.
34. Resuspend cells using vortex to break up pellet in the first tube.
35. Add 2–3 drops of fresh fix from Pasteur pipette and vortex to mix thoroughly.
36. Repeat steps 34 and 35 for the rest of the tubes.
37. Fill each tube with fresh fixative to a volume of 10 mL.

38. Recap tubes.
39. Invert tubes twice.
40. Centrifuge for 10 minutes at 1200 RPM.
41. Wash and centrifuge cell pellets 2x with fixative, vortexing each time, allowing the vortex to travel to the bottom of the tube.

D. Slide-making

Prepare slides

42. Wash each slide with dish detergent.
43. Rinse slides under hot running water.
44. Rinse with distilled water.
45. Fill container with cold distilled water.

Test culture quality

46. Make slides on one patient at a time.
47. Remove last fix wash leaving approximately $\frac{1}{2}$ inch above cell pellet depending on size of pellet.
48. Resuspend cells with Pasteur pipette.
49. Drain water from slide on paper towel.
50. Drop one drop of cell suspension on slide from 3–4 inches.
51. Drop one drop of fix on top from same height.
52. Drain slide and label with patient CG #. A second identifier (e.g., patient name, date of birth or medical record number) may be required at some facilities.
53. Dry on the bench top at an appropriate distance from the vent for drying.
54. Observe slide under inverted microscope.
55. Adjust slide-making technique (e.g., flood slide with 2–3 drops fix and drain, 2–3 drops fix on top of slide while drying, drying slide on wet paper towel, adjusting height, etc.) to obtain slides with long, well-spread chromosomes and good mitotic index.

Make slides

56. Make 8–10 slides per patient.

IV. Notes on troubleshooting

Factors to consider	Tips on technique
Culture problems (poor growth)	
Specimen date	Check date of blood taken; blood over 4 days old rarely grows well.
Incubator temperature	37.5–38°C is an optimal temperature; under or above this setting can cause poor growth.
Culture Flask	Type of flask may affect growth; changing brands or different lot #'s may be required.
KCl Time	If timing is too high, chromosomes will blast, and mitotic index will be low. If timing is too low, chromosomes will be encased or hard to spread.
Vortex	Overvortexing cultures can cause cell damage or blasting. Undervortexing cultures can cause encasement or metaphases that are hard to spread.
Temperature/humidity	Can affect changes in KCl timing. Higher temperature and humidity conditions generally reduce the KCl time required for obtaining adequate spreading of metaphase. Lower humidity and temperature conditions usually requires an increase in KCl time.
Slide-making tips	
Mitotic index	Cell concentration in fix will affect spreading and contrast. Slides should contain a large number of dark, well spread metaphases with long chromosomes suitable for analysis. Keep crossovers to a minimum.

Contrast	Darkness of chromosomes observed under phase will influence banding. Lighter (less contrast, grey) cells will band well within a few minutes on 140–150 °C hotplate, but are easier to over-dry. Darker (more contrast, black) cells generally need longer drying. Leave overnight at room temperature and band the following day.
Refractile cells	Generally do not band well and tend to overtrypsinize or have “ghost” like appearance when banded.
Spreading	Adjust height of pellet drops on slide to increase or decrease metaphase spreads.
Humidity	Check using hygrometer for humidity readings. Humidity can affect contrast of cells, e.g., too much humidity can cause encasement, making chromosomes look refractile. Adjust humidity setting and distance of slides to vent for drying. Place slides on or near wet paper towels to increase humidity and aid in spreading of metaphases or increase contrast of chromosomes.
Temperature	Adjust temperature setting in blood slide-making room.
Fixative	Multiple (3–4) drops of fix on top of cell suspension droplet on slide can aid in spreading. Each drop of fix should be added only when rainbow color starts to appear as fix is drying on the slide.

Protocol 3.3 Constitutional cytogenetic studies on peripheral blood

Anonymous Contributor

Important notice: Any adopted procedure must be thoroughly tested to ensure that results are consistent and accurate before it can be used in the clinical setting.

I. Principle

Venipuncture is the least invasive procedure for obtaining tissue for metaphase chromosome analysis; therefore, it is the first tissue source recommended for routine constitutional cytogenetic analysis. Peripheral blood requires stimulation with a T- and/or B-cell mitogen in order to yield a sufficient quantity of cells for analysis. An exception is cord blood, which can provide direct examination of dividing cells (see Note 1, Processing cord blood); however, metaphase quality is generally less optimal from cultures without stimulation.

With the exception of newborn blood, two 4 mL green top (sodium heparinized) Vacutainer® tubes are generally received. Specimens are not discarded if they do not meet this quantity, but physicians are notified. Newborn blood is set up whole, using 7 drops from a sterile plastic Pasteur pipette per 10 mL of media. All other blood samples are left untouched for 1–2 hours at room temperature so that the buffy coat can be extracted.

For routine processing, two cultures are set up: one stimulated with a T-cell mitogen and harvested ~70–72 hours, and the second is both synchronized with methotrexate (MTX) and released in BrdU before harvesting for obtaining a higher band level. Syndromes, such as Prader–Willi del(15)(q11.2–12), Langer–Giedion del(8)(q24.1), Miller–Dieker del(17)(p13), DiGeorge del(22)(q11.2), Wilms tumor del(11)(p13), Beckwith–Wiedemann dup(11)(p15), and Smith–Magenis del(17)(p11.2), may sometimes be detected with this higher band resolution culture; however, a normal result would not rule out a subtle abnormality and would thus require more sensitive testing, such as microarray or FISH. For children <12 years of age, a third culture will be set up with a B-cell mitogen for 96-hour stimulation. If time permits or harvest schedules require synchronization, mitogens may be added the morning after set-up, which also allows the cells to acclimate to the medium.

Peripheral blood on a “neonate” (up to 28 days of age) is categorized STAT; therefore, minimum culture-setup requirements change, depending upon the situation being investigated and the quantity of blood received. For example, in addition to the routine 72-hour T-cell (PHA) culture and 96-hour B-cell (PKW) culture described above, two additional cultures (T-cell stimulation for 48 hours) and (T-cell stimulation for 24 hours, respectively) are also set up, if quantity allows, so that a preliminary report can be given as quickly as possible, but no later than 3 days, and a final report within 7 days. Because the cells are in early transformation, the 24-hour culture aims for quantity over quality so that trisomies and monosomies can be detected quickly; therefore, its yield may improve by extending the incubation time in Colcemid® (e.g., 3 hours to overnight) and by harvesting the cells after they have been transformed by the mitogenic agent, generally 24 hours. If there was not enough quantity to set up a synchronized culture and the preliminary findings are “normal,” the 96-hour B-cell (PKW) culture can be converted to a higher resolution culture.

Routine chromosome analysis requires 20 cells counted, with full chromosome karyogram coverage minimally at the 550-band level for both homologues. In addition, mosaicism studies require 30 additional counts. Higher resolution studies require analysis and full chromosome karyogram coverage at the 850-band level, preferably for both homologues, but at least for one of the homologues of each set [1].

Safety precautions

ALL specimens are regarded biohazardous until cells are fixed; therefore, use proper equipment and wear proper PPE (personal protection equipment). Fixative is caustic and flammable and thus should be used and stored with proper fume ventilation, with full protection from spillage (see Note 2, Handling fixative).

II. Materials

General supplies

1. Sterile bottled water, 1000 mL
2. Sterile 5-mL snap top tubes
3. Sterile amber 1.5 mL microcentrifuge tubes
4. Sterile flasks, T-25 (Corning)
5. Sterile centrifuge tubes, 15 mL and 50 mL (Corning)
6. Glass Pasteur pipettes, 5½ in.
7. Sterile plastic Pasteur pipettes, 5¼ in.
8. Sterile pipettes, 1, 5, 10 mL (Falcon)
9. Microscope slides
10. Microscope coverslips
11. CytoSeal
12. Glass slide tray/holder

Equipment

1. Suctioning apparatus
2. Micropipettor
3. Filtering apparatus
4. Vortex
5. Biohazard centrifuge
6. Humidifier/temperature readout and controlled environment
7. 37°C/5% CO₂ water-jacketed incubator
8. Emergency electrical outlet line
9. Metal trays
10. Hot plates set to 40, 60, and 96°C (if needed for STAT cases)
11. Phase microscope
12. Timers, NIST approved
13. Water bath at 37°C or dry incubator at 37°C
14. Scales for both micro and gram weighing quantities
15. Microfilters for sterility filtration
16. Biohazard hood
17. Chemical Storage Fume Hood
18. Chemical Harvest Fume Hood
19. Access to eye wash and shower
20. Vinyl lab coat and gloves

Reagents

Setup

1. Chang Medium MF (Irvine Scientific #91005). Store in freezer. Stock expiration: see bottle label. After defrosted: 30 days. (If Chang Medium MF is not available, see Note 3, Backup medium)
2. Phytohemagglutinin (PHA) (Irvine Scientific #96691), lyophilized. Store at 4°C. Reconstitute with 5 mL sterile bottled water (*read product instructions to ensure that instructions have not changed*), store at 4°C. Expiration after reconstitution: one month.

3. Lectin from *Phytolacca americana* (pokeweed) (Sigma # L8777). Reconstitute with 5 mL Hanks' Balanced Salt Solution (Sigma #H9394). *Read product instructions to ensure that instructions have not changed*. Store at 4°C. Expiration after reconstitution: 3 months.
4. Methotrexate (MTX) (Sigma #A-6770). Store in freezer. Expiration on bottle.
 - a. *MTX Stock Solution* (1×10^{-3} M): Dissolve 10 mg of MTX in 22 mL sterile bottled water. Aliquot into five 5-mL snap top tubes, unless estimated daily requirements indicate larger or smaller aliquots. Label each aliquot with amount, reagent name, storage requirements and expiration date; labels must also either contain or be able to trace to the following information: lot #, date received, date prepared, storage requirements, stock test validation details and final date placed into production, safety warnings and initials of preparer. Store in freezer. Expiration: one year from preparation unless superseded by stock expiration date.
 - b. *MTX Working Solution*: (1×10^{-5} M):
 1. Dilute 0.1 mL MTX stock solution with 9.9 mL sterile bottled water.
 2. Aliquot 0.3 mL into amber 1.5-mL microcentrifuge tubes, unless estimated daily requirements indicate larger or smaller aliquots.
 3. Label each microcentrifuge tube with amount of aliquot, reagent name, storage requirements and expiration date; labels must also either contain or be able to trace to the following information: lot #, date stock solution was received, safety warnings, date current batch was prepared, initials of preparer, and QC test validation details for that batch, including case numbers, results and final date placed into production.
 4. Store in freezer. Expiration: 6 months from date of preparation unless superseded by stock or stock solution's expiration date.
 5. Test the MTX Working Solution on at least two samples to validate the batch's effectiveness before placing into production.
5. 5-Bromo-2'-Deoxyuridine (BrdU)
 - a. Fluorodeoxyuridine (FUDR) Stock (Sigma #F-0530), stored in chemical cabinet at room temperature. FUDR Stock Solution (4×10^{-4} M): Dissolve 0.01 g FUDR in 100 mL of sterile bottled water. Seal with Parafilm and store in 50-mL centrifuge tubes in freezer. Label as described under MTX above. Expiration: one year.
 - b. Uridine Stock (Sigma #U-3003). Stored in chemical cabinet at room temperature.
 - c. Uridine Stock Solution (3×10^{-3} M). Dissolve 0.01 g in 10 mL of sterile bottled water in a 15-mL centrifuge tube. Label as described under MTX above. Store in freezer. Expiration: one year.
 - d. 5-Bromo-2'-deoxyuridine (BrdU) Stock, 10^{-2} M (Sigma #B-5002). Store in freezer; sealed from light.
 - e. "BrdU" Working Solution (10^{-2} M):
 1. Weigh 0.03 g BrdU (10^{-2} M). Place into a small snap-top tube.
 2. Add:
 - 1 mL FUDR (4×10^{-4} M) Stock Solution
 - 2 mL Uridine (3×10^{-3} M) Stock Solution
 - 9 mL sterile bottled water
 3. Mix well.
 4. Aliquot 0.3 mL into 1.5-mL amber microcentrifuge tubes.
 5. Label each aliquot with amount of aliquot, reagent name, storage requirements and expiration date; labels must also either contain or be able to trace to the following information: lot #, date received, date prepared, storage requirements, stock test validation details and final date placed into production, safety warnings and initials of preparer.
 6. Test the Working Solution on at least two samples for batch result validation before placing into production.
 7. Store in freezer. Expiration: 6 months.
6. Hypotonic Reagents: Potassium chloride (KCl); sodium citrate

Hypotonic Solution: 8 mL 0.5% KCl + 2 mL 0.8% sodium citrate/tube.

 - a. Dissolve 5 g of potassium chloride (0.5% KCl) in 1000 mL sterile bottled water. This is a 0.067 M KCl solution. Label each stock solution with amount, reagent name, storage requirements and expiration date; labels must also either contain or be able to trace to the following information: lot #, date received, date prepared, storage requirements, stock test validation details and final date placed into production, safety warnings and initials of preparer. Store at 4°C for 2 weeks. Test the Working Solution on at least two samples to validate the batch before placing into production. Aliquot as needed and warm to 37°C before using.
 - b. Dissolve 8 g of sodium citrate (0.8% Na citrate) in 1000 mL sterile bottled water. This gives a 0.8% solution. Label each stock solution with amount, reagent name, storage requirements and expiration date; labels must also either contain or be able to trace to the following information: lot #, date received, date prepared, storage requirements, stock

- test validation details and final date placed into production, safety warnings and initials of preparer. Store at 4 °C for 2 weeks. Test the Working Solution on at least two samples to validate the batch before placing into production. Aliquot as needed and warm to 37 °C before using.
- c. Mix 2 parts 0.8% sodium citrate to 8 parts 0.50% KCl just prior to use. Keep warm.
 7. Colcemid® 10 µg/mL (Life Technologies #15210–016). Store at 4 °C. Expiration date on bottle.
 8. 1 : 3 Fixative: Add 1 part glacial acetic acid to 3 parts absolute methanol (see Note 4, Fixative quality). This solution should be made fresh periodically throughout the day. CAUTION: FLAMMABLE, CORROSIVE. Use proper PPE and proper ventilation to reduce caustic vapors.

III. Method

Setup

1. Confirm that the name and second identifier on tube matches name and second identifier on requisition.
2. Assign a case number and generate labels. Two labels should be affixed to each culture vessel.
3. Whole blood is set up immediately on neonates (<29 days of age). Add 7 drops of whole blood per 10 mL Chang MF medium in each flask. Depending on the amount of blood received, 3–5 cultures will be set up. The later cultures need not be harvested if satisfactory results can be obtained with the earlier cultures.
4. All other samples remain at room temperature for 1–2 hours in order for the buffy coat to settle out.
5. The number and type of cultures being set up will depend upon the age of the patient, or the degree of band resolution required:

72T = PHA stimulation for 72 hours. Within 70–74 hours after mitogen addition, add 0.5 µL Colcemid® for 30 minutes. This routine culture is set up for all blood samples.

72P = PHA stimulation for a 72-hour prophase harvest (0.4–0.5 mL pokeweed B-cell mitogen can also be added). This is the second culture routinely set up for all blood samples because it should provide a higher level of band definition, if needed.

24T = PHA added for 24 hours. Colcemid® is added either overnight at a half dosage (0.25 µL of Colcemid®) or early next morning at full dosage Colcemid® (0.5 µL). This longer incubation with a mitotic inhibitor (for 3 hours minimum to 24 hours) is used in order to improve the success in capturing metaphases at this early stage. This culture is only used for chromosomal studies on newborn (<28 days of age) specimens. For STAT cases, 0.4–0.5 mL pokeweed B-cell mitogen can also be added to increase mitotic yield.

48T = PHA stimulation for 48 hours. This culture is used for chromosomal studies on neonates (<29 days of age) specimens. For STAT cases, 0.4–0.5 mL pokeweed B-cell mitogen can also be added to increase mitotic yield.

96B = B-cell stimulation using Pokeweed mitogen for 96 hours. This 96B culture is used for patients under 13 years of age.

96P = B-cell prophase harvest using Pokeweed mitogen after 96 hours stimulation. This culture, similar to 72P, is used for high-resolution studies and requires cell synchronization and BrdU release prior to harvest. Samples requiring high resolution analysis will use this culture in addition to 72T and 72P.

6. Whole blood or buffy coat can be inoculated in one of two ways:
 - a. Place 10 mL Chang MF medium in each culture flask and inoculate each flask separately, or
 - b. Place all required media (10 mL x #-cultures) together into one flask and inoculate before subdividing into separate culture vessels. For example, a routine adult study, which requires two cultures – 72T and 72P, would combine 20 mL Chang MF into one flask. The entire buffy coat is added to that one flask, the inoculated medium is gently swirled for even distribution, and 10 mL inoculated medium is transferred to a separate culture vessel, thus providing a more balanced cell distribution among the different cultures.
7. Mitogen is added to coincide with harvest personnel availability; therefore, it can be added on the same day or next day. Record time mitogen is added on label, and either create or update the harvest worklist.
8. Incubate with loosened caps at 37 °C in 5% CO₂ for specified times. If the medium color turns acidic, see Note 5: Yellow medium).

Harvest pretreatment for high-resolution only

1. At 2:30 p.m. on the day before a prophase harvest, add 0.1 mL MTX Working Solution per P culture (72P or 96P).
2. On the morning of the harvest, remove inoculated medium to a sterile centrifuge tube, affix one of the flask labels to the tube, initial label (see Note 6, Following a specimen's trail) for transfer, and centrifuge at 1000 RPM for 10 min.

3. Remove supernatant and discard. Add fresh Chang MF medium (no need to add a mitogen) and resuspend pellet. *Optional:* To increase surface area for receiving nutrients during this incubation time, you can return the suspension to the flask, and save the tube for later use, or you can leave in the tube.
4. Add 0.1 mL BrdU Working Solution per P culture, mix, wrap in aluminum foil, loosen cap and return to the 37°C, 5% CO₂ incubator for 4 3/4 hours.
5. Continue with Harvest.

Harvest preparation

1. Prepare hypotonic: 8 parts KCl, 0.5% + 2 parts sodium citrate, 0.8%, per tube, warmed to 37°C. If you notice that chromosomes are consistently coming out dark and skinny, alternate ratios to 2 parts KCl + 1 part sodium citrate.
2. Prepare fresh fixative by adding 1 part acetic acid to 3 parts methanol.
3. Place clean, unscratched slides in slide tray and allow to sit 5 for minutes in clean fixative, followed with two rinses with sterile bottled water. Refill with fresh sterile bottled water and store at 4°C until ready to use. Slides can be prepared in advance.

Harvest

Three-day cultures are ready to be harvested within 2 hours pre- or post-inoculation time; however, 24-hour cultures will yield better results if they have at least one full 24-hour cycle after mitogen stimulation. Because chromosome morphology from these early harvests is generally poor, quantity rather than quality is the anticipated goal; therefore, longer incubation exposure to Colcemid, including overnight inoculation, is a realistic option for improving the quantity, not quality, of countable cells for ruling out aneuploid situations in STAT newborn cases.

1. Transfer blood to centrifuge tubes, along with a label from flask. Initial label with transfer and add Colcemid®. Incubate at 37°C for the following time interval:
 - 24T – If Colcemid® was not added for an overnight setup, add it early and harvest as late as possible to improve the number of metaphases captured.
 - 48T – Add 0.5 µL Colcemid® for 1–3 hours; harvest should not be performed until close to or after 48 hours following mitogen addition. This culture should be successful.
 - 72T – After 70–74 hours from mitogen addition, add 0.5 µL Colcemid® for 30 min.
 - 96B – After 94–98 hours from mitogen addition, add 0.5 µL Colcemid® for 30 min.
 - 72P, 96P – After PRE-HARVEST TREATMENT, add 0.5 µL Colcemid® for 8 min.
2. Centrifuge
 - 24T, 48T, 72T, 96B for 10 min at 1000 RPM
 - 72P and 96P for 8 min at 800 RPM
3. Remove as much supernatant as possible.
4. Resuspend with vortex at medium speed.
5. Add warm hypotonic with a steady stream.
6. Cover tightly, rotate tube and incubate at 37°C for 10 minutes.
7. Centrifuge as described in step 2.
8. Remove all but 2–3 mL supernatant, depending on the size of pellet. Resuspend well.
9. Moving rapidly, add 1 drop fixative, mix or vortex at low speed; follow with 2 drops fixative, mix/vortex, and 3 drops, mix/vortex. Move to the next specimen until all pellets have been allowed to rest with the initial fixation.
10. Slowly add up to 5 mL of fixative, mixing and shaking between increasing quantities. Continue with all remaining tubes.
11. After all tubes have been fixed to the 5 mL mark, add full pipettes (~2 mL), mixing between each, until reaching 10 mL. Allow to sit at least 15 minutes.
12. Centrifuge 1000 RPM for 10 min. Remove supernatant and resuspend pellet. Gently add full pipettes of fixative, mixing between pipettes. Allow to sit 10 minutes.
13. Repeat step 12 for 1 or 2 more times, until the supernatant is clear and pellet is white.
14. Centrifuge 1000 RPM for 10 min.
15. Remove supernatant until fixative is equal in size to pellet. If cell concentration is too cellular, add fresh fixative until desired concentration and make slides.

IV. Notes

1. Processing cord blood [1,2]

1. Add 1 mL whole blood to 10 mL medium in each flask.
2. Incubate 1–3 hours at 37°C in CO₂ incubator.
3. Add 50 µL Colcemid® with micropipette.
4. Incubate 1–3 hours or overnight at 37°C in CO₂ incubator
5. Pour into 15 mL centrifuge tube and spin at 1000 RPM for 10 min.
6. Take off supernatant and resuspend pellet with 10 mL of 2 : 8 hypotonic solution, as described above.
7. Incubate 10 minutes in 37°C incubator.
8. Layer 3 mL of fixative on top of hypo and blood solution and centrifuge at 1000 RPM for 10 min.
9. Remove supernatant and resuspend pellet with 1 : 3 fixative. Wait 20 minutes to 1 hour at room temperature.
10. Centrifuge at 1000 RPM for 10 minutes. Remove supernatant, resuspend pellet with 1:3 fixative and wait 10 minutes. Repeat for one more fix.
11. Make slides. Age and band slides according to standard protocol. Adjustments may need to be made to trypsin time.

2. Handling fixative

Acetic acid can cause severe burns. Although methanol may neutralize some of its toxicity, it is still a reagent that should be used with a fume hood and any contact should be immediately treated. Do not carry any bottle containing fixative by the lid, as accidents have been reported because the top was not securely closed. Thermo Fisher Scientific SDS for acetic acid provides the following first aid measures for acetic acid exposure [3]:

Eye contact: Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required.

Skin contact: Wash off immediately with plenty of water for at least 15 minutes. Immediate medical attention is required.

Inhalation: Move to fresh air. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance; induce artificial respiration with a respiratory medical device. Immediate medical attention is required.

Ingestion: Do not induce vomiting. Call a physician or Poison Control Center immediately.

3. Backup medium

Materials

1. RPMI 1640 (Life Technologies #11875-051): Store in refrigerator. Expiration on label.
2. Fetal Bovine Serum (Life Technologies #26140-012): Store in freezer. Expiration on label.
3. L-Glutamine, 100 mL (Irvine Scientific #9317). Divide steriley into 1 mL aliquots (200 mM; 29.2 mg/mL). Store in freezer; protect from light exposure.
4. Penicillin – Streptomycin (Life Technologies #1514-015). Store in freezer in 10 mL aliquots. Each aliquot may be thawed and refrozen twice. Expiration date on label.

Procedure

To a sterile bottle add:

100 mL RPMI 1640 (89.3%)
 1 mL L-glutamine (0.9%)
 1 mL Penicillin–streptomycin (0.9%)
 10 mL fetal bovine serum (8.9%)
 Stored in refrigerator. Expiration: 10 days

4. Fixative quality

The SDS for Acetic Acid describes its stability as hygroscopic, i.e., it readily absorbs moisture from the air. For smaller laboratories, therefore, we find it best to use smaller stock bottles to ensure its freshness.



I. PRINCIPLE

Setting up PHA- and/or pokeweed (PKW)-stimulated cultures depends on patient age, specimen quantity, and band resolution required. For STAT newborns (< 29 days old), whole blood is harvested at 48 and 72 hrs, and at 24 hrs, if qty allows. Synchronized harvests are also set up for higher resolution, if needed. All other peripheral blood samples use the buffy coat (~1-2 hrs) for a routine, 72-hr, PHA-stimulated (T) culture, and a 72- or 96-hr synchronized (P) culture for increased band resolution. For patients < 13 yrs, a pokeweed (96B) culture is added, and for high resolution cases, a synchronized pokeweed culture (P) is added (see Culture Set-up Table).

II. SET UP

Follow universal precautions. Use biological safety cabinets and appropriate PPE. For preparing working solutions, see Protocol 3.3.

- (1) Confirm that name and second identifier on tube matches requisition.
- (2) Assign case number and generate 2 labels per culture. Warm and aliquot medium (volume = # cultures x 10 mL). Newborn blood can be set up immediately. All other blood specimens must allow the buffy coat to separate (~1-2 hrs).
- (3) To inoculate, confirm patient ID on both specimen and culture vessels; initial. Using sterile Pasteur pipettes, evenly distribute whole blood (5~7 drops/10 mL medium) or buffy coat among culture vessels. *Optional:* If using the same medium lot, combine the total volume in 1 flask, add the buffy coat or newborn blood (5~7 drops x #cultures), and aliquot inoculated medium into separate 10 mL cultures.
- (4) The day and time that mitogen is added will depend on personnel availability at the time of harvest. Without off-hour coverage, PHA is added by 2pm on Mon, Tues and Fri, unless the specimen is STAT. Pokeweed is added Mon, Thurs and Fri. Record date and time mitogen is added on label, initial and document harvest record.
- (5) Loosen caps if incubating in a humidified, 37 °C CO₂ incubator.

III. PRE-HARVEST PREPARATION

Prophase Pre-Harvest Synchronization

- (1) At 2:30pm the day prior harvest, add 0.1 mL MTX [1 x 10⁻⁵ M] to P cultures. Reincubate.
- (2) At 8am next morning, transfer to a sterile centrifuge tube, affix one flask label to tube, initial and centrifuge at 1000 RPM for 10 min.
- (3) Remove supernatant, add 10 mL warm Chang MF (no mitogen) and 0.1 mL BrdU [10⁻² M] working solution, mix, wrap in foil, loosen cap and return to incubator for 4 3/4 hrs.

Culture Set-up Table

Pt Age	Amt/culture	24T	48T	72T	72P	96B	96P
< 29 days *if qty allows	7 drops whole blood	Y*	Y	Y	Y*	Y*	
< 13 years	buffy coat			Y	Y	Y	
13+ years	buffy coat			Y	Y		
Hi Resolution	buffy coat			Y	Y		Y

Harvest Initiation Table

Cultr	Mitogen	Pretreatment	Hrs in culture	Time in Colcemid®
24T	PHA		24	overnight or 3+ hrs
48T	PHA		48	3+ hrs
72T	PHA		70-74	30 min
72P	PHA(+PKW)	MTX (Day 2)	BrdU(Day 3) ~5 hrs	8 min
96B	PKW		94-98	30 min
96P	PKW	MTX (Day 3)	BrdU(Day 4) ~5 hrs	8 min

Danger! Acetic Acid

Flammable liquid and vapor. Causes severe burns. Use proper PPE and fume hoods throughout procedure.

Reagent Preparation

- (1) Prepare hypotonic: 8 parts 0.5% KCl + 2 parts 0.8% sodium citrate per tube; warm to 37 °C.
- (2) Prepare fresh fixative: 2 parts glacial acetic acid to 8 parts methanol per culture, per fixation step.
- (3) Place clean, unscratched slides in glass slide dish and allow to sit 5 minutes in clean fixative, followed with two rinses of sterile bottled water. Refill with fresh sterile bottled water and store at 4 °C until ready to use. Slides can be prepared in advance and stored at 4 °C.

IV. HARVEST

- (1) Transfer each flask's contents to a centrifuge tube and attach one of the labels from the flask to the tube; initial label. Add 50 µL Colcemid®; mix well. Incubate at 37 °C for the time interval specified in the Harvest Initiation Table.
- (2) Centrifuge 24T, 48T, 72T, 96B for 10 min at 1000 RPM
 72P and 96P for 8 min at 800 RPM
- (3) Remove as much supernatant as possible. Vortex at medium speed .
- (4) Add warm hypotonic with a steady stream while mixing.
- (5) Cover tightly, rotate tube and incubate at 37 °C for 10 min.
- (6) Centrifuge as described in #2. *Optional:* Newborn blood may benefit from a 2 mL pre-fix prior to centrifuging.
- (7) Remove enough supernatant to equal the pellet size. Resuspend well.
- (8) Moving rapidly, add fixative, dropwise while mixing or vortexing, until red cells begin to lyse. Move to the next specimen until all pellets have been allowed to rest with the initial fixation.
- (9) Slowly bring up to 5 mL fixative, mixing and shaking while adding. Continue until all tubes have been fixed to the 5 mL mark. Then add full pipettes (~2 mL), mix between each, until reaching 10 mL. Allow to sit at least 15 minutes. Tubes can be left overnight at RT, frig or freezer.
- (10) Centrifuge 1000 RPM for 10 min. Remove supernatant and resuspend pellet. Add fixative gradually at first, while mixing, then full pipettes after 5 mL, mixing between each pipette-full. Allow to sit 10 min.
- (11) Repeat step #10 until the supernatant is clear and pellet is white, generally 1-2 more times. Centrifuge 1000 RPM for 10 min.
- (12) Remove supernatant until fixative is equal to pellet. If needed, add more fixative until appearance is cloudy but not dense.

See Chapter 2 for slide-making procedures.

Figure 3.3 Peripheral blood preparation for chromosome analysis.

5. Yellow medium

Yellow-colored medium (which is not cloudy) in culture indicates a depletion of nutrients, generally caused either by an over-noculation of the specimen, overgrowth of cells in culture, or the presence of an early contaminant, all three being potentially toxic for the growing cells. Send the medium sample to the Microbiology laboratory for contamination testing and check white blood cell count (WBC) of the specimen. If fresh specimen is still available, reset a smaller quantity in fresh medium and quarantine the culture until results are returned from Microbiology. If the specimen is no longer available but is critical, take a small amount of cells from the original culture, and re-set in fresh medium. Rinsing the cell pellet a few times in fresh medium is generally less effective for suspension cultures, but is an option. Penicillin and streptomycin are generally present in complete medium, but a fungicide could be added if the yellow medium appears cloudy; however, it too may affect cell growth, and thus should be used with caution. Adding mitogen is probably not needed if the cells had enough time to transform, but it won't hurt if you don't know. Harvest as soon as possible.

6. Following a specimen's trail

In our laboratory, we initial a label any time the specimen is transferred from one receptacle to another. This provides a handler trail, if it were to be needed; therefore, our protocols include this reminder.

V. References and additional readings

1. ISCN (2013): *An International System for Human Cytogenetic Nomenclature*. Shaffer LG, McGowan-Jordan J, Schmid M (eds). Basel: S Karger, 2013.
2. Day W, Jalal S, Sinclair-Worley L, Kukolich H, Freeman M, Sakakini J. Direct Chromosome Analysis From Neonatal Cord Blood. *Am J Hum Genet* 1988; 31:943–946.
3. Graham I, Sutherland GR. Rapid Karyotyping of Neonates on the Basis of Data from Cord Blood. *Am J Hum Genet* 1987; 41:668–760.
4. Thermo Fisher Scientific Material Safety Data Sheet (MSDS) for Acetic Acid, CAS# 64–19–7. Revised July 30, 2010. 2nd rev.

Protocol 3.4 Blood culture and harvest procedure for microarray confirmation studies

Adapted from a procedure contributed by Oregon Health & Science University, Knight Diagnostic Laboratories Cytogenetics Laboratories

I. Principle

Blood lymphocytes are stimulated by the mitogen phytohemagglutinin (PHA) to rapidly grow and divide such that three days of culturing is sufficient to yield material of acceptable quantity. Colcemid inhibits the formation of the spindle fibers. When added to the stimulated culture it causes mitotically active cells to be arrested in early metaphase. The synchronization of the cultures with methotrexate and thymidine allows large numbers of cells to enter early metaphase near the time of the harvest. Addition of Ethidium Bromide can also produce chromosomes with greater length and resolution by intercalating with the DNA, thus inhibiting chromosome condensation.

Safety warnings

All tissue specimens should be considered hazardous. Use Biohazard handling procedures and follow Universal Precautions. Use the laminar flow hood for all steps up to harvest spin. Wear a laboratory coat and protective gloves for all steps through slide-making. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with fresh Sanimaster 4 (made fresh weekly). Cover cuts with protective bandages even when gloves are worn. Dispose of Pasteur pipettes, needles and syringes in sharps container. Wash hands thoroughly after removing gloves.

Methotrexate, Ethidium Bromide, and Colcemid are toxic chemicals and should be handled accordingly. Avoid contact with skin.

Specimen

Lymphocytes are obtained through procurement of anti-coagulated peripheral blood. Three to five milliliters of sodium heparinized whole blood is required for optimum results. Other anticoagulants, though not recommended, may be accepted. Samples should be kept at room temperature and sent to the laboratory as soon as possible for optimum viability. Samples sent by overnight mail generally yield adequate results.

II. Materials

Supplies

1. 15-mL Corning polypropylene tube (Lab Stores #64.2005)
2. Pasteur pipette, 5¾ in. (Lab Stores #63.1614)
3. Pipette bulbs, 2 mL (Lab Stores #63.0202)
4. 5-mL sterile pipettes (Falcon #7543)
5. 10-mL sterile pipettes (Falcon #7551)
6. 1-mL sterile pipettes (Falcon #7521)
7. 25-mL sterile pipettes (Falcon #7525)
8. T-12.5 blue-capped flasks (Falcon #353018)
9. Micropipettors and sterile tips (Eppendorf)
10. Sterilizing filter unit, 115 mL, 0.2 µm (Nalgene #124-0020)

Culture medium and stock reagents

11. RPMI 1640 (Gibco #11875-051)
12. Fetal Bovine Serum (Irvine #3000)
13. Glutamine 200 mM (Gibco #250030-016)
14. Gentamicin 50 mg/mL (BioWhittaker #17-5282)

Incomplete medium

- a. 50 mL fetal bovine serum
- b. 10 mL Glutamine
- c. 0.5 mL Gentamicin
- d. 439.5 mL RPMI 1640

Mix together for a total of 500 mL. Aliquot into 250 mL sterile flask or bottle and label as incomplete medium (also known as Bone Marrow Medium). Store at 4°C. Shelf life is 6 weeks.

15. Phytohemagglutinin M-Form (Gibco #10576-015)

As of 3/29/05, PHA comes reconstituted and frozen. To use, thaw 10 mL vial and dispense into four 2.5 mL aliquots. Refreeze unused aliquots and thaw just before making the next batch of complete medium. Aliquot the next 10 mL vial when the last 2.5 mL aliquot is used.

Add 2.5 mL of PHA-M to 250 mL of incomplete medium (same as Bone Marrow medium). This is now **complete** medium used to grow peripheral blood T-lymphocytes. Store at 4°C. Shelf life is 4 weeks.

16. Hypotonic Solution: KCl (Mallinckrodt #6838)

Hypotonic KCl (0.075 M): Add 5.59 g KCl to 1 liter of deionized H₂O.

Aliquot into 100 mL bottles and store at 4°C. Shelf life is 4 weeks. Warm to 37°C before using.

17. Fixative:

Methanol, Absolute (Mallinckrodt #3016)

Glacial Acetic Acid (EM Science #AX0073-9)

Add 3 parts methanol to 1 part Glacial Acetic Acid. Make fresh before each use.

18. Methotrexate: Methotrexate, 50 mg/mL (Lederle #NDC0205-5325-26)

Methotrexate (MTX) may be fatal if swallowed, or absorbed through the skin. May have reproductive effects. Wear gloves and lab coat at all times when handling.

Stock Solution: add 80 µL methotrexate to 4.4 mL sterile H₂O using a micropipette.

- Working Solution: Add 0.1 mL (100 µL) of stock to 9.9 mL of sterile H₂O for a concentration of 10⁻⁵ M. Store at 4 °C. Shelf life 12 months. Use 0.05 mL (50 µL)/5 mL culture to synchronize cultures.
19. Thymidine: Thymidine (Sigma #T-5018)
Thymidine working solution: Add 0.025 g thymidine to 100 mL Hanks' (1×) for a final concentration of 10⁻³ M. Filter sterilize with a 0.2-µm filtration unit.
Aliquot 20 mL into a T-12.5 blue-capped flask. Store at 4 °C. Shelf life 12 months. Use 0.1 mL (100 µL)/5 mL culture to release cells from methotrexate.
 20. Hanks' (1×): Hanks' 10× (Gibco #14180-020)
Add 1 part Hanks' 10× to 9 parts sterile H₂O. Store at room temperature or 4 °C. Shelf life 12 months.
 21. Colcemid: Colcemid 10 µg/mL (Gibco #15210-016). Shelf life is 12 months.
 22. Ethidium Bromide: Ethidium Bromide (Sigma #E-1510), 10 mg/mL
EB Working Solution: Add 1 mL of EB to 9 mL of Hanks' (1×). Store at 4 °C. Shelf life 12 months.
Ethidium Bromide is a potential mutagen, and is irritating to the eyes, skin, and respiratory system. Flush for 15 minutes in case of accidental contact. Wear gloves and lab coat at all times.

III. Method

Culture setup

1. Add 5 mL of Complete Culture Medium to each of three 15-mL Corning polypropylene tubes. Label one of these tubes as 2A, one as 2MA, and one tube as 2AEB. Label with patient name, date, and laboratory identification information.
2. At 4:00 to 4:30 p.m., add heparinized whole blood to 5 mL Complete Culture Medium using a 1-mL pipette or Eppendorf pipette aid. Use 0.25–0.3 mL for Newborns, 0.3–0.35 mL for individuals <15 years old, and 0.35–0.40 mL for individuals >15 years old. Cap tube and mix well.
3. Incubate cultures at 37 °C for 48–96 hours. Incubation time is dependent upon the age of the patient, type of study and urgency of the study (48 hours for fast results and all newborns and young children, 72 hours for best results for children and young adults, and 72–96 hours for adults and women who are pregnant).
4. Add 0.05 mL of 10⁻⁵ M methotrexate to cultures 2A, 2MA, and 2AEB at 4:00 to 4:30 p.m. the day before harvest. Incubate cultures at 37 °C overnight.
***Cultures 2A, 2MA, and 2AEB are methotrexate synchronized overnight and released for 5 hours.
5. Add 0.1 mL of 10⁻³ M thymidine to the 2A, 2MA, & 2AEB cultures at 8:30 a.m. the morning of the scheduled harvest. Mix well and re-incubate at 37 °C until harvest.

Blood harvest procedure

1. At 12:30 p.m. on the scheduled harvest day, add 20 µL Colcemid to the 2MA culture using a micropipettor. Mix well and incubate for 60 min at 37 °C.
2. At 1:00 p.m. on the scheduled harvest day, add 20 µL Colcemid using a micropipettor to the 2A and 2AEB cultures. Add 0.05 mL (50 µL) ethidium bromide to the 2AEB culture. Mix well and incubate for 30 min at 37 °C.
3. At 1:30 p.m., centrifuge each culture for 10 min at 1000 RPM.
4. Remove supernatant. Add 4 mL warm (37 °C) 0.075 M KCl to each tube and resuspend cell pellet. Centrifuge for 10 min at 1000 RPM. Allow cultures to stand at room temperature for 5 minutes if number of cultures in harvest is less than 9 tubes.
5. Remove supernatant. Add 4 mL warm 0.075 M KCl to each tube and resuspend cell pellet. Add 5 drops of fresh fixative to each tube and mix well. Centrifuge for 10 min at 1000 RPM.
6. **Each tube must be treated individually for this step.** Remove supernatant and resuspend cell pellet. Add 2 mL fresh fixative and mix well. Add an additional 2 mL fixative and mix well. Repeat for each individual tube. Centrifuge for 10 min at 1000 RPM.
7. Remove supernatant. Add 4 mL fresh fixative to each tube. Centrifuge for 10 min at 1000 RPM.
8. After centrifugation, cell pellet may be used to make slides, or stored in a refrigerator for later use.

IV. References and additional readings

1. Hungerford DA. Leukocytes cultured from small inocula of whole blood and the preparation of metaphase chromosomes by treatment with hypotonic KCl. *Stain Technology* 1965; 40:333–338.
2. Francke U, Oliver N. Quantitative analysis of high resolution trypsin-Giemsa bands on human prometaphase chromosomes. *Human Genetics* 1978; 45:137–165.
3. Yunis JJ. High resolution of human chromosome. *Science* 1976; 191:1268–1270.

CHAPTER 4

General cell culture principles and fibroblast culture

Debra F. Saxe¹, Kristin M. May² and Jean H. Priest¹

¹Emory University School of Medicine, Atlanta, GA, USA

²Children's Hospital at Erlanger, Chattanooga, TN, USA

4.1 Definitions of a culture

Most cultures used for genetic studies involve the growth of cells that do not undergo further differentiation. Therefore, the term *cell culture*, rather than *tissue* or *organ* culture, is the most appropriate general description. If some particular function or structure is selected and maintained, the term *cell strain* can be used.

4.1.1 Time in culture

Cultures can be defined by parameters of time. *Primary cultures* are initiated from cells, tissues, or organs taken directly from an organism. One way to start a culture is to take an excised fragment of a tissue or organ, termed an explant. A primary culture may be regarded as such until it is subcultured for the first time. The description *short term* is used for a primary culture that is not subcultured. The term *primary colony* is used if the colony starts from one cell (or a few) in suspension that attaches and divides to form an isolated cluster or colony. A *cell line* arises from a primary culture at the time of first subculture. The term *cell line* implies that cultures from it consist of numerous lineages of the cells originally present in the primary culture. If it is known to start from a single cell, the term *clone* is used. If special or unique characteristics are preserved after subculturing, it is referred to as a *cloned line* or *strain*. For various research purposes, it may be important to quantitate culture time parameters. *Subculture* or *passage number* indicates the number of times cells have been subcultured (transplanted) from one vessel to another, whereas *subculture interval* denotes the time between consecutive subcultures. *Cell generation time* denotes the interval between consecutive divisions of a cell. Time in culture can be expressed in *population doubling time*, the interval taken for the cell number to double. The corollary is *population doubling number* or population doublings, the number of times the cell population has doubled, which is the most accurate way to measure culture lifetime.

If subcultures are made following a 1 : 2 split of the original culture, subculture numbers are equivalent to population doubling number. If a 1 : 4 split is used, one subculture is equivalent to two population doublings; 1 : 8 equals three population doublings, and so on. Accurate records of split ratios are important to keep track of culture age, which then gives an indication of how long a particular culture can be maintained for study.

Cultures originating from normal solid tissue, such as skin and products of conception, are usually capable of 20–100 population doublings, depending on culture conditions due to limited in vitro lifetime of human diploid cells. However, once an initial culture becomes confluent, growth slows and eventually ceases and then recedes. Subculturing on a regular basis is necessary to keep cells dividing and healthy. A *continuous cell line* has the ability to be passaged indefinitely in vitro and has become transformed or immortalized due to changes in a gene or genes controlling normal cell growth and division. These are, by definition, abnormal cell lines. Transformed cells can be derived from in vitro transformation of normal cells or from malignant tumors or effusions transformed *in vivo*.

Cell lines can be described in various ways: whether the original tissue is normal or neoplastic, and prenatal or postnatal. It is also important to document the animal species and age, as well as the organ and/or cell type and whether the line has been

cloned. By convention, these lines are designated with no more than four letters in series, indicating the laboratory of origin and a series of numbers indicating the line, for example, NCL 123.

4.1.2 Growth characteristics

Cultures are also defined by the way they grow. *Monolayer* refers to a single layer of cells growing on a surface. The ability of cells to attach and grow is measured by *absolute plating efficiency*, or the percentage of individual cells that give rise to colonies when inoculated into culture vessels. *Suspension* denotes a type of culture in which cells multiply while suspended in medium. In some monolayer cultures the cells will also detach and grow in the medium. In another type of culture the dividing cells will *hover* between monolayer and suspension, never making a true monolayer.

4.1.3 Morphology

Cultures can be characterized by cell morphology when viewed at low magnification under an inverted microscope. Cultured cells generally do not preserve the full architecture of the tissue of origin without special conditions. They nevertheless can have a unique appearance that is maintained. The *amniocyte* (or AF-type) cultured from amniotic fluid is pleomorphic and contains a mixture of mononucleated and multinucleated cells (Figure 4.1a,b). *Epithelial* (or E-type) cells refer to polygonal cells, which form continuous sheets in a monolayer with little intercellular substance (Figure 4.1c) and poor dissociation by trypsin. *Fibroblast* cultures (or F-type) contain monolayer spindle shape cells with some irregularities. Fibroblast cultures form parallel arrays at confluence (Figure 4.1d). The term fibroblast implies that these cells make an extracellular matrix or “fibers” that generally cannot be demonstrated without special procedures. The cell type cultured from chorionic villus mesenchymal core forms a monolayer and can be referred to as *mesenchymal*. The appearance at confluence is not spindle-shaped in parallel arrays but is a less organized monolayer.

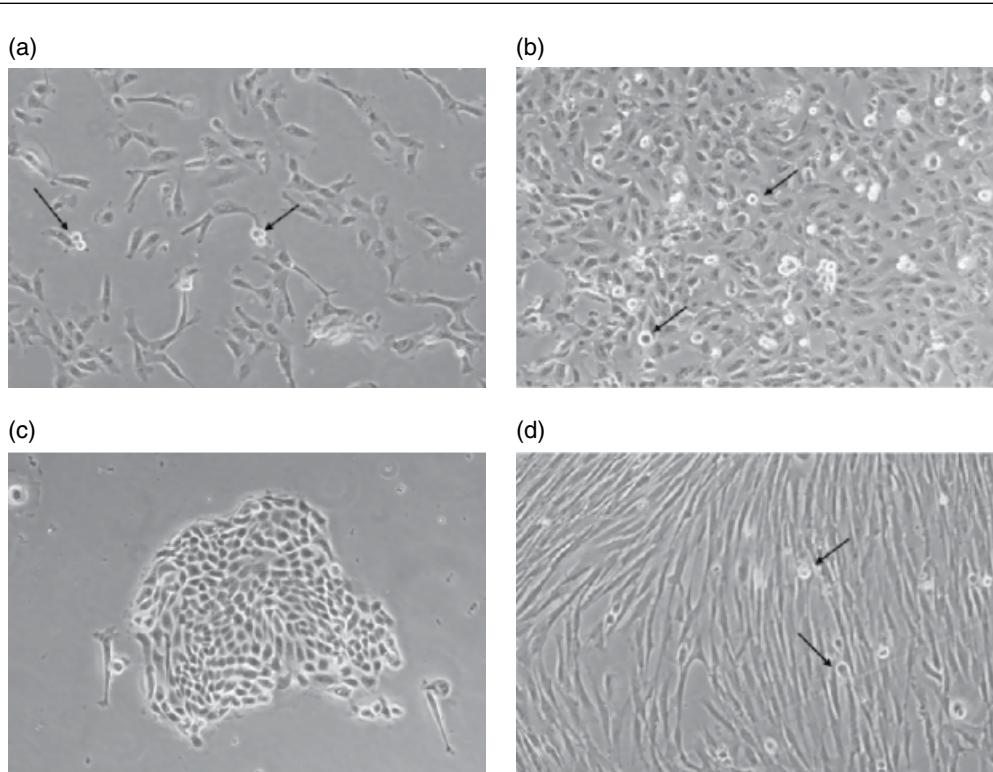


Figure 4.1 Amniotic fluid cell types. (a) AF-type cells from amniotic fluid in colony formation. (b) Confluent colony of AF-type cells from amniotic fluid. (c) E-type cells from amniotic fluid. These cells generally have a low mitotic rate and rarely yield metaphase chromosome preparations at harvest. (d) Fibroblast cells from skin biopsy. These cells have a spindle appearance. Mitotically active cells are round with a pale circle around them (examples indicated by arrows). Courtesy of Dr. Kristin May.

4.1.4 Tissue source

Culture types may also be classified by their tissues of origin. Cells suspended in amniotic fluid are recovered in suspension and can be plated out directly to form primary colonies of F-, E-, and AF-type cells growing as monolayers. The cells generally used for prenatal genetic studies are the AF type. This cell type is probably best described as an early, undifferentiated trophoblast because it may be multinucleated, pleomorphic, and in culture has functions of placenta including production of human chorionic gonadotropin (hCG) and basement membrane collagen. The F-type cells in amniotic fluid cultures can be shown to have the properties of fibroblasts. E-type cells in amniotic fluid cultures are a mixed group morphologically and do not subculture well, making them hard to define or study in culture. Primary cultures and subcultures from chorionic villi grow as monolayers with mesenchymal cell morphology and also produce type I collagen. Placenta cultures need to be set up with knowledge of cell origin, whether from chorionic plate, placental villi, or identifiable chorionic villi. Products of conception should be carefully dissected from placental tissue or maternal cells (see Figures 4.2 and 4.3). Other fetal tissue or autopsy specimens may be cultured from explants or disassociated tissue. Morphologic distinction is difficult to make once the cells attach and grow from these different locations.

Fibroblast cultures from skin biopsy (living or post-mortem) are started from explants by mincing or enzyme dissociation. Fibroblast monolayers can be maintained in serial culture, and produce type I collagen as do fibroblasts *in vivo*. Explants from other tissue or organ sources usually produce fibroblast monolayer cultures unless special conditions are provided.

Hematopoietic cells from bone marrow and lymphocytes from peripheral blood, thymus, or lymph node are usually placed in a short-term “hover” culture before chromosome analysis. These short-term methods are discussed in Chapter 11 (Cytogenetic Analysis of Hematologic Malignant Diseases).

4.2 Basic considerations in cell culture

For an *in vitro* human cell culture to thrive, it requires an environment that enhances cell proliferation, spreading, and migration. These environmental conditions include a substrate or surface for the cells to grow on, as in monolayer growth, or a liquid as in suspension cultures and an appropriate physiological environment (medium, pH, osmolarity, CO₂, temperature, and humidity). Media are often supplemented with sera and additives such as growth factors, proteins, and adhesion factors to promote cell growth.

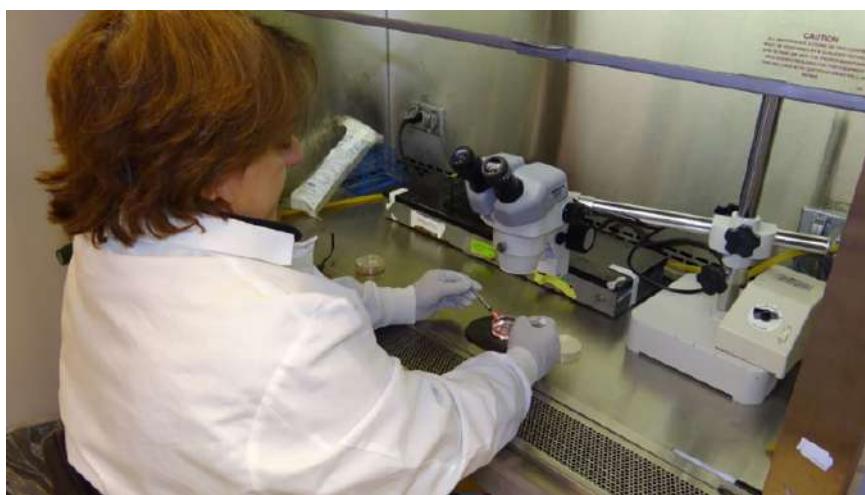


Figure 4.2 Sterile technique. Being aware of the various aspects of proper sterile technique is critical when working with patient specimens. Invitrogen provides a video that demonstrates proper sterile technique, which can be viewed at the following Web address: <http://media.invitrogen.com.edgesuite.net/Cell-Culture/videos/SterileTechnique.html?CID=ccbvid2>. The Laboratory Response Network at New York State Department of Health Wadsworth Center (<http://www.wadsworth.org/testing/lrn/>) also provides a video, “Essentials in Biosafety,” for the proper use of a biological safety cabinet. <http://www.wadsworth.org/testing/lrn/resources.html>. Select Biosafety Video at the bottom of the page. Courtesy of Cheryl D. De Mara M.Photog., FP. See insert for color representation of this figure.



Figure 4.3 Distinguishing a villus frond from maternal decidua. Villi (top) appear as branching “fronds” attached to a base. Maternal decidua (bottom) is amorphous, without the branching effect. Courtesy of Cheryl D. De Mara M.Photog., FP. See insert for color representation of this figure.

Disposable, prepared, and pre-sterilized culture supplies are used by most laboratories. These include culture dishes, pipettes, media, scalpels, and filters. It is important to date the perishable items on arrival, place in designated areas, and follow the manufacturer’s instructions regarding storage conditions and expiration dates. Most clinical laboratories use disposable tissue culture supplies and prepared media because it is ultimately less expensive and more efficient. Using glassware and making media from scratch requires additional time in testing and validation. Nevertheless, there may still be situations in which a laboratory may need to sterilize culture materials and/or prepare media. It is essential to understand the basics about sterilization, glassware washing, water for cell culture, temperature control, pH and humidity control, media, balanced salt solutions, methods to disperse cells, and antimicrobial agents. Examples and discussion of methodology in these areas are given here to advance the understanding of basic culture techniques.

In order to maintain a sterile environment, all work surfaces must be kept clean, including hoods and incubators. A good disinfectant for hoods is Conflukt® or 70% alcohol (bleach is corrosive). Cultures should be handled in a biological safety cabinet class IIA or B with a high-efficiency particulate (HEPA) air filter. Regular maintenance inspection is essential.

Aseptic or sterile technique is necessary to avoid introduction of rapidly dividing microorganisms or other cell types into the cultures. These microorganisms will not only quickly exhaust the media of nutrients, but will also excrete toxins into media that may inhibit or kill growth of the eukaryotic cells. All areas that come in contact with the cultures should be cleaned routinely with an antiseptic solution, such as a 10% bleach solution, 70% ethanol, or commercial antiseptics such as Conflukt®. Use care when choosing a cleaning agent, as some may corrode metal surfaces. Biological safety cabinets, or tissue culture hoods, and countertops should be thoroughly wiped immediately after spills and daily, before and after use. It is advisable to run hood fans at least 10 minutes before working with cultures. Ultraviolet (UV) lamps are not required in biological safety cabinets, and, indeed, they are not recommended because of potential safety hazards associated with their use. However, if they are used, staff should not be present in the room. UV exposure damages the DNA of tissues such as skin and cornea of the eye. UV lights should be cleaned weekly to remove dust and dirt that may block the light’s effectiveness, and the bulb’s intensity should be checked periodically.

4.2.1 Culture containers

Know the expected growth characteristics and requirements of your cells before beginning work with them. In general, disposable, sterile culture vessels are available commercially and include flasks, Petri dishes, tubes, roller bottles, multiwell plates, and specialized units, such as slide chambers, for growing cells directly on microscope slides. Glass vessels for reuse need special washing and sterilizing procedures described in the following sections (see 4.2.2, Sterilization and washing principles of cell

culture section). In situ chromosome analysis requires that the cell monolayer grow on a surface that can be processed easily for analysis. Glass coverslips or culture dishes that are treated to have a negatively charged surface to allow for cell attachment should be used to culture cells that grow in a monolayer. Flasks or culture tubes can be used for backup culturing if the in situ method fails and for growing cells to a higher density for other testing.

The size of the culture vessel and number of cells in the inoculate needs to be selected carefully because human monolayer cells grow poorly if inoculated at too low or too high a density. The caps of culture flasks can be loosened to allow gas exchange or tightened to form a closed system. Vented caps are also available for culture flasks. Smaller culture or Petri dishes are advantageous for providing free gas exchange and growing small volumes for rapid harvesting. Specialized culture vessels may be required for unusual types of cells.

4.2.2 Sterilization and washing principles of cell culture

There are several methods for sterilizing items used for cell culture. Methods and their applications are given later. Place items to be sterilized in one area, cover items with foil if needed, and mark with heat-sensitive tape. A thermometer should be in the oven or autoclave to record maximum temperature. Heat-sensitive tape allows you to know if the appropriate temperature was reached and maintained. Vials of biologic heat indicators may also be included as an additional check or for monthly quality control of equipment. These vials contain bacteria which change color when killed by heat at 120 °C. After sterilization, cool and store in an area used just for sterile items, and label the items as sterile with the date that they were sterilized. As a general rule, unused sterile supplies in contact with cells and cultures should be re-sterilized at intervals not to exceed 1 month.

Dry heat sterilization

Items to be sterilized are placed in an oven and the temperature is raised to 165 °C for 2 hours. If the oven is small or crowded, heating should last for 3 hours. Examples of items to be sterilized by dry air include cotton-plugged serologic pipettes, instruments in glass or metal containers, large dissecting pans that do not fit in an autoclave, and glassware covered with aluminum foil. Dry air sterilization tape is available that changes color when exposed to heat long enough to sterilize. Coverslips for in situ chromosome analysis of cultured cells can be sterilized at 100 °C for at least 1 hour.

Autoclave – fast or gravity exhaust

Items are autoclaved at 121 °C for 20–30 minutes, 15 psi. Examples of items to be sterilized by fast exhaust include instruments in cloth or paper wraps, rubber or plastic bulbs, scrapers, other items damaged by high temperature, and glassware with caps that can be damaged by dry heat. Caps on bottles should be loose during autoclaving and then tightened once they have cooled and dried. Autoclave strips are also available that change color when sterilized.

Autoclave – slow exhaust or liquid cycle

As for fast exhaust, items are autoclaved at 121 °C for 30 minutes (longer if there is a large volume of liquid), 15 psi. Examples of items to be sterilized by slow exhaust include loosely capped vessels containing liquid, not more than two-thirds full, and reusable membrane filter assemblies.

Filter sterilization

Disposable pre-sterilized filters save time, give more assurance of sterility, and can be worth the additional expense. Filters of 0.20–0.22 µm pore size should be used for sterilization. The rate of filtration is less than with 0.4 µm pore size, but small Gram-negative bacteria are not excluded by these larger pores. Pre-filters are needed if filtering particulate material or serum. If filter holders and accessory parts are reused, they should be washed immediately and rinsed. Washing instructions for items coming into contact with cells are discussed in the next section on washing culture glassware. Most laboratories, however, purchase sterile, disposable, individually packaged filters at various volumes and do not reuse them. Considerations for choosing the correct size of filter include the following:

- Small volumes (<50 mL): Filter holders of several sizes are available to fit on a hypodermic syringe for pressure filtration of small volumes of liquid.
- Intermediate volumes (100–1000 mL): Filter holders are available for suction filtration. A vacuum pump may be necessary to furnish enough suction for rapid filtration if the laboratory is not equipped with adequate wall suction.

- Larger volumes (>1000 mL): Larger filter holders that operate by pressure from a compression pump are used. The holding container and tubing upstream from the filter should be cell culture-clean but not sterile. However, sterility must be strictly maintained for the dispensing device and containers downstream from the filter.

Washing culture glassware

There may be some permanent items that come directly or indirectly into contact with cultured cells that can be recycled. Protocols for washing cell culture glassware vary in complexity, although the basic underlying rules apply.

Glassware should be washed thoroughly with a liquid or powdered phosphate-free detergent and hot water. Adequate rinsing is imperative. This should include multiple rinses with tap water and then three rinses with distilled water.

Washing and sterilizing cell culture instruments

Scalpels, forceps, and scissors that are used for tissue culture must be properly decontaminated following laboratory biohazard procedures. Immediately after use, soak briefly in decontaminating solution. Dilute (10%) bleach can be added. After soaking in a decontaminating solution, the instruments should be washed, rinsed completely, dried and then autoclaved for re-use.

4.2.3 Water for cell culture

The exact preparation of water for cell culture may vary from one laboratory to another, but certain rules must be followed for cell growth, particularly mammalian cells. Type I quality water, as defined by CAP (College of American Pathologists) or CLSI (Clinical and Laboratory Standards Institute), is recommended for clinical laboratories. This is the highest quality of water in terms of limited amounts of contaminants such as particles, bacteria, ions, and organic compounds. To ensure type I water quality, resistivity, bacterial content, particle count, and silicon content need to be monitored. Millipore and equivalent companies supply filtration systems, which deliver water that should not require additional distillation for use with cultured cells. For distilled water to qualify as type I, resistivity must be above 10 MΩ·cm; bacterial content must be below 10 CFU/mL; and silicate content must be below 0.05 mg/L SiO₂. Each filtration system will have a different method to monitor these parameters. It is essential to have regular maintenance and replacement of the filtration system cartridges. Be sure the pH is near-neutral (not acid) at the time of use. Reservoirs for water should be rinsed frequently with water freshly prepared; prolonged storage is not recommended.

Alternately, sterile cell culture water is available commercially if smaller volumes are needed. This type of water can be obtained from suppliers of cell culture media or from a hospital supply facility where it is generally termed pyrogen-free or triple distilled and is approved for *in vivo* intravenous use.

4.2.4 Temperature, pH, CO₂, and humidity control

Mammalian cell cultures require a constant temperature of 37–37.5 °C, a pH of 7.2–7.4, and a humidity level that is close to saturation. Constant temperature is maintained by air circulation, water jackets, high-efficiency insulation, or diffuse heaters in incubators if the doors are opened infrequently. The pH for human cells is usually maintained by a constant CO₂ atmosphere and a bicarbonate buffer in the culture medium.

Cells can be grown in either an “open” or “closed” system. These terms refer to the state of the culture vessel rather than the incubator itself. In an open system, the design of the culture vessel allows for free exchange of gases between the air in the incubator and the air around the culture. In a closed system, cultures in flasks are gassed with 5% CO₂ and capped tightly to maintain the pH. A solution of phenol red added to the medium by the manufacturer is a sensitive indicator of pH. The color of this indicator is pink-orange in the proper pH range; yellow is too acid, pink too alkaline. For closed systems, organic buffers (such as HEPES) may also be suitable and do not require a constant CO₂ atmosphere. These buffers are also excellent for transport media. If cells are shipped from another laboratory, it is important to know what concentration of CO₂ is needed for the medium on the cells.

There are two types of open system incubators. Both maintain the CO₂ at approximately 5%. These systems require a bicarbonate buffer and gas exchange using unsealed dishes, plates, or flasks with permeable or vented caps. Another type of incubator, termed a tri-gas, also regulates the level of oxygen by addition of nitrogen gas. The atmospheric oxygen level (approximately 20%) is much higher than physiological levels in tissues, which is generally 1–6%. A typical tri-gas incubator may have 5% O₂, 5% CO₂, and 90% N₂. Amniocytes have been shown to grow more quickly and form larger colonies in a

tri-gas system with 5–7% O₂ and 5–7% CO₂. Fibroblasts, lymphocytes, and other cell types thrive in an open system, single-gas incubator that only regulates CO₂ levels. The use of water pans in open systems is imperative to maintain 80–95% humidity and reduce evaporation in Petri dishes and multi-well plates. Antimicrobial agents such as copper salts may be added to the water pans if recommended by the manufacturer.

It is best to limit the number of times that the incubator door is opened in order to maintain a constant environment. Some incubators automatically infuse with CO₂ every time the door is open without measuring the CO₂. This may change the pH if the door is opened frequently within a short time frame. Frequent opening and handling may also lower the temperature, which slows growth. The amount of time that cultures are removed from the incubator to assess growth or perform media changes should be kept to a minimum. Even in brief trips out of the incubator, flasks or Petri dishes should be kept on a 37°C warming plate to avoid cooling the cultures.

For all types of incubators, frequent cleaning/decontamination according to the manufacturer's instructions are essential. A regular maintenance schedule should be developed by each laboratory. Many manufacturers offer HEPA filters in CO₂ incubators to reduce contamination during incubation. Copper-lined chambers with copper shelves and fixtures also reduce fungal growth and other contaminants and can be worth the extra expense.

As part of laboratory quality maintenance, regular incubator checks for the level of CO₂ and oxygen should be performed with a Fyrite testing system or a similar product. It is important that the incubators be equipped with an alarm system to alert the laboratory staff when temperatures and gas concentrations are out of range or when CO₂ or N₂ tanks are empty. Most laboratories have backup tanks of CO₂ and N₂, which is automatically switched on immediately after the first tank is emptied. If a backup is not connected, cultures grown in an open system will change pH within a few hours after the gas tanks are emptied. Incubators should be connected to an emergency power supply or generator so that the cells are not damaged when power outages occur.

4.2.5 Media

Defined media for the culture of mammalian cells are available commercially in sterile liquid or powder form, but standard recipes may also be used for assembling the ingredients. It is always important to use the right kind of water (see the previous section on water for cell culture) if a medium is prepared from scratch or is reconstituted from a commercially available powder and sterilized. The growth of mammalian cells usually requires additives to the defined medium, such as L-glutamine and serum, which contain growth factors, proteins, and adhesion factors that promote growth.

A concentration of approximately 10–20% fetal bovine serum (range of about 5–30%) is added, depending on composition of the defined medium and type of cell to be grown. Human cells at low density or specialized cultures may require higher serum concentrations. Once cell lines are established, the serum concentration can often be decreased. Different lots of serum may vary in ability to support growth. Defined serum substitutes are also available for various cell types, as well as media that already contain supplements. Serum and other media components must be tested for sterility before use, and complete medium must be tested for its ability to support cell growth before use by performing cell plating efficiency (see Protocol 4.16, Plating efficiency of serum). Serum that is purchased commercially is prescreened for mycoplasma and filtered with a 0.1 µm filter membrane, which eliminates the need to employ heat inactivation. In fact, heat inactivation of serum may adversely affect growth factors [1]. Serum should always be shipped frozen, thawed once, aliquoted, stored frozen, and added to medium just before use. Most complete media with unstable additives, such as serum, glutamine, and antibiotics, have a relatively short shelf life and generally should be used within 2 weeks. The manufacturer's recommendations should be followed. It is important to avoid leaving medium out in the light for any length of time. There is a possibility that fluorescent lights may create free radicals, which could make the media unstable over time.

Formulations for defined media are available in catalogs from commercial suppliers. Different cell types require different additives to support or enhance growth. For instance, serum added to media may favor normal cell growth over malignant cells. Cell lines that are purchased or obtained from another laboratory should be maintained in the same medium in which they were first established. Examples of appropriate media to support growth of various cell types are listed here.

- Alpha MEM: Eagle's minimal essential medium (MEM) modified to contain non-essential amino acids, B vitamins, and salts.
- Dulbecco's MEM and McCoy's 5A: media modified to contain additional amino acids, vitamins, and non-essential amino acids suitable for general cell growth.
- Ham's F10 and F12: media that include higher concentrations of vitamins, amino acids, minerals, and glucose and are designed to support clonal growth of mammalian cells.
- RPMI 1640: medium that was originally defined for suspension (lymphoblast) cultures.

- AmnioMAX and Chang: complete media designed for clonal and monolayer growth cultures, such as for human amniotic fluid cells.
- PB-MAX and Chang MF: complete media, with and without T-cell stimulant, respectively, which were designed predominately for growth of human suspension lymphocyte blood cells.
- MarrowMAX and Chang BMC: human stromal cell conditioned medium and ORIGEN giant cell tumor-conditioned medium, respectively, which were designed for suspension (lymphoblast) cultures of bone marrow cells.
- Medium 199: medium that is low in folate and useful for the demonstration of folate-sensitive fragile sites.

Labels must provide enough information to be able to identify its contents, and to be capable of tracing its contents to its initial source. CAP (July 2011, CYG. 33100) has defined the minimum required fields to be placed on reagent labels (see Figure 4.4), which include content and quantity or concentration, storage requirements, precautionary labels, and preparation and expiration dates. Each laboratory may adopt its own set of additional requirements, which would be defined in the procedure manual for that laboratory.

4.2.6 Balanced salt solution

Balanced salt solutions (BSSs) are needed to rinse cells free of serum that contains trypsin inhibitors before trypsinization, during processing for examination of chromosomes, during other types of specialized cell procedures, and as buffers in defined media.

College of American Pathologists	Revised 07/28/2015
REAGENT LABELING	
COM.30300 REAGENT LABELING	Phase II
Reagents, calibrators, controls, stains, chemicals, and solutions are properly labeled, as applicable and appropriate, with the following elements. <ol style="list-style-type: none"> 1. Content and quantity, concentration or titer 2. Storage requirements 3. Date prepared, filtered or reconstituted by laboratory 4. Expiration date 	
<i>NOTE: The above elements may be recorded in a log (paper or electronic), rather than on the containers themselves, providing that all containers are identified so they are traceable to the appropriate data in the log. While useful for inventory management, labeling with "date received" is not routinely required. There is no requirement to routinely label individual containers with "date opened"; however, a new expiration date must be recorded if opening the container changes the expiration date, storage requirement, etc.</i>	
<i>This requirement also applies to the labeling of chemicals used in the laboratory to prepare reagents or during the preanalytic and analytic phases of the testing process. Requirements relating to precautionary labeling for hazardous chemicals are included in the Chemical Safety section of the Laboratory General Checklist.</i>	
Evidence of Compliance: <ul style="list-style-type: none"> ✓ Written policy defining elements required for reagent labeling 	
REFERENCES <p>1) Department of Health and Human Services, Centers for Medicare and Medicaid Services. Clinical laboratory improvement amendments of 1988; final rule. <i>Fed Register</i>. 2003(Jan 24):7164 [42CFR493.1252(c)]</p>	

Figure 4.4 Reagent labeling (as defined by CAP checklist COM.30300 Phase II revision 7/28/2015). The College of American Pathologists (CAP) has developed accreditation standards that have been adopted by over 7000 laboratories worldwide. This figure demonstrates the five essential elements required either on each reagent label or in a paper or electronic log which can be traced to every associated container. Neither "Date Received" or "Date Opened" is required by CAP, but upon opening a reagent, *the new expiration date, if it changes*, must be recorded, along with any other affected parameter, such as storage requirements, etc. The CAP program provides a checklist that defines detailed and focused laboratory standards, and it uses professionals within their specialized areas to perform onsite inspections of associated laboratories. Medicare and Medicaid Services (CMS), the Joint Commission (TJC), and many state certification requirements have all recognized this program as a reliable and effective overall assessment for the management and operation of laboratories within their specialties. The standards demonstrated in this figure have been based on 2015 revisions and may become overridden or changed in subsequent releases. Adapted from College of American Pathologists.

A pH in the range 7.2–7.4, as well as isotonicity, should be maintained by these solutions. Balanced salt solutions are available commercially in both sterilized and nonsterilized forms and are widely used by clinical laboratories. These include phosphate-buffered saline (PBS), Earle's solution, and Hanks' solution. PBS is deficient in calcium and magnesium, which aids dissociation of cells (as a diluent for trypsin) and helps to prevent clumping of fresh cells during processing for chromosomes.

4.2.7 Dispersion of monolayer cells for subculture

Trypsin is commonly used to disperse cells enzymatically from a monolayer into suspension. The correct strength and time of application of this enzyme is critical to ensure good growth following trypsinization. It is best to subculture at 80–90% confluence when cells are actively dividing. Trypsin is inactivated by serum, which contains protease inhibitors. Therefore, the monolayer cells to be subcultured must be rinsed one or two times with BSS (Ca^{2+} and Mg^{2+} free) before trypsinization. Afterward, the addition of medium containing serum prevents further action of the enzyme. The concentration of trypsin should be determined by the lowest concentration that will remove cells in 5–10 minutes. A concentration of 0.25% trypsin alone or 0.05% trypsin-EDTA is generally recommended for subculturing of human cells. Different preparations of trypsin are available commercially and vary in purity. Highly purified trypsin preparations are not recommended for routine subculturing.

Some types of human cells are more difficult to disperse, particularly overly confluent monolayers, phase III amniotic fluid (AF) cells at the end of their growth potential, or epithelial-type cells. These cells may require washing with additional PBS or supplementing the trypsin solution with collagenase, dispase, or a chelating agent such as EDTA. Mechanical methods that help to remove cells from monolayer include shaking or hitting a culture container, using a scraper, or forcibly pipetting the trypsin solution against the bottom of the flask after the cells are dispersed enzymatically. Petri dishes and multi-well plates that are treated to allow for cell harvest or removal without enzymatic treatment, by lowering the temperature from 37°C to 20°C, are also available.

4.2.8 Antimicrobial agents and monitoring for contamination

Antimicrobial agents are optional but are usually added to collection medium for specimens to be cultured and to primary cultures for treatment of early contamination. Many commercial media already contain antibiotics. If medium is prepared in the laboratory, it must first be checked for sterility, which includes filtration, incubation at 37°C for 48 hours, and careful examination for turbidity, before antimicrobial agents are added.

Cell culture media provides an excellent environment for growth of bacteria and fungi. Generally the best way to monitor for contamination is by observing the appearance of the media, including a change in pH (usually acidic) or an increase in turbidity. Inspection of cultures through an inverted microscope can reveal cells with indistinct margins in monolayer, an increased number of detached cells or loss of the cell layer entirely. Fungal growth may be apparent microscopically. As a general rule, it is unnecessary to identify the offending organism(s). Suspect cultures should be isolated, or decontaminated with 10% bleach and discarded. Treatment with doses of antimicrobial agents at the higher end of the recommended range may be indicated if the culture does not have a backup. In routine cytogenetic studies it is usually a good idea to harvest as soon as possible if bacterial contamination is suspected and another clean culture is unavailable.

It is important to carefully select the concentration and antimicrobial agent, since some are toxic at effective concentrations. Fungicides, in particular, may slow or inhibit cell growth. Most cell culture supply companies manufacture various combinations of antibacterial antibiotics, such as penicillin and streptomycin with either neomycin or amphotericin (final concentrations in medium: penicillin 50–100 U/mL, streptomycin 50–100 µg/mL, neomycin 100–200 µg/mL, amphotericin 0.25 µg/mL). Other antimicrobial agents include the following:

- Kanamycin (10–100 µg/mL final concentration in medium), gentamicin (50 µg/mL), and polymyxin B sulfate (100 U/mL) are broad-spectrum antibiotics that can be used if organisms are resistant to a combination of penicillin and streptomycin.
- Nystatin, for example, Mycostatin (50–100 µg/mL final concentration in medium), and amphotericin B, for example, Fungizone (2.5 µg/mL) both have antifungal (antimycotic) activity.

Mycoplasma is smaller than bacteria or fungi and cannot be seen through the microscope, removed by filtration, or treated with common antibiotics. It may slow cell growth and cause chromosome abnormalities without causing other visible changes often associated with culture contamination. Sources for mycoplasma include poor sterile technique and contaminated tissues used to initiate cultures. Test kits are available commercially.

The bottom line for all cell culture is that good sterile technique takes care of most contamination before it starts!

4.3 Fibroblast culture

4.3.1 Tissue sampling and transport

Various tissues can be grown in culture for chromosomal analysis or other testing. The basic considerations are the same regardless of tissue type. Sterility in recovering and handling the specimen is essential. Microorganisms have a much quicker doubling time and will spend the medium quickly, thereby slowing or stopping mammalian cell growth. If possible, collection of the material should be performed under surgical sterility. The tissue should be placed immediately into a sterile container with transport medium containing antibiotics and buffered salts, preferably including HEPES. If pH-buffered transport media are not available, most other mammalian culture media will suffice. If none of the above are available, isotonic phosphate buffer solutions (PBS) can be substituted, but may cause some cell damage. Specimens that are placed in PBS should be transferred to an appropriate medium immediately upon receipt.

Smaller specimens can be placed in sterile leak-proof containers, preferably in transport media containing antimicrobial agents. Larger specimens can be packed well in strong plastic bags (preferably double) or larger plastic containers. Biosafety rules for any carrier (postal service, air, local courier) require that the specimen be additionally wrapped in absorbent material and placed in one or two strong leak-proof and insulated outer containers. Biohazard must be marked on the outside. Cold packs may be enclosed with a larger specimen.

- Skin is frequently used to establish monolayer fibroblast cultures from a living individual. Every laboratory has somewhat different details in the skin biopsy protocol, but general principles are the same. These include the following: (a) preparation of the biopsy site such that cells will not be killed by the surgical preparation; (b) sterile sampling of dermis so that fibroblast cells can grow out in primary and then serial cultures; (c) a method to suspend and disperse the cells in a culture container. Pinch and punch biopsy techniques are both satisfactory. Procedural details are given in the protocols section of this chapter (see Protocol 4.1, Solid tissue collection for establishing cultures).
- In determining which tissues to collect for products of conception, it is advisable to obtain chorionic villi and fetal tissue whenever possible from abortus or intrauterine fetal demise cases, as villi often remain viable longer than fetal tissues. If villi are not available, cord or internal organs, such as spleen, lung, and kidney, grow well in culture. It is important to notify the referring physician not to send an entire fetus if it is more than about 12 weeks of gestation, if your facility does not have the appropriate means to discard the unused tissue. Specimens collected under nonsterile conditions should be rinsed several times and soaked for 20–30 minutes with BSS containing antimicrobial agents to reduce the risk of culture contamination.
- Cells from ascites fluid, intrauterine cystic hygromas, pleural effusions, and urine can be cultured similarly to fibroblast cultures if viable mesenchymal cells are present. These fluids should be collected and placed in sterile transport tubes.

4.3.2 Tissue sampling to exclude mosaicism

Reasons for sampling more than one organ or tissue, or multiple sites from the same tissue, are determined by the problem to be solved, such as potentially limited cell viability in abortus material, or suspected chromosomal mosaicism. The well-recognized diagnosis of confined placental or fetal mosaicism necessitates attention to the choice of where to take samples within both placental and fetal tissues. Explants from chorionic villi, amnion, and chorionic plate will sample developmentally distinct portions of the placenta. In the case of some postnatally diagnosed syndromes that involve possible mosaicism, study of multiple skin sites or areas of different skin texture and color is often indicated. Tissue from each site should be cultured and analyzed separately.

4.3.3 Specimen setup

Specimens should be examined to determine the quality and type of tissue received. The recorded information should include a description of the specimen, whether or not it contained clots, maternal tissue, or budding villi, as well as special conditions that the tissue was exposed to, such as extreme temperatures. These records are valuable when tracking quality assurance issues. They are of particular importance in such specimens as tumors or hydatidiform moles in POC (products of conception) specimens, where it may aid in the analysis and interpretation. If maternal tissue contamination is a possible problem, the tissue should be thoroughly cleaned of maternal material prior to mincing, to avoid growing maternal cells.

Multiple primary cultures should be initiated for a patient from each type of tissue received. This is important in chromosome analysis for excluding mosaicism. The cultures should be initiated in two different types of media or in different lots of the same medium, and each culture vessel should be labeled in a way that allows for tracking details of its set-up and culture progress (e.g., patient name, laboratory identification number and “CS1” for coverslip) [1].

If large samples are obtained, there is a temptation to start with too large a sample, which makes cell dispersal or attachment more difficult. Tissue samples should first be minced into fine pieces. The cultures can then be initiated by either allowing cells to migrate from adherent tissue fragments (explants) to the culture dish or by dispersion with proteolytic enzymes, such as collagenase, trypsin, and/or hyaluronidase. Not only do the cells grow up faster after enzymatic treatment, but contamination seems to be less. Achieving sterility in the primary cultures can be a problem with autopsy and abortus tissues.

Enzyme treatment digests proteins involved in cell surface adhesion and/or the extracellular matrix. The tissues can then dissociate into a single cell suspension with gentle agitation. These single cells will eventually attach to the substrate and grow. Crude trypsin is the most common enzyme used for disaggregating, because it is tolerated by a variety of cells and is effective for many tissues. In some cases, disaggregating with trypsin may damage epithelial cells or be ineffective on fibrous tissues. The intracellular matrix of these tissues contains collagen; therefore, collagenase is particularly effective for disaggregating several normal epithelial and malignant tissues. Crude collagenase (with contamination of nonspecific proteases) can be used alone or sequentially with trypsin. Once the tissue is disaggregated, the residual trypsin activity can be quenched by the addition of medium containing serum, and collagenase can be removed by centrifugation. It is very important not to leave cells in proteolytic enzymes for more than 20–30 minutes without checking the amount of disaggregation. These enzymes will begin to breakdown cell membranes and lyse the cells if allowed to be in contact with the cells too long.

Once the cells are in a single cell suspension, they can be seeded in a variety of culture vessels. If cytogenetic testing is required, the cultures can be seeded on coverslips in Petri dishes or in flasks. If biochemical assays are being performed, or if the cells are being transported to another laboratory for testing, it is best to seed the cultures in flasks. If the specimen is adequate, multiple cultures are set up. In the case of enzyme-treated cells, both single-cell suspensions and untreated minced fragments may be set. The protocol section describes the techniques in detail.

4.3.4 Routine handling and maintenance of monolayer cultures

Cell suspensions are generally put into the culture vessels in a minimum amount of medium in order to hasten cell attachment. Once cells have been seeded into a flask or on a coverslip, they begin to attach to the surface of the vessel. This may occur quickly, within 1–2 days, or take much longer. Factors involved in rates of cell attachment include tissue source (placenta grows more quickly than skin) and cell viability (tissue from a fetal demise may grow slowly or not at all or the specimen may have been exposed to adverse temperatures in transport). Tissue explants must be put in with a sufficient volume of media to keep them moist but not enough to make them float. Additional medium is added to cultures approximately 1–3 days after the initial seeding (this is also known as “flooding” the cultures). Complete media changes are performed 2–4 days after flooding, depending on the number of unattached cells. The longer waiting period is necessary when there are many unattached cells. Subsequently, cultures are generally maintained by media changes every 2–4 days, depending on the cell type and growth rate. A medium change is recommended 12–20 hours before a harvest to stimulate growth. After the cells have attached and begin to show growth, their progress should be assessed on a daily basis by viewing the cultures under an inverted microscope. All observations should be noted in the culture record.

Subculturing may be required to obtain a large number of cells or to maintain a cell line. This is performed by scraping cells mechanically or by releasing them with trypsin from the vessel surface of a flask or coverslip and dividing the cell suspension between two or more culture vessels (see 4.1.1, Time in culture for population doubling determination). Many cell types fail to grow well if overdiluted. Human cultures are generally subcultured at split ratios of 1 : 2 or 1 : 4. This often has the effect of stimulating a wave of growth such that the cells may be harvested soon after subculturing. Similarly, trypsinizing cells in a culture that has a few healthy but slowly growing colonies can stimulate growth. In this case, rather than subculturing, the cells are released from the culture surface, given fresh medium and returned to the same culture vessel without subculturing.

To track a culture in the laboratory, it is necessary to use two identifiers on each culture container and all records. This can be the laboratory accession number and patient/cell line name or initials, or identification number. It is valuable to include consecutive culture numbers on each culture vessel grown from one specimen. For quality control, these unique culture numbers should also be recorded in subculture and cell freezing records in order to identify a culture that has been analyzed or has been sent to another laboratory. Clinical laboratories are required to keep records on each culture vessel. These records should include laboratory accession number and a second identifier, tissue source, name, age, diagnosis, date received, and setup information. Many computerized laboratory information systems provide specific sections to record culture information that is recommended or required by various regulating bodies, such as CLIA (Clinical Laboratory Improvement Amendments), CAP, and ACMG (American College of Medical Genetics), including initial specimen condition, set up, maintenance, freezing/storage and disposal. If a commercial system is not available, the laboratory is responsible for maintaining a culture record book or separate sheets that would describe each vessel's complete culture history.

4.3.5 Preparation of cultured cells for analysis

For chromosome analysis

Primary cultures are preferred for chromosome analysis since they are more likely to represent the *in vivo* chromosome complement. Cells should be in log phase growth (when many rounded, refractory cells are present) when harvested. Coverslips are usually ready for harvest when four moderate or a minimum of five small colonies with rounded cells are present. Flasks should be harvested when they are approximately 50% confluent; however, this depends on the type of cell involved. Overly large coverslip colonies or overly confluent flasks will not generate good quality metaphase cells.

Two methods can be employed to increase the length of the chromosomes. One is by addition of a DNA intercalating agent, such as ethidium bromide, during Colcemid® exposure. Cells growing on coverslips are then arrested in prometaphase/metaphase using Colcemid®, treated with hypotonic solution, fixed, dried, and stained. Monolayers on the surface of a culture vessel need to be suspended, as for routine subculture, and then processed as a cell pellet. The second method is synchronization of cell division for the purpose of arresting cells in prometaphase (see later).

For cell synchronization and phasing

Many procedures are available for the synchronization or phasing of mammalian cells to make a culture more uniform in progression through the cell cycle. For chromosome analysis, it is useful to sample the cells at a time when many are in mitosis.

When a cell monolayer reaches confluence, the cells are arrested and will move into S phase (synthesis period) somewhat together when subcultured. This principle is utilized in cytogenetic procedures to achieve a maximum number of metaphases for examination. Cells can be synchronized prior to the addition of Colcemid® by blocking cells in S phase with methotrexate and then releasing the block by replacing with thymidine-rich medium. Medium change will also stimulate the cells to progress together through the cell cycle toward metaphase.

One synchronization procedure that is applicable to monolayer fibroblast cultures and uses an inhibitor of DNA synthesis (BrdU) followed by release of the block is given in the protocols section. The synchronization achieved in this procedure and others is short-lived and never approaches 100%, due to the wide range of behavior for individual cells within the whole population. Any procedure applied to the culture as a whole does not take into account the state of individual cells. Furthermore, cells starting a portion of the cell cycle together will not stay together long if they are moving through the cycle at different individual rates.

For biochemical protocols

Monolayers from culture vessels are put into suspension by mechanical or enzymatic treatment according to protocols for individual tests and laboratories. It is important to maintain culture records so that source of cells, culture life, and so on can be traced. It may be necessary to select control cultures when the cells to be tested are set up. If multiple flasks are required, it must be made clear that additional time will be necessary to grow them up. Always plan to maintain backup cultures, particularly if cells are sent to another laboratory for testing. Anticipate ahead of time, if possible, the likelihood that cells will need to be frozen for permanent storage, and freeze them at an early culture age.

For molecular genetics protocols

Laboratories performing molecular genetics usually want to isolate their own DNA. The ideal situation is to have cytogenetic and molecular laboratories working closely together. If the specimen is large enough to give the molecular laboratory tissue or uncultured cells, only a backup culture will need to be maintained. The molecular testing method will dictate the amount of cultured cells needed. This is generally expressed in a minimum number of flasks (T-25, T-75) at a specific confluence level. It is important to know this information when establishing primary cultures. As above, a backup culture should always be maintained in the originating laboratory in case the first cultures are lost or damaged in shipment or if an insufficient amount of DNA or RNA was extracted. Tissue biopsies can be stored in liquid nitrogen or cell pellets can be placed in lysate buffer and stored at -20 °C for future DNA extraction. Storage of biopsied tissue for DNA is valuable especially in cases where the tissue may fail to grow in culture. Transporting DNA, which is more stable than tissues, is not a biohazard. Storage of DNA when the type of testing is uncertain allows for recovery at a later date when the tissues or individuals are unavailable.

4.3.6 Tracking culture age

Most clinical laboratories only track the number of days in culture, the number of subcultures, and the split ratio, since most cultures are destroyed shortly after reporting. However, for some types of testing and for some research applications it may be important to track the age of a culture in more detail. It is convention that the age of a culture should be described by the number of population doublings, rather than by the time in culture or by the number of subcultures (passages) unless the split ratio is specified. The routine subculture record does not take into account number of doublings required during the time between first growth in primary culture and first subculture. This figure is determined by the number of viable cells from the explants and cells in the container used for first subculture. A general estimate is in the range of 20 population doublings. Because of the limited in vitro lifetime of human cell cultures (20–100 population doublings in standard culture conditions), it is important to estimate culture age in order to predict how long the culture can be used. If cells are frozen in liquid nitrogen as early as possible in their culture lifetime, older cells in culture can be discarded and younger ones retrieved from storage.

4.3.7 Freezing and storage of cell lines

The basic principle of successful cryopreservation is a slow freeze and quick thaw. Cultured cells can be cryopreserved for long periods of time using at least 20% serum and dimethylsulfoxide (DMSO) or glycerol to protect the cell from damage caused by the formation of ice crystals.

Human monolayer cultures in log phase of growth can be suspended as single cells, frozen at a controlled rate (1–3 °C/minute), and stored for prolonged periods of time at a minimum of –130 °C in liquid nitrogen (about –190 °C in the liquid phase) or in a specialized mechanical freezer that maintains a temperature of –130 °C. Cells can be frozen at –80 °C for a shorter period of time. The freezer maintains a constant temperature below –130 °C, but liquid nitrogen tanks create a gradient from the top to the liquid level as the nitrogen evaporates. All storage vessels should include alarms that at least warn of low liquid nitrogen levels or temperatures rising above –80 °C. A liquid nitrogen storage vessel should not be allowed to become less than half full before it is replenished.

Heavy insulated gloves should be worn at all times when racks or canisters are handled. The room should be well ventilated when the refrigerator is opened or filled (a buildup of nitrogen can replace essential oxygen in the air), and extreme care should be taken to avoid spilling liquid N₂ on the skin because it burns.

For the most controlled freeze, a biological freezing apparatus, including control unit, freezing chamber, chart recorder, liquid nitrogen transport/storage cylinder, and transfer hose, is needed. A convenient method to approximate a 1 °C/minute rate of cooling is to use a Nalgene freezing container. Isopropanol at room temperature is added to the double-walled container, samples are placed in the inner compartment, and the unit is placed in a –70 °C freezer for at least 4 hours. Frozen samples may then be transferred to liquid nitrogen. Cell retrieval after storage is better if the rate of freezing is well controlled. When these devices are unavailable, cells can be placed in a Styrofoam container and put at –20 °C for a minimum of 2 hours, then transferred to a –70 °C freezer as above. Recommendations for the preparation of cells for freezing are given in the protocols at the end of the chapter (see Protocols 4.25, Cryopreservation; 4.26, Cryopreservation with Nalgene cryogenic container; 4.28, Freezing tissue cultures (cryopreservation)).

Programmed freezing

Carefully read the detailed instructions that accompany the freezing equipment. A chart recorder is used to record the rate of cooling. The freeze program allows a controlled drop of 1 °C/minute to –40 °C and then a rapid reduction to –80 °C. At this point the ampules are transferred rapidly to racks, which are inserted into holding canisters in the liquid nitrogen refrigerator or ultra low mechanical refrigerator system. The type of storage varies with the style of storage refrigerator.

Storing and thawing frozen cells

The most common storage containers are 2–4-mL freezer vials that are easy to use and store in boxes or on metal canes for suspension in liquid nitrogen. However, recently a self-contained apparatus has become available which adds both cells and frozen media to a specially made flask, which mix when cells are thawed. The most efficient liquid nitrogen refrigerators (or Dewars) hold a large number of stackable aluminum or cardboard storage boxes, each containing a large number of ampules. Mechanical refrigerated systems maintain a constant temperature, decrease cross contamination, and avoid the possibility that cells in the top of the nitrogen container rise to temperatures above –130 °C. The level of liquid nitrogen should be checked and more added at 1- to 2-week intervals, depending on the type of storage refrigerator and environmental conditions. An alarm system should be installed to alert personnel of low liquid nitrogen levels.

It is important to know exactly which ampule is needed and where it is. Remove the ampule as quickly as possible, and place it in a water bath at 37°C. Thawing occurs rapidly (no longer than 3 minutes) without any damage to the cells. Specific instructions on thawing cells are listed in the Protocols section. Culturing a test vial ensures that the frozen cells are viable. Growth characteristics should be observed for 10 days and recorded in the freezer record.

Storage records

Record keeping is easily maintained by numbering the location of each vial within a specific box location. An accurate and up-to-date record of what cells are stored and where they are stored is essential. Ideally these records should be on an electronic database or spreadsheet with a back up storage plan. Records need to be updated whenever a vial is added or withdrawn.

4.3.8 Transport and shipping cultured cells

There are two main ways to transport monolayer cultures over long distances. First, as a cell monolayer, the culture must be protected from drying by filling the container entirely with culture medium. The closed cap should be sealed tightly with parafilm, and the vessel should be placed in an insulated container to protect the cells from temperature extremes. Second, in a frozen ampule, the suspended cells must be prevented from thawing with dry-ice packaging until the ampule reaches its destination. All shipments should be packaged with adequate padding and sent by the fastest carrier, as determined by the individual laboratory location.

4.4 Lymphoblastoid cell lines

Lymphoblastoid cell lines are created by immortalizing B lymphocytes with Epstein–Barr (EB) virus. B lymphocytes are isolated from whole blood and infected with a specific titer of viruses recovered from cell lines such as B95-8 or Akata. These cell lines spontaneously release high titers of the virus into the medium that can be used for infection. Lymphocytes are cultured in the presence of the virus for several weeks before they become immortalized. The lymphoblastoid cells begin to grow in suspension as small loose aggregates, which can be easily dissociated by gentle agitation or pipetting. The transformed cells thrive at a cell density of about 200,000–500,000 viable cells per milliliter, and should be split when the density reaches about 10^6 cells/mL. At this density, the medium is very acidic and appears yellow with a phenol indicator. If cells are left at this pH, viability and cell growth will decrease. These cells are very sensitive to cell density, and should be monitored carefully. It is important not to split or feed the cells too early but to wait until the medium is yellow. If the cells are growing slowly, the initial density could be increased to 300,000–500,000 cells/mL.

Besides cell density and pH, lymphoblastoid cells are very sensitive to temperature, L-glutamine depletion, surface-to-air ratio, and length in culture. The medium should contain 5–15% fetal bovine serum and freshly added L-glutamine and should be pre-warmed to 37°C just prior to feeding. In order to maintain the appropriate surface-to-air ratio, lymphoblastoid cells are usually grown in an upright T-25 flask, with no more than 20 mL of medium. Although these lymphocytes have been immortalized, they should not be maintained continually in culture. Rather, they should be frozen in aliquots and thawed as needed.

Glossary

Amphotericin: either of two polyenic antifungal substances obtained from a soil bacterium of the genus *Streptomyces* (*S. nodosus*), especially amphotericin B.

amphotericin. (n.d.). *Merriam-Webster's Medical Dictionary*. Retrieved October 07, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/amphotericin>

Ascites: the accumulation of serous fluid in the peritoneal (abdominal) cavity.

ascites. (n.d.). Dictionary.com Unabridged. Retrieved July 10, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/ascites>

Cystic hygroma: a mass that commonly occurs in the head and neck area. A cystic hygroma occurs as the baby grows in the womb. It forms from pieces of material that carry fluid and white blood cells. Such material is called embryonic lymphatic tissue. After birth, a cystic hygroma usually looks like a soft bulge under the skin. The cyst may not be recognized at birth. It typically grows as the child does, and sometimes is not noticed until the child is older.

Retrieved 7/30/2011 from <http://www.nlm.nih.gov/medlineplus/ency/article/000148.htm>

dH₂O: double distilled water

Hydatidiform mole: (1) a mass in the uterus that consists of enlarged edematous degenerated chorionic villi growing in clusters resembling grapes, that typically develops following fertilization of an enucleate egg, and that may or may not contain fetal tissue hydatidiform mole.

(n.d.). *Merriam-Webster's Medical Dictionary*. Retrieved July 31, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/hydatidiform mole>.

(2) in human pregnancy, abnormal growth of the chorion, the outermost vascular membrane that in a normal pregnancy would enclose the embryo and ultimately give rise to the placenta. In the situation in which the hydatidiform mole develops, the embryo is usually either absent or dead. The mole, a collection of sacs (cysts) containing a jellylike substance, resembles clusters of grapes and can attain a great size. Most of the moles are expelled in about the 20th week of pregnancy. The molar pregnancy is usually terminated by suction curettage. In a few cases, the mole spreads into the muscle of the uterus and causes bleeding. If this is severe, the obstetrician may remove the mole by surgery. In extremely rare instances, hydatidiform moles develop into choriocarcinomas, which are highly malignant tumors.

hydatidiform mole. (n.d.). © *Encyclopedia Britannica, Inc.* Retrieved July 31, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/hydatidiform mole>

Pleural effusions: the body produces pleural fluid in small amounts to lubricate the surfaces of the pleura, the thin tissue that lines the chest cavity and surrounds the lungs. A pleural effusion is an abnormal, excessive collection of this fluid.

Retrieved July 26, 2011 from <http://www.nlm.nih.gov/medlineplus/ency/article/000086.htm>

Reference

1. Hyclone (2) Art to science. *Lab* 1996;15(1):1–5.

Additional readings

1. Barch M, Knutsen T, Spurbeck J, eds. *The AGT Cytogenetics Laboratory Manual*, 3rd ed. Philadelphia: Lippincott-Raven Press, 1997, Chapter 4 and Appendix.
2. Bhardwaj U, Zhang YH, Rangwala Z, McCabe ER. Completely self-contained cell culture system: from storage to use. *Mole Genet Metab*, 2006;89(1-2):168–173.
3. Dunn B, Burns K. *Cytogenetic Symposium*, 2nd ed., 2005; Chapter 5: Principles of long term culture and harvest.
4. Freshney RI. *Culture of Animal Cells: A Manual of Basic Technique*, 4th ed. 2000; New York: Wiley-Liss.
5. Held K, Sonnichsen S. The effects of oxygen tension on colony formation and cell proliferation of amniotic fluid cells in vitro. *Prenat Diagn* 1984; 4(3):171–179.
6. Jakoby WB, Pastan IH, eds. Cell culture. *Methods in Enzymology*, vol. 58. San Diego: Academic Press, 1979, Section I, Basic Methods.
7. Lam K, Crawford D. Establishment of lymphoblastoid cell lines. *Methods in Cell Science* 1995; 17(2): 67–74.
8. Menasha J, Levy B, Hirchhorn K, Kardon NB. Incidence and spectrum of chromosome abnormalities in spontaneous abortions. *GIM* 2005; 7(4): 251–263.
9. Rooney DE, Czepulkowski BH, eds. *Human Cytogenetics: A Practical Approach*, vol. 1, *Constitutional analysis*, 2nd ed. New York: IRL Press 1992, Chapter 3.
10. Priest JH. *Medical Cytogenetics and Cell Culture*, 2nd ed. Philadelphia: Lea and Febiger 1977; Chapters 5, 12, and 14.
11. Sigma Aldrich at www.sigmaldrich.com. 2006.
12. Smith C. Trouble in the hood: culturing difficult cell types. *Nature Methods* 2005; 2: 385–391.
13. *Standards and Guidelines for Clinical Genetics Laboratories*. (2006). The American College of Medical Genetics, Laboratory Practice Committee, 9650 Rockville Pike, Bethesda, MD 20814.
14. Verma RS, Babu A. *Human Chromosome: A Manual for Basic Techniques*, New York: McGraw-Hill Inc, 1989: Chapter 2.

Contributed protocols section

IMPORTANT: No protocol included in this manual should be used clinically unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements of all chemicals used in the laboratory.

NOTE: For coverslip harvests, see Protocol section in Chapter 5.

Protocol 4.1 Solid tissue collection for establishing cultures

Miscellaneous contributors

A. Skin biopsy

Contributed by Tara Ellingham and Daynna Wolff, Medical University of South Carolina, Charleston, SC

When obtaining a skin biopsy from a living individual, care must be taken to thoroughly cleanse the target area with 70% ethanol (and air dry). Sterile technique should be practiced. A punch biopsy, a procedure used for standard investigation of skin disorders, is a good method for ensuring that the plug of obtained tissue reaches through the epidermis to the dermis. Cells will grow from the dermal layer.

On a baby or small child, it is preferable to obtain the biopsy from the upper lateral area of the buttocks. On an adult, the under surface of the forearm about 5–6 inches below the elbow joint and lateral to the large veins of the arm is often used. Collect 0.2 cm³ tissue in sterile transport medium containing antibiotics provided by the laboratory. If transport medium is not available, the tissue may be collected in sterile saline or Ringer's solution.

B. Products of conception

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

Specimens from products of conception are often difficult to obtain by sterile techniques. Every attempt should be made to minimize contamination.

Provide a minimum of 1 cm³ of abortus tissue. If fetal tissue cannot be identified, chorionic villi or placental membranes are preferred. Chorionic villi will remain viable longer than fetal tissue in the event of an intrauterine fetal demise. If both fetal tissue and villi are present, both should be set up in separate cultures. Do not send the entire fetus if it is more than 8 weeks gestation. Most laboratories are not set up for proper disposal of large or whole fetal samples.

Use aseptic technique to collect tissue. Rinse with sterile saline and place specimen in a screw-capped, sterile tube containing Hanks' Balanced Salt Solution with added antibiotic, antimycotic, or other similar transport media.

For lung, tendon, or fetal tissue from products of conception, it may be necessary to dissect an appropriate size sample from the original submitted tissue sample which may be very large in late gestation fetuses. In most cases, 0.5 cm³ is sufficient; however, it is best to be sure that enough tissue is present to ensure culture success, especially if viability is low.

C. Autopsy material

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

Specimens collected at autopsy should be taken from areas protected from external contamination. Inner tissues, such as lung, diaphragm or thymus, are good choices. The tissue must be taken before it is fixed in formaldehyde or frozen. If material has been fixed in formaldehyde, it can be dissociated and used for FISH analysis. Whenever possible, the tissue should be placed in sterile transport media containing antibiotics or in sterile saline solution.

D. Solid tissue collection and transport

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

Ideally, tissue specimens of at least 0.5–1 cm³ are collected with sterile methods into closable containers with sterile culture medium supplemented with serum and antibiotics (transport medium). If transport medium is unavailable, sterile Ringer's solution (lactated or non-lactated) or sterile isotonic saline are acceptable. Specimens are delivered to the laboratory as soon as possible (up to 2 days after collection), well protected from temperature extremes.

Skin samples should be deep enough to include the dermis layer. Abortus specimens most likely to be successful include skin, tendon, fascia, heart, lung and gonad. In early products of conception, chorionic villi and membrane may be used in

addition to fetal parts, if present, and tend to survive better than fetal parts after fetal death. However, keep in mind that the karyotype from extraembryonic tissue may not represent the fetus proper. The use of multiple tissue sites for culturing will increase the likelihood of culture success. To address potential confined placental mosaicism, particularly in the case of intrauterine growth retardation (IUGR), multiple (2–4) placental sites should be sampled.

Protocol 4.2 Solid tissue transport and sendout media

Miscellaneous contributors

A. Transport medium A (without Fungizone)

Contributed by Kristin May, Children's Hospital at Erlanger, Chattanooga, TN

Media must have been previously tested for sterility and ability to support growth (see Note 1, Media testing for sterility and growth support).

I. Materials

1. RPMI 1640, 100 mL, Gibco®/Invitrogen. Storage at 2–8 °C.
Expiration: manufacturer's date.
2. L-Glutamine/Pen/Strep, Sigma, 200 mM L-glutamine/10,000 U penicillin/10 mg streptomycin in 0.9% NaCl. Storage: –5 to 20 °C.
To prepare aliquots:
 - a. Thaw once.
 - b. Divide into 1.2 mL aliquots.
 - c. Label tubes LGPS (for *L*-Glutamine/*Pen*/*Strep*), and preparation/expiration dates. Document all required reagent labeling in media logs.
 - d. Freeze.
 - e. Expiration: same as manufacturer's original date.

II. Method

1. Combine all components in the original medium bottle under sterile conditions in biological cabinet.
2. Label "Transport Medium A" and add CAP required reagent information (see Figure 4.4).
3. Expiration: 2 weeks stored in refrigerator (2–8 °C).

III. Notes

1. **Media testing for sterility and growth support.** In general, media lots should be tested for sterility and growth support before being approved for in vitro culture usage. The following tests should be performed before any antibiotic or antimycotic reagent is added.
 - a. Media that has been prepared in the laboratory must first be filtered (see chapter section 4.2.2 on Filter sterilization);
 - b. A small sample of medium either from new lot(s) or filtered from (a) is added to a test culture vessel and incubated at 37 °C;
 - If serum is present in the medium, set up a second culture vessel for growth support evaluation (see protocol 4.16, Plating efficiency of serum);
 - If medium that contains serum has been prepared in the laboratory, a third culture vessel is set up in order to test for mycoplasma presence, using either a mycoplasma testing kit or see Protocol 4.15, Mycoplasma testing;
 - c. After 48 hours, the medium is carefully examined for turbidity. If it did not contain serum and is clear of any turbidity, proceed to using the medium. If it contains a new lot of serum, plating efficiency (see Protocol 4.16, Plating efficiency of serum) must also be completed before the batch can be used for in vitro culturing. Mycoplasma testing is additionally required if the medium, containing serum, was prepared in the laboratory.
 - d. Antibiotics and/or antimycotic solutions are added only after the above testing is complete and demonstrates that the medium is free of contaminants and that it satisfactorily supports in vitro cell growth (see 4.2.8, Antimicrobial agents and monitoring for contamination).

B. Transport medium B (with Fungizone)

Contributed by Kristin May, Children's Hospital at Erlanger, Chattanooga, TN

Note: Media batch must have been previously tested for sterility and ability to support growth. (See Protocol 4.2A, Note 1, Media testing for sterility and growth support).

I. Principle

- Used only if products of conception, autopsy material or tissue from IUFD (intrauterine fetal demise) is not received in transport medium with Fungizone AND if the specimen will not be set up immediately.

II. Materials

- RPMI 1640, 100 mL, Gibco®/Invitrogen. Storage at 2–8 °C. Expiration: manufacturer’s date.
- L-Glutamine/Pen/Strep, Sigma, 200 mM L-glutamine/10,000 U penicillin/10 mg streptomycin in 0.9% NaCl. Storage: –5 to 20 °C.
To prepare aliquots:
 - Thaw once.
 - Divide into 1.2 mL aliquots.
 - Label tubes LGPS and prep/exp date (see Figure 4.4).
 - Freeze.
 - Expiration: same as manufacturer’s original date on package
- Fungizone (amphotericin B), Gibco®, 250 µg/mL

Aliquot procedure

- Divide into 0.5 mL aliquots and store frozen (–5 to –20 °C)
- Aliquots thawed at room temperature.
- Expiration date same as manufacturer’s date on bottle.

III. Method

- Combine all components in the original medium bottle under sterile conditions in biological cabinet.
- Add 0.5 mL Fungizone (amphotericin B).
- Label “Transport Medium B + Fungizone” and add CAP required reagent information (see Figure 4.4).
- Expiration: 2 weeks stored in refrigerator (2 to 8 °C).

C. CVS/skin transport media

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Materials

- CO₂ Independent Media (by adding 12.5 mL HEPES)
- L-Glutamine/Pen-Strep (29.2 mg L-glu, 5 mg/mL Pen, 5 mg/mL Strep)
- Amphotericin (250 µg/mL)
- Gentamicin (10 mg/mL)

II. Method

- For smaller quantities, combine 100 mL CO₂ Independent Media with 1 mL L-glu/pen-strep solution, 0.5 mL amphotericin and 0.25 mL gentamicin. For larger quantities, combine 450 mL CO₂ Independent Media with 5 mL L-glut/pen-strep solution, 2.5 mL amphotericin and 1.25 mL gentamicin.
- Label all tubes with date prepared.
- Aliquot 25 mL into 50 mL polypropylene centrifuge tubes.
- Store in refrigerator at 4° to 8 °C or freeze at -5 to -20 °C.
- If frozen, media expires one year from date prepared. If refrigerated, media expires 3 months from date prepared.

D. CVS transport medium

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Materials

1. Ham's F10, Gibco
2. Gentamicin sulfate (50 mg/mL) BioWhittaker
3. Sodium heparin, **preservative free**, 1000 USP units/mL

II. Method

1. To 100 mL of Ham's F10 medium in a T-75 tissue culture flask, add 1 mL gentamicin and 1 mL sodium heparin.
2. Aliquot 10 mL/tube into sterile screw cap centrifuge tubes.
3. Store at 4°C. Expiration: 3 months.

E. POC transport media

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Materials

1. CO₂ Independent Media (by adding 12.5 mL HEPES)
2. L-Glutamine/Pen-Strep (29.2 mg L-glu, 5 mg/mL Pen, 5 mg/mL Strep)
3. Gentamicin (10 mg/mL)

II. Method

1. Prepare media.
Formula I
For smaller quantities, combine 100 mL CO₂ Independent Media with 1 mL L-glu/pen-strep solution, 0.5 mL gentamicin.
For larger quantities, combine 450 mL CO₂ Independent Media with 5 mL L-glu/pen-strep solution, 2.5 mL gentamicin.
Formula II
For smaller quantities, combine 100 mL MEM-Alpha with 15 mL Fetal Bovine Serum and 1 mL L-glu/pen-strep solution.
For larger quantities, combine 500 mL MEM-Alpha with 75 mL Fetal Bovine Serum and 5 mL L-glu/pen-strep solution.
2. Label all tubes with date prepared and specimen type.
3. Aliquot 25 mL into 50 mL polypropylene centrifuge tubes.
4. Store in refrigerator at 4 to 8°C or freeze at -5 to -20°C.
5. If frozen, media expires one year from date prepared. If refrigerated, media expires 3 months from date prepared.

F. Solid tissue transport medium

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Materials

1. Ham's F10 or MEM, Gibco®
2. Fetal bovine serum, Irvine
3. L-Glutamine (584 mg/mL), Gibco®
4. Gentamicin sulfate (50 mg/mL), Bio Whittaker

II. Method

1. To a 75-cm² screw-cap tissue culture flask, add 20 mL fetal bovine serum, 2 mL L-glutamine, 0.2 mL gentamicin, and 180 mL F10 or MEM.

2. Aliquot 5 mL/tube into sterile screw cap test tubes or centrifuge tubes.
3. Store at 4°C.
4. Expiration in 3 months.

G. Send-out medium for biochemical testing

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Materials

1. Ham's F10 or MEM (Gibco®)
2. Fetal bovine serum, Irvine
3. Gentamicin sulfate (50 mg/mL), BioWhittaker

II. Method

1. To 100 mL of F10 or MEM, add 10 mL fetal bovine serum and 0.1 mL gentamicin. Use to fill flasks for shipment.
2. Store at 4°C.
3. Expiration: 3 months.

Protocol 4.3 Tissue culture reagents

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

Label all solutions with contents, date, expiration date, and technologist's initials.

A. Collagenase

I. Materials

1. Hanks' 10× BSS solution (HBSS), Irvine
2. Sigma Type IV-S, 536 units/mg *or* Worthington Type I collagenase, 210 units/mg.

II. Method

- A. Sigma
 1. Reconstituted with 10 mL sterile Hanks' saline or incomplete medium.
 2. Use 0.20–0.25 mL in 5 mL incomplete medium.
 3. Expiration 1 month.
- B. Worthington
 1. Dilute with incomplete medium to a final concentration of about 1 unit per mL, rounding up to the closest multiple of 10 (e.g., for 142 units/mg, use 150 mL medium).
 2. Filter through a vacuum filtration unit with 0.2 µ pore size.
 3. Aliquot into 4 mL snap cap sterile tubes and freeze.
 4. Shelf life 1 year.

B. Hanks' balanced salt solution (HBSS, BSS, Mg²⁺/Ca²⁺-free)

I. Materials

1. Hanks' 10× BSS solution (HBSS), Irvine
2. Sodium bicarbonate solution, 7.5%, Gibco®
3. Sterile double distilled water

II. Method

1. To a large 750 mL screw-top tissue culture flask, pipet 50 mL of Hanks' 10× BSS (balanced salt solution). Fill to 500 mL with sterile double distilled water.

2. Adjust pH with 0.4 to 0.7 mL of sodium bicarbonate (7.5% solution) until solution is no longer amber, and not pink, but a medium red color (acid pH looks yellow, and basic pH looks pink).
3. Store at 4°C.
4. Expiration in 1 month.

C. Hanks'-trypsin solution

I. Materials

1. Trypsin-EDTA, Gibco®
2. Hanks' 10× BSS solution (HBSS), Irvine

II. Method

1. Add 2 mL of 10× trypsin-EDTA solution (kept in freezer in 2 mL aliquots) to a 25-mL flask.
2. Add Hanks' 1× to 30 mL.
3. Expiration in 7 days.

D. Harvest medium

I. Materials

1. Fetal bovine serum, Irvine
2. Ham's F10 or MEM medium, Gibco®

II. Method

1. Add 50 mL fetal bovine serum (in freezer in 50 mL aliquots) to a 750 mL screw-cap tissue culture flask. Fill to 500 mL with medium.
2. Store at 4°C.
3. Expiration in 1 month.

E. Stock hypotonic solution

I. Materials

1. Fetal bovine serum, Irvine
2. Sterile double distilled water

II. Method

1. Make a 4 : 1 dilution of bovine serum:
 - a. To 20 mL of serum, add 80 mL of double distilled water in a 250-mL screw cap tissue culture flask.
 - b. For use, dilute as described under F. Working hypotonic, prenatal specimens.
2. Store at 4°C.
3. Expiration in 2 weeks.

F. Working hypotonic, prenatal specimens

I. Materials

1. Stock hypotonic, as above (4:1 dilution of fetal bovine serum)
2. 0.075 M KCl solution (0.56% w/v solution of KCl in double distilled water)

II. Method

1. Dilute the stock hypotonic solution 3:1 with 0.075 M KCl (e.g., 30 mL of KCl to 10 mL stock hypotonic solution).
2. Expiration in one (1) day.

G. Fixative

I. Materials

1. Methanol, Methyl Alcohol (Mallinkrodt Chemical)
2. Glacial Acetic Acid (Fisher Scientific)

II. Method

1. Prepare 3 parts methanol to 1 part glacial acetic acid.
2. Expiration in one (1) day.

H. Test broth medium

I. Materials

1. Tryptose phosphate broth, 100 mL bottles, Sigma
2. 2,3,5-Triphenyl tetrazolium chloride indicator, Sigma
3. Stock indicator: 1% solution of triphenyl tetrazolium chloride (100 mg/10 mL dH₂O).
4. Filter sterilize with 0.2-μm filter. Store in refrigerator.

II. Method

1. Add 2 mL of stock indicator to 100 mL of tryptose phosphate broth.
2. Aliquot 5 mL into sterile screw cap test tubes or centrifuge tubes.
3. Store at 4°C.
4. Expiration 6 months.

I. Chang medium (Irvine)

I. Materials

1. Chang B liquid medium (100 mL), Irvine Scientific
2. Chang C lyophilized supplement, Irvine Scientific
3. L-Glutamine (584 mg/mL), Gibco®
4. Gentamicin sulfate (50 mg/mL), BioWhittaker

II. Method

1. To 100 mL Chang medium, add supplement, 2 mL of L-glutamine, and 0.1 mL gentamicin.
2. Store at 4°C.
3. Expiration in 10 days.

J. Ham's F10 or MEM complete medium

I. Materials

1. Ham's F10 or MEM, Gibco®
2. Fetal bovine serum, Irvine
3. L-Glutamine (584 mg/mL) Gibco®
4. Gentamicin sulfate (50 mg/mL) BioWhittaker

II. Method

1. To a T-75 tissue culture flask, add 2.5 mL L-glutamine, 0.25 mL gentamicin, 50 mL fetal bovine serum, and q.s. to 200 mL with Ham's F10 or MEM.
2. Store at 4°C.
3. Expiration 2 weeks.

Protocol 4.4 Phosphate buffer solution deficient in Ca²⁺ and Mg²⁺

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Principle

PBS is a balanced salt solution which is a combination of salts used to control pH and osmotic pressure. It can be added to media or used as a solution to add reagents such as Colcemid® or trypsin. Although cells can be transported or treated for a short time in PBS, other nutrients such as amino acids and growth factors are needed to culture cells.

II. Method

1. Add 16g NaCl, 0.4g KCl, 4.23g Na₂HPO₄·7H₂O to 2200 mL of water (distilled). With most autoclaves, even on slow exhaust, the final volume is close to 2000 mL after autoclaving.
2. The pH should be 7.4±0.04. Check with a pH meter. Adjust with NaOH or HCl.
3. (a) Dispense solution into hard glass bottles about two thirds full (or other containers appropriate for cell culture and autoclaving); or (b) filter-sterilize into sterile containers (skip autoclaving and cooling steps) and test for pH using sterile technique to remove aliquot.
4. Autoclave for 30 minutes, with slow exhaust, and caps loose and covered with aluminum foil.
5. Allow to cool and label.
6. Check pH again after sterilization (use sterile technique to remove an aliquot).

Protocol 4.5 Solid tissue and fibroblast culture setup

Contributed by Lauren Jenkins, Kaiser Permanente San Jose, CA

I. Principle

Tissue from products of conception (POC) as well as solid tissue from an autopsy or living individual can be cultured as a monolayer for genetic testing. The tissue is minced and enzyme digested into a near single cell suspension and then transferred to a culture dish and allowed to grow.

II. Materials

General stock

1. Sterile T-25 flasks, 25 cm²
2. Sterile Petri dishes: 60×15 mm, 100×15 mm, 35×10 mm with coverslip (MatTek Corp)
3. Sterile plastic centrifuge tubes, 15 mL
4. Sterile plastic serological transfer pipettes: 1 mL, 5 mL, 10 mL
5. Sterile disposable scalpel #21
6. Sterile forceps
7. Sterile tuberculin syringes, 1 mL
8. Sterile syringe, 12 mL
9. Powder-free nitrile gloves

Reagents

1. Pronase
2. Complete Alpha MEM – expires 14 days after date made

100mL or 450mL	MEM-Alpha
15mL or 75mL	Fetal Bovine Serum
1mL or 5mL	L-Glu/Pen-Strep mix

III. Methods

Explant flask method (fetal site, if available)

1. One flask will be the explant, and one will be the pronase flask. More flasks might be needed depending upon the size of the sample and number of sites available. Label all flasks.
2. Using a 1 mL tuberculin syringe, lift a small piece from the minced sample and place into the labeled, explant flask. Firmly press the piece against the flask floor with the needle. Avoid using large pieces.
3. Repeat this process until 10–15 explants have been placed. If sample is small, place 5–10 explants in the center area.
4. Stand the flasks on end. Carefully add 3 mL complete medium into the explant flask and 2 mL medium into the pronase flask(s), using the appropriate media.
5. Cap the flask and stand the explant flask on labeled end on the 37°C warming hot plate. This allows the explants to attach and the medium to stay warm.

Enzymatic disaggregation

Note: For each sample site, the following disaggregating steps can be performed in tandem, but care must be taken to maintain the integrity of each separate sample.

1. Pipet 0.5 mL working pronase onto remaining minced sample, gently stirring sample with the pipette to mix.
2. Keep the pronase/sample area centered in the dish, not allowing pronase to spill out over the entire dish floor. Cover and move dish to 37°C warming plate for 10 minutes for first check and thereafter every 5 minutes (but no more than 30 minutes) until single cells are evident. The firmer the tissue, the more pronase is required. An average time is between 10–20 minutes. When there are single cells and/or the tissue has an applesauce appearance, the sample can be rinsed.
3. Remove dish from slide warmer and rinse disaggregated sample with 3 mL medium. Pipet sample up and down into the dish bottom to further separate cells.
4. Transfer rinse and specimen into the appropriate conical tube.
5. Draw 6 mL medium into a pipette. Expel 3 mL into tube with disaggregated sample. Use the remaining 3 mL in pipette to rinse dish by allowing the medium to flow over the dish from top to bottom once. Remove rinse and place into the same conical tube as the first rinse.
6. Cap tube and place in centrifuge.
7. At this point, check the explant flask. If explants look somewhat dry (not moist), do the following in order to rinse the explants briefly. Turn the flask slowly, allowing medium to gently flow over explants. Fluid will end up in the top of the flask, and the explants that are on the bottom of the flask will now be facing up. This allows the explants to maintain a small amount of medium, yet not be flooded with medium, which might lift explants.
8. Centrifuge all tubes for 10 minutes at 1400 RPM.

Seeding vessels

9. Once spun, remove tube and discard fluid above pellet.
10. Working with one site at a time, resuspend pellet by flicking bottom of tube. Add 0.5 mL medium per flask to be seeded. Pipet suspension into site's vessel(s).
11. Check flasks under inverted scope for cell density.
12. If cell density is very low, consider combining same site vessels. Too low a density of cells can significantly slow growth. Overabundance of cells requires checking for pH change due to the medium support required.
13. Check all vessels made with original tubes before placing in incubators.
14. Put flasks in the closed incubators, checking to be sure the flask caps are closed tightly.
15. Slowly invert the explant flask to right-side-up, secure the cap, and place in incubator.

Protocol 4.6 Solid tissue setup and processing

Contributed by Urvashi Surti, Magee Women's Hospital, Pittsburgh, PA

I. Principle

Solid tissue specimens are set up in culture in order to determine if a chromosome abnormality is present. The types of tissues normally received include: tumors, chorionic villi from spontaneous abortuses, placental tissue, amnion, chorion, decidua, cord tissue, skin, and organ tissues (i.e., lung, kidney, spleen) from autopsy. Specimens may originate in surgical pathology,

autopsy, or labor & delivery. Medical staff working in both the operating and emergency rooms send tissues directly to surgical pathology. Tissue samples are also received from outside institutions.

The tissue (if villi) is first examined under a stereo-dissecting microscope in order to separate any maternal tissues from fetal tissues. The specimen is then dissociated enzymatically and the cells plated out in culture vessels in media to promote cell attachment and growth. All samples are set up in coverslip/Petri dish culture vessels and placed in media in two separate incubators so that backup cultures are available in the event of an incubator failure. Two separate lot numbers of media are also used to prevent total loss of a sample in the event of media contamination or other media-related culture failure. When sufficient growth of cultures has been attained, the cells are arrested in the metaphase stage of cell division and “harvested” to obtain chromosomes for analysis.

NOTE: UNIVERSAL PRECAUTIONS GUIDELINES MUST BE FOLLOWED WHEN HANDLING PATIENT SAMPLES!

Gloves and lab coats should be worn at all times when handling tissue specimens. Sterile conditions should be maintained at all times when handling tissue samples. All supplies needed for specimen set-up should be assembled prior to beginning the set-up procedures.

II. Materials

General stock

1. Two (2) conical 15 mL centrifuge tubes with screw cap for each specimen type (multiple tissues on a patient should each be set up in separate tubes)
2. Several sterile 5 mL and 10 mL serological pipettes
3. Medium size sterile glass Petri dish (one per tissue type)
4. Sterile Petri dish (1–2 per tissue type)
5. Prepackaged, sterile coverslips in Petri dishes (the number of coverslip cultures is dependent on the size of the sample - see set-up procedure)
6. Sterile forceps (two per patient) and sterile scissors (sterile disposable safety scalpel if necessary)
7. Sterile glass Pasteur pipettes and bulb
8. Whatman (or similar) markers for labeling cultures
9. Plastic beaker containing 0.525% sodium hypochlorite (bleach) solution for fluid waste
10. Centrifuge tube rack
11. Two lots of culture media for tissues from the refrigerator (see Reagent Preparation section of this manual - aliquot the amount of media that you think you will need for set-up and prewarm it in a 37°C incubator during specimen preparation)
12. Two Scienceware stak-a-tray racks (Fisher Scientific) with trays, and colored tape

Reagents

1. Trypsin Solution Preparation (0.1%)
 - a. Weigh out 0.5 g of trypsin – Worthington Biochemicals (Trypsin 2× TRL – 1 gram bottles).
 - b. Dissolve the trypsin in 500 mL of HBSS.
 - c. Filter the solution with a 0.22 µm filter under sterile conditions.
 - d. Prepare labeled 5 mL aliquots and freeze.
 - e. NOTE: This solution expires in one (1) month.
2. Collagenase Solution Preparation (0.1%)
 - a. Weigh out 0.5 g of collagenase - Worthington Biochemicals (Collagenase Type 1 – CLS-1, 125 U/mg, 1 gm). This preparation should be in the range of 100 - 300 U/mg.
 - b. Dissolve completely in 500 mL HBSS.
 - c. Filter using a 0.22 µm filter under sterile conditions.
 - d. Prepare 5 mL aliquots and freeze.
 - e. NOTE: This solution expires in one (1) month.

III. Method

Setup procedures

*NOTE: If multiple tissues are received on one patient, ALWAYS set up villi tissue and at least one fetal tissue. If the sample received is to confirm an abnormality diagnosed prenatally, ONLY dissociate the fetal tissue. If the fetal tissue does not attach,

the villi may be set up as well. If the prenatal diagnosis involves mosaicism or IUGR (Intrauterine Growth Restriction), the villi and fetal tissue should both be set up in culture. A mosaicism diagnosis may also require setting up multiple placental sites and/or multiple fetal organs.

1. All tissue specimens are to be set up under a laminar flow hood once they have been dissociated in order to maintain conditions of sterility.
2. Label the sterile conical centrifuge tubes with the accession number assigned to that specimen, the patient name, and the type of tissue (i.e., lung, villi, etc.).
3. Remove the tissue(s) from the tubes that they were initially placed in when obtained and place specimen(s) in a sterile Petri dish. Add Hanks' balanced salt solution (HBSS) with 3x antibiotics.
4. Under the stereo-dissecting microscope in the tissue culture room, carefully clean villi specimens using sterile forceps to make sure that they are free of maternal decidua and blood clots. Discard any remaining maternal tissue or blood clots in a red biohazard trash container (**Note: in cases of suspected molar pregnancy, you must separate and clean both villi AND decidua tissues**). The cleaned villi are then transferred to a labeled conical tube with sterile forceps. If the cleaned tissue will not be dissociated immediately, place the specimen in a labeled tube with sterile Hanks' (HBSS) without antibiotics. Refrigerate the specimen in fresh media if kept overnight. If skin is received, it should also be examined carefully to be sure that a full thickness is present. The presence of the innermost layer of the skin is necessary to provide viable cells for culture.
 ** NOTE: The previous cleaning step is especially important when setting up villi or placental samples in order to prevent maternal cell contamination. Newer technologists should ask for assistance from a more experienced tech until villi recognition training has been completed and sufficient experience is gained in distinguishing maternal vs. fetal tissue types. All technologists should ask for a second opinion from another experienced technologist when dealing with an unusual or suspicious sample or if a prolonged time has elapsed since the completion of a rotation in this area.
5. All tissue specimens (except chorionic villi) should first be minced finely with sterile scissors. Only if necessary, a sterile disposable scalpel can be used to mince the tissue. Transfer the minced tissue by pipette into a labeled, sterile 15 mL centrifuge tube (one tube per type of tissue). The tube containing the tissue may be centrifuged at 1000 RPM for 5–10 minutes to remove excess Hanks'.
6. Add the 5 mL aliquot of trypsin to the conical tube containing the specimen and mix by inverting the tube multiple times. Less than 5 mL of trypsin may be used for dissociating very small specimens. Since trypsin can degrade with time and temperature changes, thaw the vial of trypsin and use it within a half hour.
7. Incubate the tube at 37°C for a MAXIMUM of 15 minutes, checking earlier if the tissue appears to be less than optimal. Macerated specimens should be checked after approximately 5 minutes. Tumor tissues and other very connective type tissues (especially skin) usually require the full time in trypsin.
 * NOTE: DO NOT let the tissue remain in trypsin until COMPLETELY dissociated! The purpose of the trypsin is to soften and prepare the tissue for collagenase dissociation. If a large portion of the tissue appears to be falling apart, it is better to leave a few larger chunks than to over dissociate and potentially kill the cells!
8. While sample(s) is (are) incubating, the tissue set-up record sheet should be prepared. Notes should be made regarding the size and condition of the sample.
9. At the end of the trypsin incubation, remove tube(s) from incubator and with a sterile disposable pipette, add 5 mL sterile HBSS *without* antibiotics to each tube. Invert the tube(s) several times to break up the tissue.
10. Centrifuge the sample for 10 min. at 1000 RPM. Remove and discard supernatant into a waste container. Add 5 mL collagenase and invert the tube several times to mix the pellet. Less than 5 mL of collagenase can be used for smaller samples. Especially tough tumors may be incubated overnight in collagenase. These types of tumors should first be discussed with the Laboratory Director to determine if a change in protocol is warranted.
11. Place tissue in a 37°C incubator for 5–15 minutes. Make sure to check earlier if the tissue is less than optimal. Five minutes may be all that is needed for macerated specimens. Tissues that are more connective in nature may require further incubation (up to 15 minutes). These samples may also be agitated by inverting the tube to help in breaking up larger pieces.
12. At the end of the collagenase incubation, add 5 mL sterile HBSS without antibiotics; mix well with a sterile Pasteur pipette or invert the tube. Split the sample into two pre-labeled 15 mL conical centrifuge tubes. Make sure to transfer all of the tissue, splitting it as evenly as possible into 2 tubes.
13. Spin the tubes for 10 minutes at 1000 RPM. If multiple tissue types are received, label 4–5 coverslips per tissue type per patient (depending upon the size of the cell pellets at the end of the dissociation process). If only one tissue type is received, set up a maximum of 8 coverslips. Fewer coverslips may be set up on small specimens and 4–5 coverslips should

be set up on cases only requiring 5 counts. Label cultures with the patient name, accession number, date, and culture letter (on both the lid and bottom of the dish). Culture letters are assigned alphabetically.

****NOTE:** Newer technologists should consult with more experienced techs when evaluating cell pellet size until adequate experience is gained! When in doubt - ask for help!! After plating the suspension into the first coverslip, check for appropriate cell density under the inverted microscope. If the cell density is too high, set up more dilute coverslips for the remaining cultures. If the cell density appears to be low, consider respinning the tube and removing some of the surplus media above the cell pellet.

****NOTE FOR MOLAR PREGNANCIES:** For tissues of suspected molar pregnancies, set up 8 cultures of villi and 5 cultures of cleaned decidua.

14. Remove the caps from *one* specimen (if multiple samples are being set up) and, using a sterile pipette, carefully remove the supernatant without disturbing the cell pellet. Discard the supernatant into the plastic waste beaker. If the cell pellet is not compact in the tip of the tube, it may be easier to pick up the pellet with a pipette and transfer it to another labeled tube before discarding the supernatant.
15. For each tissue type, add 0.5 mL of "A" tissue/ CVS media for each coverslip to be set up from the first tube, and 0.5 mL of "B" media for each coverslip to be set up from the second centrifuge tube.
16. Resuspend the cell pellet in the medium using a separate pipette for each tube. Place 0.5 mL of the suspension onto each coverslip to be set up, making sure that the liquid covers the entire surface area of the coverslip. Each specimen should be completely plated before another specimen is set up. Make sure that the specimen number and *type of tissue* on the centrifuge tube matches the number and type of tissue on the culture vessels!
17. All coverslips should be split into two separate incubators. Half of the primary cultures are placed in the "A" incubator. The other half of the primaries should be put in the "B" incubator. This ensures that all cultures on a specimen would not be lost in the event of an incubator system failure. The outside of the each incubator door is labeled as to its contents. The incubators are kept at 37°C with 5.5% CO₂ and 5% O₂ levels. All coverslips set up on a given day are kept on a separate tray that should be labeled with the set-up date on colored tape. Record dates of flooding/media changing on this tape.
18. Record the total number of each type of culture on both the tissue set-up record sheet and the specimen record in the GIS database in the computer. Label the set-up record not only with the number of coverslips, but also with the tissue type that is plated in the dish. This is the responsibility of the technologist.
19. When all tissues to be set up are completed,
 - a. Empty the waste beaker into the sink and flush with a large amount of cold running water.
 - b. Discard all used plasticware in the red biohazard trash.
 - c. Either return the media aliquoted for the day's setup to the 37°C incubator, or if all tissues have been set up for the day, discard as described above.
 - d. Wipe down the surface of the hood with a paper towel, using the designated lab disinfectant spray.
 - e. Discard worn set-up gloves in the biohazard trash.
 - f. Rinse glass Petri dishes thoroughly with water, disinfect with bleach solution and follow your laboratory's SOP (standard operating procedure) for the sterilization process.
 - g. Rinse used dissection tools with water and soak in a beaker with the designated lab disinfectant. After soaking, the instruments should be sonicated for 10 minutes, rinsed again with water and placed in a rack to air dry. When dry, dissecting tools should be packaged and autoclaved for sterilization.
20. **If there is extra tissue that was not used for dissociation,** place the excess in a labeled tube in the refrigerator in the 'excess tissue' rack. Make sure to indicate on the specimen set-up sheet that excess tissue is in the refrigerator. The TC Tech will evaluate the cultures for growth and discard the excess tissue, if it is not needed, or inform a tech on the tissue rotation that the excess requires additional setup, if growth is poor.

Protocol 4.7 Flask and coverslip setup for POC/fibroblast cultures

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Principle

Cells from many different types of solid tissues may be cultured for chromosome analysis. In most cases, it is necessary to mince the tissue or enzymatically disaggregate the cells to allow the fibroblasts or epithelial cells to attach to the culture flask and grow. Once growth is established, the cells may be subcultured to coverslips for *in situ* culturing and harvest.

II. Materials

General stock

1. Sterile T-25 culture flasks
2. Sterile Petri dishes, 35 × 100 mm, with coverslips
3. Sterile Petri dishes, 100 × 20 mm (large)
4. Sterile iris scissors
5. Sterile fine tipped forceps

Reagents

6. Transport Media (see Protocol 4.2 E, POC transport medium)
7. Culture Media for POC
AmnioMAX-C100 complete – expires 10 days after date made

100mL or 450mL	Basal
15mL or 75 mL	Supplement
1mL or 5mL	L-Glutamine (29.2 mg/mL)

Complete Alpha MEM – expires 14 days after date made

100mL or 450mL	MEM-Alpha
15mL or 75 mL	Fetal Bovine Serum
1mL or 5mL	L-Glu/Pen-Strep mix

8. Collagenase, type IIS, 50 mg (Sigma).
 - a. Store in the freezer before rehydration.
 - b. Reconstitute with 6.2 mL of complete medium (MEM). Final concentration should be 8 mg/mL.
 - c. Aliquot in 1 mL amounts and freeze.
 - d. Label tube with date, concentration, expiration date of 6 months and preparer's initials, and lot number.

III. Method

1. Remove specimen from transport medium using sterile techniques and place in a 100-mm Petri dish. If tissue was received in saline or no media, transfer the tissue to POC transport medium and allow it to soak for at least one hour.
2. If bloody, transfer tissue into another 100-mm Petri dish filled with sterile PBS. Clean off any blood clots or excess blood. Do not leave tissue in PBS for a long period of time. In this case, transfer tissue to sterile complete MEM medium.
3. A portion of the tissue being set up should be saved in a cryo vial with at least 1 mL of complete MEM medium. This vial should be placed in a refrigerator for FISH studies if the primary culture does not grow. The tissue should be ~0.5 cm to 1 cm. If there is an insufficient amount of tissue for culture, do not save a portion for FISH.
4. Cut the specimen for culture into very small pieces using sterile scalpels or scissors. Pieces should be approximately 1 mm square or less. If material is adequate, two (2) flasks and four (4) coverslips should be set up on each product of conception sample. If there is more than one type of tissue received, set one tissue as your primary source and set the other tissue in an extra minced flask.
5. Place about 5–10 pieces of minced tissue in the bottom of a flask. Place the flask on end for 20 minutes and then gently add 2 mL of complete MEM medium to the flask. All flasks are open systems; therefore, tighten a vented flask cap or loosen a plugged flask cap. Place in the 37°C/5% CO₂ incubator designated for minced POC flasks.
6. The second flask should be labeled Flask #2. If chromosome analysis is required, also label four coverslips (3–6) for set up with the collagenase (conc.) cell suspension.
7. To the aliquoted 1 mL vial of collagenase, add minced tissue pieces, or to the Petri dish containing the minced pieces, add the 1 mL vial of collagenase and place the mixture in a 15 mL centrifuge tube.
8. Incubate in a 37°C water bath for 8–10 minutes.
9. After 8–10 minutes of digestion, check if the cells in the aliquoted vial or centrifuge tube are in suspension. The medium will appear very cloudy and will become more turbid as the vial is shaken.

10. When digestion has progressed as desired, add 4 mL of complete MEM medium to the centrifuge tube or transfer the contents of the aliquoted vial to a centrifuge tube, and add 4 mL complete medium.
11. Centrifuge at 800–1000 RPM for 10 minutes.
12. Carefully aspirate the supernatant and discard. Gently resuspend the pellet and add the appropriate amount of media needed to set up 1 flask and 4 coverslips (4 mL), if indicated. NOTE: If the specimen does not require chromosomes for analysis, do not set up coverslips. Only a flask with minced, collagenased tissue should be set up and placed in the properly designated 37°C/5% CO₂ incubator.

Protocol 4.8 Coverslip setup for solid tissue biopsy specimens

Contributed by Kristin May, Children's Hospital at Erlanger, Chattanooga, TN

I. Principle

If it is often necessary to culture cells from a solid tissue biopsy for cytogenetic, biochemical, or molecular study. In order to provide cells with free access to the culture surface, cells from the biopsy must be dispersed either enzymatically or mechanically. Enzymatic dispersal is performed by treating the minced tissue fragments with a collagenase solution. The collagenase treatment will cause individual cells to dissociate from the fragments. Cultures will usually grow more rapidly and can be harvested by the *in situ* method if tissue is disrupted by proteolytic agents such as collagenase.

Specimen: Solid tissue specimen, including products of conception, skin biopsy, fetal tissue.

II. Materials

General stock

1. Sterile 25 cm² flasks (T-25)
2. Sterile tissue culture flasks (250 mL) – for Alpha MEM media
3. Sterile plastic centrifuge tubes, 15 mL
4. Sterile Pasteur pipettes
5. Sterile plastic serological transfer pipettes: 1 mL, 5 mL, 10 mL
6. Powder-free nitrile gloves
7. Sterile disposable scalpel #21
8. Nalgene filter syringe
9. Sterile Petri dishes: 60 × 15 mm, 100 × 15 mm, 35 × 10 mm with coverslip (MatTek Corp)
10. Sterile forceps
11. Sterile syringe, 12 mL

Reagents

1. Transport medium A (without Fungizone)

Note: Medium must have been previously tested for sterility and ability to support growth. See Protocol 4.2A, Note 1, Media testing for sterility and growth support.

Reagent	Volume
RPMI 1640, 100 mL Gibco®/Invitrogen Storage: 2–8 °C Expiration: manufacturer's date	100 mL
L-glutamine/Pen/Strep – 200 mM L-glutamine, 10,000 U penicillin, 10 mg streptomycin in 0.9% NaCl Source: Sigma Storage: –5 to 20 °C	1.2 mL
Aliquot procedure	
• Thaw once	
• Divide into 1.2 mL aliquots; Label tubes “LGPS” and prep/exp date	
• Freeze; Expiration same as date on original package	

Procedure for making transport medium A

1. Combine all components in the original medium bottle under sterile conditions in biological cabinet.
2. Label “Transport Medium A” and add CAP required reagent information.
3. Expiration: 2 weeks stored in refrigerator (2–8 °C).

2. Transport medium B with Fungizone

Notes:

1. *Used only if products of conception, autopsy material or tissue from IUD is not received in transport medium with Fungizone AND if the specimen will not be set up immediately.*
2. *Medium must have been previously tested for sterility. (See Protocol 4.2A, Note 1, Media testing for sterility and growth support.)*

Reagent	Volume
Fungizone (Amphotericin B) Gibco®, 250 µg/mL	0.5 mL
<i>Aliquot procedure</i>	
<ul style="list-style-type: none"> • Divide into 0.5 mL aliquots and store frozen (−5 to −20 °C); • Expiration date same as manufacturer’s date on bottle; • Aliquots thawed at room temperature. 	

Procedure for making transport medium B

1. Follow procedure for Transport Medium A.
2. Add 0.5 mL of Fungizone (amphotericin B).

3. Alpha MEM complete medium

Note: Media must be previously tested for sterility and ability to support growth. (See Protocol 4.2A, Note 1, Media testing for sterility and growth support).

Reagent	Volume
Alpha MEM Medium with Earle’s Salts, 500 mL Source: Irvine Scientific Stored in refrigerator Expiration: manufacturer’s date	100 mL
<i>Fetal Bovine Serum, 100 mL</i>	
Source: GIBCO®/Invitrogen Storage: −5 to −20 °C	
<i>Aliquot procedure</i>	
<ul style="list-style-type: none"> • Thaw at room temperature; Mix well • Aliquot 10 mL into 15 mL conical centrifuge tubes • Label tubes and freeze • Expiration: same as manufacturer’s date 	
L-Glutamine/Penicillin/Streptomycin (LGPS), 100 mL Source: Sigma Storage: −5 to 20 °C	1 mL
<i>Aliquot procedure</i>	
<ul style="list-style-type: none"> • Thaw once • Divide into 1.2-mL aliquots and label tubes “LGPS” and CAP required reagent information (see Figure 4.4); • Store in freezer; • Expiration date same as date on original package. 	

Procedure for making complete alpha MEM medium

1. Mix the above in a 250 mL sterile flask and label.
2. Store in refrigerator and use within 14 days.

4. AmnioMAX complete medium

Note: Media must be previously tested for sterility and ability to support growth. See Protocol 4.2A, Note 1.

Reagent	Volume
AmnioMAX-C100 Supplement Gibco®/Invitrogen Store in freezer -5 to -20 °C, thaw in 37°C water bath	15 mL
AmnioMAX-C100 Basal Medium Gibco®/Invitrogen Stored in refrigerator, 2–8 °C	90 mL

Procedure for making complete AmnioMAX

1. Add supplement to basal medium bottle.
2. Label with preparation/expiration dates.
3. Test complete media for sterility and ability to support growth before use.
4. Store at 2–8 °C in amnio refrigerator for up to 10 days.

5. Collagenase type 1A

Source: Sigma Aldrich

Store in freezer

III. Method

1. If specimen does not arrive in time to establish cultures on the same day, place the specimen in transport medium with Fungizone (if not already in it) and refrigerate overnight.
2. Make sure that name on specimen, paperwork, and labels match. Pour contents of specimen tube or container into a sterile 60 × 15 mm or 100 × 15 mm sterile Petri dish, depending on the amount of specimen received.
If specimen is very large, several small sections should be excised with a sterile scalpel and sterile forceps, and then put into a 60 × 15 mm sterile Petri dish. Approximately 5 mL of transport media should be added. Proceed to step 4.
3. Transfer approximately 5 mL of transport medium into a second sterile 60 × 15 mm Petri. More Petri dishes with media may be needed to wash bloody tissues. Using a sterile scalpel and forceps, cut off enough of the original tissue to be able to set up at least 4 flasks and/or 8 coverslip cultures. A piece approximately the size of a pencil eraser will set up 2 flasks and 2 coverslip cultures. The specimen should be obtained from various sections of the original sample. Avoid very bloody portions or wash (by swishing) away blood with transport medium. Wash and transfer to fresh Petri dish with transport medium until tissue is clean.
 - a. If more than one piece of tissue is received from a single patient and the tissue is designated from different sites (i.e., skin, fascia), each tissue should be set up separately into approximately 2 flasks and/or 4 coverslips.
 - b. Note on the set-up sheets what was received and what was set up – *be specific* (i.e., villi, skin, decidua), and note the condition of specimen.
 - c. If the specimen is a POC or IUFD, carefully clean the specimen to obtain villi and/or fetal tissue. View under inverted microscope to further identify viable tissue. Villi will remain viable longer than fetal tissue. It is very important to obtain CLEAN villi!
 - d. If the tissue is a POC and there is no identifiable fetal tissue or villi (but there IS tissue and not just clots), obtain a second opinion from another technologist. If there is agreement that no fetal tissue or villi is present, then the lab director is to be notified and a recommendation will be given to the doctor's office that we did not set up the specimen. Appropriate paperwork is to be filled out.
 - Place the specimen in the refrigerator to await the response from the doctor's office.
 - If the MD office says to cancel the testing, the specimen will be discarded.
 - If the MD office says to continue OR if they do not respond in 24 hours, we will set up the specimen.

4. The tissue section from step 3 should be transferred to the 60×15 mm dish containing transport medium (step 2). Each large piece should be finely minced using sterile forceps and scalpel.
5. One piece of tissue should be placed in a sterile conical tube with fresh media. The tube should be labeled with the patient's name and lab number. This tissue is placed in a refrigerator and will be used for aneuploidy FISH screening if the specimen fails to grow. This should not be done if the specimen is extremely small. In that case, all villi and/or fetal material should be placed in culture.
6. Divide the entire contents of each dish evenly into two 15-mL sterile conical centrifuge tubes. This can be done using a 10 mL pipette. Label each centrifuge tube with the specimen #, patient's last name, type of tissue, and media designation: one tube should be designated for Alpha MEM and a second for AmnioMAX. Lightly cap.
7. Weigh out collagenase to achieve a final concentration of 1.0 mg/mL in each tube.
8. Collagenase should be carefully transferred into another sterile Petri dish and enough transport medium (RPMI 1640) added that is equal in volume to the total volume of fluid in the centrifuge tubes.
9. Allow the collagenase to dissolve for a few seconds and then aspirate the collagenase solution into a syringe.
10. Remove the needle from the syringe and attach a syringe filter to the barrel.
11. Into each centrifuge tube, carefully dispense (through the filter) a volume of fluid equal to the volume in each centrifuge tube.
12. Recap the tubes and incubate at 37°C for approximately 30 minutes. Shake the tubes to check for cloudiness every 10 minutes, which indicates the collagenase is working. You want to see a cloudy suspension but not to the point where there are no intact pieces of tissue left.
13. Add approximately 5 mL complete Alpha MEM to the tube so designated and 5 mL AmnioMAX to the other tube so designated. This addition of complete media will stop the action of the collagenase.
14. Centrifuge all tubes at 1000 RPM for 9 to 10 minutes.
15. Label two 25-cm³T-culture flasks and at least two 35×10 mm culture dishes with coverslips for each centrifuge tube that has been prepared. Label all flasks with the patient's name, lab number, type of media, and setup date; sequentially label each flask A, B, C, D, etc. Each coverslip culture should be labeled with the same information but sequentially labeled as 1, 2, 3, 4, etc.

NOTE: Specimens that are to be grown for reference lab testing only need to have flask cultures initiated. Coverslip cultures are not necessary. At least 2 and preferably 4 flasks should be set up, depending on the size of the specimen.

16. After specimens are centrifuged, remove supernatant with a sterile pipette, leaving approximately 2 mL of tissue suspension. Resuspend and then allow the larger chunks to settle for a few seconds. With a sterile pipette, aspirate approximately 0.5 mL of the suspension and carefully apply to each of the 4 coverslips. The suspension should be confined to the coverslip area.
17. All of the remaining suspension – including larger pieces of tissue – should be evenly divided (once again, using a sterile pipette) into each of two flasks so designated by previous labeling. Enough additional medium should be added to each flask to ensure that the bottom surface of the flask is covered but that the tissue pieces are not floating – approximately 3 mL total fluid.
18. Incubate all flasks at 37°C in a horizontal position with caps slightly loosened.
19. After cultures are set up, a “tissue culture worksheet” should be filled out with all the appropriate patient information. The sheets are kept in the Pending Cases Notebook in the tissue culture area until finished, then moved to the Completed Notebook.
20. Clean up work area. Decontaminate with 70% alcohol. Any remaining tissue is stored in its original container in refrigerator until the case is completed.

IV. Additional reading

Priest J. General cell culture principles and fibroblast culture. In Barch MJ, Knutsen T, Spurbeck J, eds. *The AGT Cytogenetics Laboratory Manual*, 3rd ed. 1997; Philadelphia: Lippincott-Raven; 173–197.

Protocol 4.9 Solid tissue (fibroblast) culturing and harvesting

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

Cytogenetic analysis of solid tissue (skin, products of conception, abortus tissue, etc.) is clinically useful for the diagnosis of cytogenetic abnormalities when blood is not obtainable or when blood-tissue mosaicism is suspected. For products of conception

and abortus tissues, the cytogenetic findings may help to determine the cause for the pregnancy loss and/or recurrence risk. In addition, solid tissue cultures are used to confirm the prenatal diagnosis of a chromosomally abnormal fetus.

Safety warnings

All tissue specimens should be handled as biohazardous, using Universal Precautions. Use the laminar flow hood for all steps up to harvest spin. Wear a laboratory coat and protective gloves for all steps through slide-making. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with Sanimaster 4 (made fresh weekly) or 70% ethanol. Cover cuts with protective bandages even when gloves are worn. Dispose of Pasteur pipettes, needles and syringes in sharps container. Wash hands thoroughly after removing gloves.

Colcemid is mutagenic, tumorigenic, embryotoxic and teratogenic with acute overexposure. Wear gloves and lab coat at all times when handling. Avoid contact with skin or inhalation, flush for 15 minutes if accidental contact.

Specimen requirements

See Protocol 4.1(D) for specimen collection instructions. Unacceptable specimens include dried-out or frozen specimens, or specimens fixed in formalin, which all cause the death of the cells and preclude culturing. In case of receipt of unacceptable specimens, notify referring physician immediately.

Log specimen into laboratory accession book and onto fibroblast flow sheet and log sheet.

Caution: Wear protective gloves and coat.

II. Materials

A. Materials for setup and culturing solid tissues

General stock

1. 35 cm² Corning sterile plastic Petri dishes
2. Sterile forceps and scissors
3. Sterile scalpel handles and disposable blades
4. Sterile 25 cm² Corning tissue culture flasks
5. Sterile cotton-plugged Pasteur pipettes
6. Sterile graduated pipettes: 1 mL, 5 mL, and 10 mL
7. Inverted microscope
8. CO₂ incubator at 37°C

Reagents

1. Media: Ham's F10 Complete:
 - a. 20% fetal bovine serum (FBS)
 - b. 0.1% gentamicin (50 mg/mL)
 - c. 1% L-glutamine (200 mM)
 - d. Shelf life 14 days. Store at 4°C.
2. MEM Alpha, complete:
 - a. 20% FBS
 - b. 0.1% gentamicin
 - c. 1% L-glutamine
 - d. Shelf life 14 days. Store at 4°C.
3. Chang C, complete:
 - a. Supplement from supplier
 - b. 0.1% gentamicin
 - c. 1% L-glutamine
 - d. Shelf life 5 days. Store at 4°C.

4. Hanks' 1× sterile saline:
 - a. 50 mL of 10× Hanks' brought to 500 mL with sterile distilled water.
 - b. Shelf life 30 days.
 - c. Store at room or refrigerator temperatures.
5. Collagenase
 - a. Sigma type IV-S, 536 units/mg (#C1889-50MG), reconstituted with 10 mL sterile Hanks' saline or incomplete media. Use 0.20–0.25 mL in 5 mL incomplete media.
 - b. Worthington Type I Collagenase, (#LS004196), 210 units/mg. Dilute with incomplete medium to a final concentration of about 1 unit per mL, rounding up to the closest multiple of 10 (e.g., for 142 mg/mg, use 150 mL medium). Filter through a vacuum filtration unit with 0.2 µ pore size. Aliquot into 4 mL snap cap sterile tubes and freeze. Shelf life 1 year.

B. Materials for subculturing fibroblast cultures from solid tissues

General stock

1. Sterile 1 mL and 5 mL pipettes
2. Corning tissue culture flasks, either 25 cm² or 12.5 cm²

Reagents

1. Hanks' 1× saline: 50 mL of 10× Hanks' is diluted up to 500 mL with sterile distilled water. (Hanks' 10×: Irvine Scientific). Shelf life 1 month.
2. Hanks'-trypsin: Defrost 2 mL aliquot of trypsin-EDTA solution and dilute it up to 30 mL with Hanks' 1×. (Gibco® Trypsin-EDTA 10×). Make fresh daily.
3. Appropriate media, depending upon tissue (see above note on media).

C. Materials for harvesting fibroblast cultures from solid tissues

General stock

1. Test tube rack
2. 1 and 5 mL sterile disposable plastic pipettes
3. Polypropylene centrifuge tubes with screw caps (use 50 mL tubes for 75 cm² flask harvest and 15 mL tubes for 25 cm² flask harvest)
4. Micropipettor with sterile tips

Reagents

1. Colcemid®, 10 mcg/mL, Gibco®. Shelf life 6 months after opening.
2. Hanks' 1× solution as above.
3. Fresh Hanks'-trypsin solution: defrost a 2 mL aliquot of trypsin-EDTA solution and dilute it up to 30 mL with Hanks' 1×.
4. Harvest Medium: Ham's F10 with 10% FBS, shelf life 1 month at 4°C.
5. Hypotonic solution:
Stock = 1 : 4 FBS/sterile distilled water, shelf life 1 week at 4°C
Working solution = 1 : 3 stock hypotonic/0.075 M KCl. Make daily and discard excess.
6. Fixative: 3 : 1 methanol-acetic acid made fresh before use.

III. Method

A. Setup and culturing solid tissues

There are two methods for establishing solid-tissue cultures: the *collagenase method* which dissolves the matrix and releases the cells from the tissue, and the *explant method* in which very small minced up fragments of tissue are allowed to attach to the plastic flask and grow from the edges out. If time permits, the collagenase method is usually preferred. If time is limited, or if the sample is very small, the explant method may be substituted.

CAUTION: Some tissue specimens may be too fragile to withstand collagenase treatment. To be safe, back-up tissue should be set aside for use within the explant method.

Tissue preparation

1. Rinse tissue in Hanks' 1× saline.
 - If sample is skin, remove any excess fat.
 - If sample is from an abortus, dissect fetal tissues apart using forceps and scissors or scalpel, and place in Petri dishes.
 - If sample includes villi, clean and set up as per CVS technique.
2. Go to step 1 for the collagenase method, and step 2 for the explant method.

Step 1: Collagenase method

1. Pipet 5 mL unsupplemented Ham's F10 medium into one sterile 35 mm² Petri dish. Place tissues in the dish; use about 5 mm² of tissue per dish. Add 0.2 mL collagenase to dish. Note time.
2. Incubate at 37°C with 5% CO₂; check the sample every half hour. The smaller the pieces, the less time is required. Check tissue dissociation on an inverted microscope. Tissues should appear translucent and cellular. If not, re-incubate further.
3. Add cell-medium suspension to a sterile centrifuge tube and pipet vigorously 20–30 times to break any clumps. Cap tubes and centrifuge at 1000 RPM for 10 minutes.
4. Label flasks with GL #, flask #, date, tissue type, patient name, and, if necessary, medium type.
5. Decant supernatant, resuspend in 1–5 mL medium (A and C flasks: Ham's F10; B and D flasks: MEM) depending on the pellet size. Seed 1 mL of suspension in a flask. Gas with 5% CO₂, 5% O₂, and 90% N₂ for 5–10 seconds, close tightly, and incubate at 37°C. Check for growth, and feed with 2 mL of media at 3–5 days.

Step 2: Explant method

1. Transfer cleaned, appropriate tissues into Petri dishes (use two different media as above). Moisten with 1 mL of medium, and mince each tissue with scissors or scalpel into fragments as small as possible.
2. Transfer fragments with the 1 mL of medium into flasks and rotate flask to allow cell suspension to cover the surface of the growth area. If there is more than 1 mL of medium, cells will not be in contact with growth surface, and may not attach. Remove excess medium, if necessary.
3. Label flasks with GL #, flask #, patient name or first three initials, date, and type of tissue.
4. Gas with 5% CO₂, 5% O₂, 90% N₂ for 5–10 seconds; tighten cap.
5. Leave undisturbed in incubator for 3–5 days. Then check cultures for growth and feed as follows: If good growth is observed, pour off medium and feed with 3 mL of fresh medium. If no growth is observed, pipet off the medium and gently replace with 1 mL of fresh medium. Re-gas cultures which are not growing.
6. Cultures will yield better harvests if subcultured first. Subculture when 3 or more colonies are seen per flask that fill the lower-power field and appear mitotic.

B. Subculturing fibroblast cultures from solid tissues

1. Fibroblast cultures are subcultured when growth is such that when transferred, the subcultured flask reaches confluence in 3–5 days.
2. Pour off medium into a bottle with a funnel and alcohol or bleach inside.
3. Rinse flask growth surface twice with 2 mL Hanks' 1× solution and pipet off into discard bottle. Add 1 mL of Hanks'-trypsin solution and incubate on the warming plate or in the incubator for 1–5 minutes, until cells are rounded up and floating. Rap flask sharply on the countertop to encourage cell detachment.
4. Depending upon the mitotic activity of the cultures, select a 25 cm² flask for rapidly-dividing cells.
5. Pipet cells a few times against the culture flask to encourage single cell suspension. Pipet an appropriate volume of cells into the new flask. Volume to be transferred depends upon cell density and activity, and varies from about 0.1 mL to 0.9 mL.
6. Label flask with culture designation (A, B, C, etc.), GL #, patient's name or initials, and date, plus tissue or origin. Feed both flasks with fresh medium to bring volume to a total of 4 mL in the 25 cm² flasks. Gas cultures and re-incubate.
7. Record subcultures on flow sheet on the tissue culture hood.
8. After the study has been reported out through chromosome conference and no further clinical work is necessary, cultures are discarded.

C. Harvesting fibroblast cultures from solid tissues

1. Add 3 µL of Colcemid® per mL of medium (e.g., add 9 µL of Colcemid® to a 3 mL culture). Incubate at 37°C for 2.5–5 hr., depending on growth rate.
2. Decant medium into an appropriately sized centrifuge tube.
3. Add 2 mL pre-warmed Ca/Mg-free Hanks' BSS to culture, rinse, decant, adding to the centrifuge tube.
4. Repeat step 3.
5. Add 1 mL trypsin-EDTA solution to culture vessel. Place on warming tray no longer than 5 minutes. Shake or tap vessel vigorously to loosen cells, if necessary.
6. Add cell suspension to the centrifuge tube (see step 3).
7. Rinse vessel with 2 mL harvest medium (F10 with 10% FBS); add this fluid to the cell suspension.
8. Repeat step 7.
9. Centrifuge the tube at 1000 for 10 minutes. Aspirate supernatant down to approximately 0.1 mL.
10. Resuspend cells by tapping tube with your finger until clumps are dispersed.
11. Begin timer for 15 minutes. Add 3 drops of hypotonic (room temperature) to the tube. Mix, add up to 1/2 mL, mix. Incubate at room temperature for remaining time on timer.
12. Add hypotonic up to 3 mL, mix. Incubate at 37°C for 15 minutes.
13. Pre-fix by adding 5 to 10 drops fix into the hypotonic.
14. Mix.
15. Centrifuge at 1000 RPM for 10 minutes. Aspirate to 1.0 mL.
16. Mix pellet gently but thoroughly before addition of fixative.
17. Add 3 drops fix (3 : 1 methanol–acetic acid), mix; add to 1/2 mL, mix; add to 3 mL. Allow to sit for 20 minutes before next step.
18. Centrifuge as before, remove supernatant. Add fresh fix, resuspend, and make slides.

IV. Additional reading

Lee EC. Cytogenetic analysis of continuous cell lines. In: Barch M, ed. *ACT Cytogenetics Laboratory Manual*, 2nd ed. Raven Press, 1991.

Protocol 4.10 Fibroblast culture maintenance: media feeding and changing

Contributed by Urvashi Surti, Magee Women's Hospital, Pittsburgh, PA

I. Principle

Cultures should be left undisturbed for a few days after setup in order to attach and proliferate. After a few days the nutrients will be spent and waste will accumulate, changing the pH. At this point the media should be replaced for continued growth.

II. Materials

General stock

1. 15 mL sterile plastic centrifuge tubes
2. Sterile Pasteur pipettes
3. Sterile plastic serological transfer pipettes: 1 mL, 5 mL, 10 mL
4. Powder-free nitrile gloves

Reagents

5. Media: Two different lot numbers or two different media which are standard for the specific cell type

III. Method

Two days after culture setup (before flooding)

1. Cultures are checked under the inverted microscope for evidence of cell attachment. If no attachment is noted, the coverslips are not flooded unless the media is no longer contained on the coverslip.
2. Then add 1.5 mL of medium back onto the original coverslip.
3. Complete media change is performed two days after flooding, with 1.5 mL of "A" or "B" media.
4. Complete media changes are then performed twice a week with 1.5 mL of "A" or "B" media until the cells are ready for harvest.

Routine maintenance

5. After 24–48 hours, add 2 mL of fresh media to each flask that was setup. Handle the flasks gently so as not to dislodge the tissue pieces. After 24 hours, add 1.5 mL media to each of the coverslips set up.
6. Put coverslips into the normal routine of media changes beginning on day 4.
7. Put flasks into normal routine of media changes on alternate days. Coverslips are changed on Monday, Wednesday, and Friday.
8. Pieces should be attached within 24–48 hours and single cell growth can be seen in about 5 days.

Growth assessment

9. When the patches are becoming dense or diffuse growth is present over much of the flask, trypsinize the culture and disperse or subculture to coverslips for harvest. It is acceptable to trypsinize and disperse or subculture coverslips if the growth is patchy and dense.
10. Back-up flasks should be kept for 2 weeks after the final report date.

POC/maternal cell determination

11. If a POC result yields 46,XX, cells should be trypsinized from at least one confluent backup flask and placed in a 15 mL centrifuge tube for possible maternal contamination studies.
12. Spin the centrifuge tube at 800–1000 RPM for 10 minutes. After centrifugation, place the cell pellet in the refrigerator (2–8 °C) and keep for 20 days after reported date.
13. If requested by referring physician, these cell pellets will be used to perform maternal cell contamination studies on POCS that were presumably not set up from fetal tissue and hence, yielded a 46,XX karyotype.

Protocol 4.11 Routine subculture of fibroblast cultures

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Principle

Flask cultures or backup Petri dishes of attached cells may be trypsinized and subcultured to other flasks or to coverslips for in situ culturing and harvesting. This is generally done when the initial in situ coverslips have not yielded the required number of colonies or if there is an abnormal colony.

Cells are enzymatically removed from the original culture vessel. The cell suspension is then diluted in culture media, split, and re-seeded into the original culture vessel. The remaining cells are seeded into (a) new culture vessel(s) thereby producing multiple cultures from one original culture. These cultures can then be used to produce chromosome preparations to complete a case or for additional staining and in-situ fluorescent hybridization techniques. When only a few dense colonies are present in a flask or Petri dish, trypsinization and dispersal may stimulate growth of the culture. Subculturing and dispersal is used frequently to produce confluent growth for metabolic and DNA studies or for freezing down cultures.

II. Materials

General stock

1. 2, 5, 10 mL disposable sterile pipettes (VWR)
2. Sterile coverslips (Clay Adams)
3. Petri dish (VWR)
4. T-25 vented flask (Fisher)
5. T-75 vented flasks (Fisher)
6. 15 mL polypropylene centrifuge tube (Fisher)
7. 37.5 °C/5% CO₂ incubators

Reagents

8. Phosphate Buffered Saline (PBS) (Invitrogen)
Store at room temperature. See expiration date on bottle.
9. Trypsin-EDTA 0.0540 (Invitrogen)
Store frozen. Check expiration date on bottle.
Best to thaw, and freeze in aliquots of 2 mL.
Use 0.5 mL in each T-25 flask or Petri dish to disperse cells.
Add 1 mL trypsin for T-75 flask.
10. Appropriate media.

III. Method

1. If you are subculturing a flask to another flask, determine the type of split. This will depend on whether the culture needs to be seeded heavily or not.
2. Label 15 mL centrifuge tube with the culture # and sub # of the flask/Petri dish and patient's initials.
3. Using an appropriate pipette, remove medium and discard. Using a new pipette, rinse Petri dish with 2 mL sterile PBS, the T-25 flask with 5 mL sterile PBS, or the T-75 flask with 10 mL sterile PBS and discard rinse.
4. Add 0.5 mL of trypsin-EDTA solution (0.05% working solution) to the flask or Petri dish. Close cap and place flask in incubator. Set timer for 5 minutes. Incubate at 37.5 °C and 5% CO₂ until cells are visibly detached.
5. Using an appropriate pipette, rinse the Petri dish with 2 mL of appropriate fresh medium. If you are trypsinizing a T-25 flask, rinse with 4 mL of appropriate fresh medium. If you are trypsinizing a T-75 flask, rinse with 10 mL of medium. Remove all but 0.5 mL of the cell suspension in the flask or Petri dishes. This will ensure continued growth of your backup culture.
6. Place cell suspension and 2 mL of fresh medium into 15 mL centrifuge tube and spin for 10 minutes at 800–1000 RPM. If you are subculturing to a flask, obtain appropriate vented flask and label with patient name and lab ID number. Based on the split being performed, place the cell suspension in the new flask and add appropriate fresh media to bring total to 4 mL for T-25 and 10 mL for T-75.
7. Refeed original T-25 flask with 4 mL medium, original Petri dish with 2 mL medium or add 10 mL medium back to a T-75 flask. Place all sources back into appropriate 37.5 °C/5% CO₂ incubators.
8. **For subculturing on coverslips**, obtain 35 mm Petri dishes with coverslips and label the top and bottom of each Petri dish with the culture number, subculture number, date trypsinized and patient's initials. Obtain 15 mL tube from centrifuge. Remove the supernatant to just above the pellet. DO NOT disturb the pellet. If no visible pellet, remove supernatant to the 0.2 mL mark on the tube.
9. Using appropriate media, resuspend cell pellet with 0.5 mL medium for each coverslip (e.g., if setting up two coverslips, add 1 mL media). Place 0.5 mL cell suspension onto each coverslip.
10. Incubate at 37.5 °C with 5% CO₂.
11. After 24 hours add 1.5–2.0 mL fresh medium; observe for cell growth (rounded up cells and doublets) and harvest when ready.

Protocol 4.12 Manual harvest for flasks

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Principle

Chromosome studies on monolayer cell cultures such as neonatal kidney cells and fibroblast cultures require different conditions for preparation and harvesting. These tissues can be harvested directly from the flasks rather than by an *in situ* culturing method.

II. Materials

Reagents

1. Trypsin, (0.05%). Store in freezer (-20 °C). Check expiration date on bottle. Store working solution in freezer with an expiration date of 6 months from date made.
2. Hypotonic Solution, 50% KCl (0.075 M) to 50% Na Citrate (0.8%). Label bottle as non-sterile with a 1-month expiration date.

To make hypotonic reagents:

- a. 0.075 M KCl: add 2.1 g KCl to 375 mL 3× dH₂O.
 - b. 0.8% Na Citrate: add 3.2 g Na Citrate to 400 mL 3× dH₂O.
 - c. Filter sterilize.
 - d. Add equal portions of each of these two to make a 50 : 50 hypotonic solution, pre-warmed to 37 °C.
3. Colcemid® solution, 10 µg/mL Gibco® KaryoMAX Colcemid® solution in PBS (Invitrogen). Store in the refrigerator. Check expiration on bottle. Use directly from bottle. No dilutions necessary.
 4. Cold fixative: 3 : 1 methanol–glacial acetic acid. Must be made fresh each time and refrigerate to keep cold.
 5. Complete Alpha MEM: Store in refrigerator. Expires 14 days after date made.

To make complete Alpha MEM, combine the following reagents:

100 mL or 450 mL	MEM-Alpha
15 mL or 75 mL	Fetal Bovine Serum
1 mL or 5 mL	L-Glu/Pen-Strep mix

III. Method (using sterile procedure)

1. Add 50 µL Colcemid® solution to a culture flask containing 5 mL of medium.
2. Incubate flask for 5 hours at 37 °C, 5% CO₂.
3. Pipet medium into a pre-labeled 15 mL centrifuge tube and set aside.
4. Pipet 1.5 mL of trypsin-EDTA solution into flasks and rinse by pipetting the solution over the cells several times. Transfer the trypsin solution to the same centrifuge tube as in Step 3.
5. Pipet 1.5 mL trypsin EDTA solution into the flask and over the cells. Incubate for 5 minutes at 37 °C, 5% CO₂.
6. Monitor the cell adhesion every 1–2 minutes. As soon as most of the cells are detached, rinse thoroughly with the contents of the centrifuge tube. Be sure to break up the clumps of cells.
7. Pipet 5 mL of medium into the same centrifuge tube. Spin at 800 RPM for 10 minutes.
8. Aspirate off most of the supernatant, being careful not to disturb the cell pellet.
9. Gently add 5–10 mL of hypotonic (depending on pellet size) and resuspend thoroughly.
10. Incubate at 37 °C for 30 minutes.
11. Add 2 mL of fresh fixative to the cell suspension and resuspend (conditioning fix).
12. Centrifuge at 800 RPM for 10 minutes.
13. Aspirate off most of the supernatant. Do not disturb the cell pellet.

14. Gently add 8–10 mL of cold fixative on top of the cell pellet. Cap and let set for 30 minutes at room temperature.
15. Resuspend well, and then spin for 10 minutes at 800 RPM.
16. Remove supernatant; add back 5 mL of fixative. Resuspend and spin for 10 minutes at 800 RPM.
17. Repeat centrifugation and remove supernatant. At this point the cells can either be stored or slides can be made. If cells are stored, resuspend in 5 mL of fixative. If slides are made, resuspend in about 0.5–1.5 mL, depending on the pellet size and desired concentration of cells.

IV. Note

For chromosome analysis and reporting of fibroblast cultures, see *ACMG Standards & Guidelines* at http://www.acmg.net/StaticContent/SGs/Section_E_2010.pdf

Protocol 4.13 Treated media for contamination

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

Culture media

I. Materials

Wash treatment medium

To 100 mL of complete MEM, add:

4 mL Gentamicin (4% concentration)

Treated culture medium

To 100 mL of complete MEM add:

0.5 mL Gentamicin (0.5% concentration)
1.0 mL Amphotericin (1% concentration)

Complete Alpha MEM – expires 14 days after date made

100mL or 450mL	MEM-Alpha
15mL or 75mL	Fetal Bovine Serum
1mL or 5mL	L-Glu/Pen-Strep mix

II. Method

1. Treat bacterially-contaminated cultures with Wash Treatment (4% Gentamicin) for one hour. NOTE: Treatment with 4% antibiotics for more than one hour may be harmful to the cells.
2. Wash culture and replace with Treated Culture Medium (0.5% Gentamicin).
3. Monitor frequently.

Protocol 4.14 Fungizone–mycostatin solution for treatment of fungus/yeast contaminated cultures

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Method

If a contaminated culture shows growth, decant the medium and rinse the flask with fibroblast nutrient medium containing 10% Fungizone or mycostatin. Repeat rinse and feed culture with the same medium. Repeat rinses with subsequent feedings. Care should be taken to properly dispose of medium and rinses to avoid contamination of other cultures.

Protocol 4.15 Mycoplasma testing

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

Species from the genus *Mycoplasma* are a serious source of contamination in tissue cultures. These organisms, which lack cell walls, do not normally produce turbidity in cell cultures and therefore, cannot be detected visually. Mycoplasmas are resistant to antibiotics that act on the cell wall, making them difficult to eliminate from tissue cultures. When present in a culture, mycoplasma interfere with investigational cell parameters. Mycoplasma can induce chromosomal abnormalities and alter the antigenicity of cell membranes. They can affect cellular metabolism by competing for nutrients and influence cell fusion procedures.

Safety warnings

All tissue specimens should be handled as biohazardous, using Universal Precautions. Use the laminar flow hood for all steps. Wear a laboratory coat and protective gloves for all steps. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with Sanimaster 4 (made fresh weekly) or 70% ethanol. Cover cuts with protective bandages even when gloves are worn. Wash hands thoroughly after removing gloves.

Acetic Acid is flammable as a liquid and a vapor. Avoid contact with skin, inhalation or ingestion. Acetic acid may be fatal if swallowed. Causes severe eye and skin burns. In case of contact, flush skin or eyes for 15 minutes, remove to fresh air in case of inhalation. Do not induce vomiting if swallowed. Use gloves, lab coat, and goggles at all times.

Methanol is EXTREMELY flammable, may cause flash fires. May be fatal or cause blindness if swallowed. Methanol is harmful if inhaled or absorbed through skin. It cannot be made nonpoisonous. In case of contact, flush skin or eyes for 15 minutes, remove to fresh air in case of inhalation. Seek medical attention if swallowed. Use gloves, lab coat, and goggles at all times.

II. Materials

General supplies

1. 35 cm² Corning sterile plastic Petri dishes

Equipment

2. Inverted microscope
3. Fluorescent microscope

Reagents

1. Myco Testing Media
 - a. MEM Alpha (Gibco®)
 - b. 10% FBS (Irvine Scientific) Shelf life 30 days. Store at 4°C.
2. Hanks' Balanced Salt Solution (Sigma) without calcium chloride, without magnesium, without magnesium sulfate, without phenol red. Store at 4°C.
3. bisBenzimid Stain (Sigma); Store in -20°C; do not expose to light.
4. Fixative: 3 : 1 Methanol–Acetic Acid
 - a. Methanol, Methyl Alcohol (Mallinkrodt Chemical)
 - b. Glacial Acetic Acid (Fisher Scientific)

III. Method

Mycoplasma stain

Stock solution

1. Dissolve 5 mg Bisbenzimid stain in 50 mL 1× Hanks' to make the stock solution;
2. Store at 4°C for 5 years.

Working solution

1. Use 0.5 mL of stock solution and dilute to 100 mL with 1× Hanks' to make stain solution;
2. Store at 4°C for 5 years.
3. Positive control for DNA staining is the stained nuclei of the cells; can compare to unstained cells for negative control.
4. Stain slide for 30 minutes and rinse in 1× Hanks'. All nuclei should stain and mycoplasma will appear as very small extranuclear dots or rods. Most other microbial contaminants are larger.

***Mycoplasma stain must not be exposed to light; cover storage containers in foil.**

Protocol 4.16 Plating efficiency of serum

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Method

1. Record the control or lot number serum to be checked.
2. Select a T-75 flask of 'just-confluent' cells. Rinse twice with PBS (see section above on Balanced Salt Solutions), 5 mL each time. Add 1.5 mL of 0.25% trypsin, and incubate at 37°C until cells come off and are suspended singly. Add 5 mL of medium (see text under dispersion of monolayer cells) containing 15% serum to be tested (usually fetal bovine serum).
3. Suspend cells well with a sterile culture pipette and perform cell counts by inoculating both sides of a standard hemocytometer twice or follow manufacturer's instructions for automated cell counting. On each side of the chamber, count five large squares (four corners and one center, 1 mm each) for a total of 20 mm² for 20 squares of 2 mm³ volume (the depth of the hemocytometer is 0.1 mm). Divide by 2, and multiply by 10³ to obtain the number of cells/mL. (Recall that 1 mm³ = 1 × 10³ mL.)
4. Using sterile technique, make up a series of cell dilutions using 10 mL plastic culture tubes and a 1 mL plastic disposable pipette. Suspend cells in the medium with serum to be tested. Be sure cells are well suspended before each dilution.
 - a. First cell dilution (should be 1 × 10⁶ cells/10 mL); from number of cells/mL = n (determined from step 3), solve for mL of cell suspension = mL to be added to the first dilution: $n/1 = 1 \times 10^6$. Add calculated number of mL of cell suspension to make 10 mL in medium containing 15% serum.
 - b. Second cell dilution (should be 2 × 10³ cells/10 mL): Add 0.2 mL of first cell dilution to 9.8 mL medium containing 15% serum.
 - c. Third cell dilution (should be 2 × 10³ cells/10 mL) of the second cell dilution to 9.0 mL medium containing 15% serum.
1. Add 0.5 mL of final cell suspension (100 cells per dish) to each of 10 60-mm plastic culture Petri dishes containing 4 mL of medium with a minimum concentration of 15% serum to be tested. Immediately after adding the cells, agitate the Petri dish gently to ensure good distribution. Be sure the pH of the medium is maintained at the correct color of phenol red indicator (pink-orange), adjusting with CO₂ as needed. Incubate at 37°C for one (1) week undisturbed. Check for macroscopic appearance of cell colonies. Further incubation might be indicated.
2. Use the following staining and counting procedure for cell colonies (nonsterile): Pour off medium. Add 1–2 mL PBS. Swirl. Pour off. Repeat. Fix for 5 minutes by adding 5 mL of 95% ethanol. Pour off. Stain. Rinse with distilled water. Air-dry dishes in an inverted position. Count the number of colonies by placing the dish on a counting page that is ruled in small squares. Record the colony count for each dish, and obtain an average by dividing by 10. Express as a percentage.

Example: Average count is 20. Plating efficiency is 20/100 = 20%.

Protocol 4.17 Routine replication plating for human diploid cells

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Method

1. Prepare a suspension of single cells from a monolayer culture according to routine trypsinization procedure. Rinse with PBS, aspirate all off, add 5 mL 0.25% trypsin to a T-75 flask, and incubate at 37°C until cells are rounded and detached as single cells.
2. Add 8.5 mL of complete medium, suspend cells well, perform a cell count, and make appropriate dilutions if an exact number of cells is required to inoculate replicate cultures (see Protocol 4.16, *Plating Efficiency of Serum*, on quantitative

plating of single cells). As a general rule, one confluent T-75 flask contains about 5×10^6 to 6×10^6 diploid human fibroblasts, so that an approximation can be made without a cell count.

3. Calculate the total number of cells required for the entire experiment or cell study from the number of culture container and the number of cells to be placed in each.
4. Suspend cells in the total amount of medium required for the study, which may be calculated from the number of culture containers needed and the amount of medium to be placed in each.
5. Dispense aliquots of cell suspension to each culture container (dish or flask). Work quickly, and keep cells well suspended by frequent pipetting. Place containers in incubator at 37°C at the proper CO₂ concentration as soon as possible to maintain the correct pH and temperature.

Protocol 4.18 Cell counting chamber method

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Method

1. Count cells with 10× objective on a standard light microscope with the light intensity low or with the condenser lowered such that it is out of focus but will increase the refractile appearance of unstained cells. Under these conditions, staining of cells is not necessary. Counting can also be done on an inverted phase microscope. However, if staining is desired, use the protocol for trypan blue stain (Protocol 4.19).
2. Cultured diploid fibroblasts will vary considerably in size when they are rounded up following trypsinization. They still retain a clear margin, appear refractile, and may have a disguisable nucleus. Keep in mind the morphologic criteria for cells under these conditions to avoid counting debris (noncellular material).
3. Follow a rule for counting liners (cells falling on the lines of the squares) such as counting upper and right but not lower and left. Use of a digital counter may increase speed.
4. If counts on one side of the chamber differ markedly from counts on the other or if the cell distribution looks uneven, re-inoculate the chamber and count again. Repeated inoculations should be avoided because the total cell counts will decrease in number, presumably due to cell loss in the pipette. Use siliconized or nonadherent plastic pipettes to decrease adherence of cells.
5. The cell suspension should exactly fit the counting area of the chambers and should not spill over into the wells around the sides of the counting area ("flooding the chamber").
6. The protocol for studying the plating efficiency of serum in this section (Protocol 4.16) gives an example of how to perform cell counts. Remember to keep track of the dilution factor of the cell suspension inoculated into the hemocytometer.

Protocol 4.19 Cell viability by dye exclusion

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

1. Place 0.5 mL of cell suspension in a small tube.
2. Add 0.1 mL of 0.4% aqueous trypan blue and mix thoroughly.
3. Allow the solution to stand for 10 minutes or incubate for 5 minutes at 37°C.
4. With a Pasteur pipette, fill a hemocytometer.
5. Count the total cells and unstained cells. The non-stained cells are those which are viable.

Protocol 4.20 Mitotic index

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Principle

A mitotic index may be determined from a chromosome preparation if metaphases are not too well spread (broken). Hypotonic treatment spreads the metaphases and makes them easier to identify. If the mitotic rate in the culture is to be determined, treatment of the cells with metaphase arresting agent prior to slide preparation is contraindicated or should not exceed about 30 minutes. Hypotonic treatment of cells prior to fixation is not an absolute requirement because mitoses can be identified when they are not well spread.

II. Method

Routine method for monolayer cells

1. Pour the medium from cultured monolayer cell (in dishes or flasks) and rinse with PBS. Add 0.25% trypsin, 0.5 mL for 60-mm Petri dishes and 5 mL for T-75 flasks (see Note 1, Modification for a small specimen).
2. Incubate at 37°C until the cells are suspended (usually at least 5 minutes).
3. Pipet the cell suspension into a plastic centrifuge tube containing 5 mL of medium. The serum in the medium will stop the trypsin reaction from harming the cells.
4. Centrifuge at 800–1000 RPM for 6–8 minutes (or longer if necessary to form a distinct cell button).
5. Decant the supernatant with one quick pour, or aspirate without disturbing the cell button (see Note 2, Modification with hypotonic treatment to increase cell spreading).
6. Add at least 2 mL 1 : 3 fix (glacial acetic acid–absolute methanol), freshly prepared.
7. Fix for at least 30 minutes (leave overnight if more convenient).
8. Centrifuge at 1000 RPM for 10 minutes, and aspirate the supernatant.
9. Add a few drops (up to about 0.5 mL for a large cell button) of 45% acetic acid. This step increases cell spreading.
10. Prepare one or more slides by the drying method. Simply place a drop of cell suspension on a clean slide, and allow the drop to run down the slide. Several drops may be placed side by side. Allow excess fixative to run off the bottom of the slide onto blotting paper. Dry slides in a slanted position.
11. When slides are thoroughly dry, stain them with Giemsa stain for 3–5 minutes. Rinse them quickly with running tap water, wipe off the backs, and air-dry them.
12. Examine at least 1000 cells. Be sure they are representative of the cells in the entire culture to be studied. Preferably make up slides of the entire specimen, and sample multiple locations on the slides. Record the number of metaphases per total number of cells (metaphases plus interphases), and express this as a percentage. Analysis is usually performed with a 40× high dry microscope objective.

III. Notes

1. **Modification for a small specimen:** Follow the preceding steps 1–5, making the volumes smaller as needed. Fill the narrow portion of a long Pasteur pipette (disposable, siliconized) with 50% acetic acid, and then, without releasing the bulb on the Pasteur pipette, draw up the cell pellet from the bottom of the centrifuge tube. Cells and fixative will mix in the wider portion of the pipette. Wait a short time and then express the fixative and cells onto clean slides as described under the preceding step 10.
2. **Modification with hypotonic treatment to increase cell spreading:** Follow steps 1–5 of the routine method for monolayer cells. Add at least 2 mL of 0.075 mol/L KCl at room temperature. Allow this to stand at room temperature for 15 minutes.

Protocol 4.21 Growth rate-estimation of mean population doubling time during logarithmic growth

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Method

1. Use **Protocol 4.17, Routine replication plating for human diploid cells**, for cell counts.
2. Select just-confluent cultures of diploid monolayer fibroblasts.
3. Inoculate 5×10^5 cells in 20 mL of complete medium in each of eight T-75 flasks, or 2×10^5 cells in 4 mL of medium in each of eight 60 mm Petri dishes. This cell inoculation should allow for at least two days in logarithmic growth before saturation density is reached. If too many cells are used, the cultures become confluent too soon; if too few cells are used, this type of culture will undergo a prolonged lag phase (little or no growth) prior to logarithmic growth.
4. Incubate at 37°C with CO₂, adjusted for proper pH.
5. Perform cell counts on duplicate flasks or dishes at 24-hour intervals from the time of replicate plating for a total of four intervals. The entire analysis takes five days.
6. Plot cell counts (vertical log scale) against time in culture (horizontal scale) on semi log paper. Include the initial number of cells at time = 0, and plot five intervals.

Interpretation

Cells are in log growth when a straight line can be drawn through three plots. Determine the time (horizontal scale) for the cell number to double (vertical scale) during log growth of the cells. This time approximates the mean population doubling time, which in turn approximates the mean cell cycle time.

Protocol 4.22 Maintenance of fibroblast cultures as non-mitotic population

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Method

1. Subculture a confluent monolayer fibroblast culture at a 1:4 split ratio, and grow it to confluence (about 1 week) in complete medium with 10–15% FBS.
2. Decant the growth medium, and replace it with medium containing 0.5% FBS. Incubate at 37°C with medium changes twice weekly.
3. Cultures can be arrested in this manner for as long as 6 months with complete recovery of proliferative capacity. However, such long-term storage is more efficiently maintained in liquid nitrogen (see chapter section 4.3.7, Freezing and storage of cell lines).
4. Recover cells from the nonmitotic state by subculturing them at a 1 : 2 split ratio into medium containing 10–15% fetal bovine serum.
5. Human fibroblast cultures can be maintained as monolayers at 37°C for 5–6 weeks with no handling at all. The medium becomes very acidic, but cells may still be retrieved by subculture after this time. When the pH of the medium becomes alkaline, retrieval is no longer possible.

Protocol 4.23 Synchronization at S-phase with BrdU

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Principle

This procedure synchronizes the growth of cell cultures in order to maximize the mitotic index at harvest. Both BrdU and Colcemid® are added together the evening before harvesting by arresting cells at prometaphase. Addition of thymidine the morning of harvest then releases the cell block on the arrested cells and allows cells to continue in the cell cycle in synchrony. Thus, most of the cells enter division at the same time. Careful timing for the addition of Colcemid® after the addition of thymidine is needed to capture these cells in prometaphase to metaphase.

II. Materials

1. BrdU solution: Dissolve 0.03 g BrdU in 10 mL HBSS. Allow dissolving completely and filter sterilize. Store at -20°C in 0.5 mL aliquots covered in foil or in a dark box.
2. Thymidine solution 10 mg/mL: Dissolve 100 mg thymidine in 10 mL PBS, place in incubator until completely dissolved. Filter sterilize. Store at -20°C covered in foil or in a dark box.
3. Colcemid® 1 : 10 solution: Add 0.5 mL of 10 µg/mL Colcemid® to 4.5 mL HBSS. Store at 5°C for 1 week.

III. Method

Select cultures the day before harvesting. Cultures should be growing well with a high mitotic index, but should not be too dense.

For coverslips

1. Using a micropipette, add 40 µL of BrdU and 80 µL 1:10 Colcemid® solution per 2 mL of culture media.
2. Incubate at 37°C for 13–16 hours.
3. Harvest according to coverslip harvest procedure (see Protocol 5.2 in Chapter 5).

For T-25 flasks

1. Using a micropipette, add 80 µL of both BrdU and 1:10 Colcemid® solution per flask.
2. Incubate at 37°C for 13–16 hours.
3. Harvest according to flask harvest procedure (see Protocol 4.12).

Protocol 4.24 Making direct FISH preparations from abortus tissue

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Principle

A small sample of each POC (products of conception) specimen received for chromosome analysis is reserved for FISH testing, which is only performed on those specimens that have not grown sufficiently for chromosome analysis after 14 days of culture. By this method it can be determined if aneuploidy exists for chromosomes 13, 15, 16, 18, 21, 22, X and Y in an otherwise unsuccessful analysis. These probes were chosen based on the incidence of these aneuploidies typically found in products of conception.

II. Materials

Preparation before FISH

1. Petri dish, 10 mm (VWR)
2. Sterile scalpels, disposable
3. Syringe, 1 mL
4. Pipettes (VWR)
5. PBS (Invitrogen): Store at room temperature. See expiration date on bottle.
6. Siliconized slides: double circle and one pre-labeled single circle
7. For MEM and collagenase preparation, see Protocol 4.7, Flask and coverslip setup for POC/fibroblast cultures.

III. Method

A. Specimen retrieval

1. Label 10 mm Petri dish with patient name and ID number.
2. Empty specimen from tube into Petri dish. Pipet off all liquid.
3. With 2 disposable, sterile scalpels, mince tissue thoroughly.
4. Add 1 mL (pre-aliquoted) POC collagenase.
5. Put in an open system incubator for approximately 1 hour. Time will depend on the breakdown of the tissue.
6. Label centrifuge tube with patient name and ID number.
7. Pipet all of specimen into the centrifuge tube, looking for clumps.
8. If clumps are found, break up by repeatedly drawing specimen into a 1 mL syringe.
9. Add 4 mL complete MEM medium for POCs to stop digestion.
10. Vortex centrifuge tube.
11. Centrifuge for 10 minutes at 800 RPM.
12. Refrigerate until ready to make slides.

B. Slide preparation

13. Aspirate off supernatant and add 5 mL PBS, resuspending the pellet.
14. Centrifuge for 8 to 10 minutes at 800 RPM.
15. Aspirate off supernatant, leaving enough PBS to create a cloudy suspension when pellet is resuspended. The amount of PBS left in the tube will increase as the size of the pellet increases.
16. Using a pipettor, spread 30 µL of specimen in each circle of two pre-labeled double circle siliconized slides and one pre-labeled single circle siliconized slide.

For the remainder of this procedure, see Chapter 16, Protocol 16.6 FISH on direct preparations from abortus tissue.

Protocol 4.25 Cryopreservation

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

I. Materials

Freezing medium: Add 5 mL (10%) sterile DMSO to 45 mL of either complete MEM 1640 or complete RPMI 1640.

II. Method

Preparation of cells for freezing

1. Build up cells by subculturing from backup dish to T-25 to T-75 flask. It is ok to use two T-25 flasks instead of subculturing them into a T-75.
2. Trypsinize one (1) T-75 flask or two (2) T-25 flasks following the subculture protocol (see Protocol 4.11, Routine subculture of fibroblast cultures).
3. Make up freezing media while flasks are in trypsin.
4. When cells are dislodged, add 10 mL complete medium to the T-75 flask (or 4.0 mL to each T-25 flask). Rinse flask by pipetting medium 2–3 times over entire surface of flask.
5. Pipet off the medium with the cells from the flask and place into a 15 mL centrifuge tube, labeled with patient information and date. Leave 0.5 mL of cell suspension in flask and add appropriate media to flask (for example, total of 10 mL for T-75 and a total of 4–5 mL for a T-25).
6. Keep this backup flask until the freeze process has been successfully completed.

Use of freezing chamber

1. Centrifuge the cell suspension at about 800–1000 RPM for 10 minutes.
2. Discard supernatant.
3. Add 6 mL freezing media to the cell pellet and resuspend well.
4. Dispense 1.5 mL cell suspension in each of 4 cryovials labeled with patient information and freeze date.
5. Dispense 1.5 mL of freezing media (without cells) into one cryovial labeled “Probe.”
6. Place “Probe” cryovial on holder in freezing chamber and hook up with the probe. Place your racks with cryovials in the freeze chamber and close door.
7. Hook up liquid nitrogen tank to freezing chamber.
8. Turn chart drive on and place a pen in the drive to record temperature changes.
9. Turn switch of freezing chamber to “start” and turn liquid nitrogen tank on. Set a timer for 10 minutes.
10. When timer goes off, turn switch of freezing chamber to “freeze ampules.”
11. After about 55 minutes, amber light indicating “end of freeze” comes on and an audible alarm sounds.
12. Turn switch to “unload.” Turn off liquid N₂ tank. Open chamber door and remove racks.
13. Turn switch to “off”.
14. Take top off liquid nitrogen storage tank and pull up one rack of boxes, then select box to be used and place vials in correct positions.
15. Remove 4th vial and thaw cells rapidly at 37°C in H₂O bath; using sterile technique place in 15 mL centrifuge tube and add 10 mL media. Centrifuge at around 800–1000 RPM for 10 minutes. Remove supernatant and resuspend pellet in 5 mL fresh MEM medium. Place suspension in a T-25 labeled with patient information on it.

Next day

16. Check test flask next day for confluency. If confluent, note in freezing record. Check culture again after 3 days for contamination. If culture is healthy, note in freezing record.
17. If culture is contaminated or did not grow, discard all 3 vials from liquid nitrogen storage tank. Go back to original T-25 and build-up and repeat protocol.

Safety

18. Use gloves and safety goggles when using DMSO. It is a skin and eye irritant.
19. DMSO is easily absorbed in the skin. In case of dermal contact, wash area with soap and copious amounts of water. In case of eye contact, rinse eye at eye wash station for 15 minutes.

20. Liquid nitrogen can cause terrible burns. Hand protection and goggles (not safety glasses) are to be worn when dispensing and handling liquid nitrogen.
21. Asphyxiation -- nitrogen is not poisonous; the air is already ~78% nitrogen (oxygen makes up about 21%, and trace gases the remaining 1%). However, if sufficient liquid nitrogen is vaporized so as to reduce the oxygen percentage to below 19.5%, you are at risk of oxygen deprivation. Rapid venting can cause near-total displacement of normal air, leading to a local concentration of about 100% nitrogen. Simple asphyxiants such as nitrogen do not have good warning properties!
22. Cryotubes containing samples stored under liquid nitrogen may explode without warning. Tube explosions are thought to be caused by liquid nitrogen entering the tube through minute cracks and then expanding rapidly as the tube thaws.

III. Additional reading

1. Verma RS, Babu A. *Human Chromosomes*. NY: Pergamon Press. 1989; 218–219.

Protocol 4.26 Cryopreservation with Nalgene cryogenic container

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

For Proper Use: Nalgene cryogenic controlled-rate freezing container is kept at 4°C before use. The chamber of the container is filled with isopropyl alcohol (IPA) and the vials are placed in the holder provided with the container. This will ensure slow freezing and preserve the viability of the cells. Make sure the level of IPA reaches line designating 250 mL. Each container can hold 18 vials.

I. Method

Two flask cultures will yield 4 vials of 1.5 mL/vial of frozen cells.

1. Transfer 50 mL of dense culture from flask to a 50 mL tube. Wash the remaining cells in the flask with room temperature RPMI 1640 and transfer to 50 mL tube. If volume of flask is more than 50 mL, separate culture into two 50 mL tubes of equal volume and centrifuge simultaneously. If continuing culture for DNA extraction, set a few mL of cells aside in the same flask.
2. Spin for 10 minutes at 1300 RPM.
3. Immediately discard the supernatant from the centrifuged cells.
4. Re-suspend the cell pellet in 5.4 mL RPMI 1640 + 20% FBS (should be cold). If you have 2 tubes, combine the cells into one tube.
5. Break up cell clumps, mix well, and transfer 10 µL of cells to a 0.5 mL tube for the cell count and viability determination. Keep rest of the cells on ice.
6. Determine the cell # and viability according to Protocol 4.18, Cell counting chamber method, and Protocol 4.19, Cell viability by dye exclusion. If cell count is more than 5×10^6 and viability is more than 85%, continue with cryopreservation.
7. Label 2 mL cryovials with alcohol-resistant lab marker, listing specimen ID, “LCL” if LCL, date of cryopreservation.
8. Add 0.6 mL of DMSO to the cells and mix well. Aliquot 1.5 mL/cryovial.
9. Immediately transfer cryovials in Nalgene cryogenic controlled-rate freezing container (see Note 1, *Liquid nitrogen dangers* and Note 2, *Asphyxiation*).
10. Place the container in -70 °C immediately. Freeze overnight.
11. Transfer the vials to liquid nitrogen storage tanks within 24–72 hours (see Note 3, Explosive warnings).
12. Record vial location in liquid nitrogen log book.
13. Enter vial location, cell count and viability values.

Retrieving cells from storage

NOTE: Cells which have been preserved and frozen in media containing dimethyl sulfoxide (DMSO) should be thawed rapidly and cultured. The cryopreserving media must be thoroughly removed before culturing the cells.

1. Warm complete media to room temperature prior to use.
2. Label a T-25 flask and a 15 mL centrifuge tube for each cell line to be thawed.
3. Remove the vial of cryopreserved cells from the freezing tank and thaw rapidly in the 37°C water bath.
4. Using a sterile pipette, transfer the cell suspension from the vial to a labeled 15 mL sterile centrifuge tube.
5. Add 10 mL of complete MEM to centrifuge tube. For lymphoblasts, add 10 mL of complete RPMI to centrifuge tube.
6. Centrifuge the cell suspension at 800 RPM for 10 minutes.
7. Aspirate off the supernatant and discard.

8. Gently resuspend the cell pellet in 4 mL of complete medium. For lymphoblasts, resuspend cell pellet in 2 mL RPMI.
9. Transfer to a properly labeled T-25 flask. If the cell pellet is large enough, it can be split into two T-25 flasks.
10. Place in the 37°C incubator set at 5% CO₂.

Evaluation

11. After 24–48 hours, check for cell attachment.
12. If there are many cells attached, change medium.
13. If attached cells are sparse, change only 1/2 the medium.
14. Flask should have media changed regularly.
15. When the flask shows active growth and nears confluence, it may be subcultured to other flasks or to coverslips for *in situ* culture and harvest. (See Protocol 4.11, Routine subculture of fibroblast cultures.)
16. Lymphoblast cultures should only be fed when media is yellow.
17. All information concerning the storage and reanimation of samples is written on the Freeze records form.

Important safety notes

1. **Liquid nitrogen dangers.** Liquid nitrogen can cause terrible burns. Hand protection and goggles (not safety glasses) are to be worn when dispensing and handling liquid nitrogen.
2. **Asphyxiation.** Nitrogen is not poisonous; the air is already about 78% nitrogen (oxygen makes up about 21%, and trace gases the remaining 1%). However, if sufficient liquid nitrogen is vaporized so as to reduce the oxygen percentage to below 19.5%, you are at risk of oxygen deprivation. Rapid venting can cause near-total displacement of normal air, leading to a local concentration of about 100% nitrogen. Simple asphyxiants such as nitrogen do not have good warning properties!
3. **Explosive warnings.** Cryotubes containing samples stored under liquid nitrogen may explode without warning. Tube explosions are thought to be caused by liquid nitrogen entering the tube through minute cracks and then expanding rapidly as the tube thaws.

Protocol 4.27 Lymphoblastoid lines

Contributed by Nirupama Masse and Stephanie Sherman, Emory University School of Medicine, Atlanta, GA

Handling safety for Epstein-Barr virus

Before you begin processing blood, please read the following.

Caution:

1. Always wear a lab coat and gloves when handling blood, Epstein–Barr virus (EBV) or growing tissue culture flasks.
2. Entire procedure is processed in **Biological Safety Hood** using sterile techniques. Biological Safety Hood should be running for at least 10–15 min. before you begin to use it. Wipe the surfaces first with Conflik® and then with 70% alcohol before use.
3. Assume that all blood we receive is infected with HIV. Clean up any spill with 10% bleach, followed by 70 % alcohol. (Bleach will rust the stainless steel surface).
4. Discard all used pipettes and supernatants in 10% bleach solution in red bucket. After an hour transfer these pipettes to a biohazard container kept by the side of Biological Safety Hood. At the end of the day discard the decontaminated liquid waste in the sink and let the water run for 5 minutes.
5. Biohazard bags when full are transferred to incineration boxes.
6. All blood vacutainer and tips should be placed in the biohazard sharps container kept in sterile guard hood. When this box is full, it should be placed in the biohazard bag box.
7. Keep the door of the tissue culture room closed while working in the hood to maintain the air flow in the room.

Epstein–Barr virus culture

I. Principle

Epstein–Barr virus (EBV) is a lymphotropic herpes-virus. Epstein–Barr virus has a double-stranded DNA genome consisting of 172,000 base pairs which is linear in virus particle, and exists as a circular episome inside the nucleus of infected cells. In vitro, Epstein–Barr virus can selectively convert B-cell lymphocytes into a continuous lymphoblastoid cell line.

Transforming virus can be obtained from marmoset cell line B95–8 (no longer available from ATCC). EBV binds selectively via B-cell surface receptors. Following EBV exposure, B-cell lymphocytes undergo lymphoblastoid transformation.

- Cryopreserved stock of EBV is maintained in liquid nitrogen.
- 10–15 vials are available at all times for culture.
- 2 flasks of active EBV cultures are maintained at all times for transformation needs. Unless contaminated, these flasks can be maintained on a regular feeding schedule for at least one year.
- In January of every year, a new aliquot of EBV is started from cryopreserved cultures of B95–8.
- 50–100 mL of virus-saturated supernatant is filtered and kept in 5 mL aliquots at –70 °C. This can be used if the culture gets contaminated and lymphoblast cell lines (LCLs) need to be set up while you are growing fresh culture.
- Flasks with EBV culture are labeled as EBV Monday and EBV Friday. These flasks are split once a week on Monday and Friday, respectively. The supernatant is kept at 4 °C. EBV is active for a week at this temperature. Half of the culture is removed and kept for EBV use and flask is replenished with equal volume of complete medium. Usually 25–30 mL is removed and 25–30 mL is added.

Preparation of EBV for transformation

For Monday–Thursday LCL setup, use Monday EBV flask.

For Friday–Sunday LCL setup, use Friday EBV flask.

1. Remove 25–30 mL of EBV supernatant and transfer to a 50 mL tube. Centrifuge at 1300 RPM for 10 min.
2. With a 20 mL syringe, aspirate 15–20 mL EBV supernatant. Attach a 0.45 µm syringe filter to the syringe. Gently push the EBV supernatant through and collect it into a 50 mL tube. Use 2 mL/T-25 flask setup. Keep the unused portion at 4 °C.

II. Materials

Media preparation

Complete media preparation

500.00 mL	RPMI 1640 with L-glutamine
90.00 mL	FBS (Heat Inactivated) by Invitrogen
3.00 mL	Penicillin/Streptomycin
3.00 mL	Amphotericin B
0.12 mL	Gentamicin sulfate
596.12	Total Volume

Complete media is to be used for initial LCL setup and first 3–4 weeks thereafter, or until the cell line is established.

Bovine growth serum (BGS) growth media preparation

500.00 mL	RPMI 1640 with L-glutamine
90.00 mL	Bovine growth serum by Hyclone
3.00 mL	Penicillin/Streptomycin
3.00 mL	Amphotericin B
0.12 mL	Gentamicin sulfate
596.12	Total Volume

BGS growth media is to be used once the cells are transferred to T-75 flasks and the cell line is established.

Materials for cryogenic process

- Cryogenic storage boxes
- Racks to hold cryovial tubes
- Liquid nitrogen tanks (Dewars)

Reagents and consumables

Product	Source	Order #	Storage	Expiry
DMSO Dimethyl Sulfoxide	Sigma	D2650	ambient	See bottle
RPMI 1640	Invitrogen	11875-093	2° to 8 °C	See bottle
FBS	Irvine Scientific	3000	-20 °C	See bottle
Nunc Cryovials	VWR	66021-986		N/A
15 mL polypropylene centrifuge tubes	Fisher	430052		N/A
Liquid Nitrogen	Specialty Gas	168 or 209		N/A

- Freezing media – 45 mL 20% FBS complete RPMI 1640 media and 5 mL DMSO

III. Method

Determining blood volume for transformation

If the blood volume is between 4.0 and 5.0mL

1. Transfer the blood to a 15-mL tube.
2. One circle of dry blood spot is made on the blood blotting card. Affix the label on the card. Allow it to dry for at least 30–60 min. before transferring to envelope.
3. Transfer 0.5 mL of blood to a 2 mL Eppendorf tube. Follow the *Processing 0.5 mL whole blood for DNA* protocol for this tube.
4. Transfer 1 mL of blood to a cryovial for -80 °C freezer storage.
5. Spin the 15 mL tube at 1800 RPM (666g) for 15 min., program # 3 (or at 4500 RPM, 3300 g, for 5 min. program #7).
6. Without disturbing the blood layer, remove plasma layer from the top and discard. Remove the buffy layer (1–1.5 mL) and transfer to a 15 mL tube.
7. Fill the tube with PBS to the 15 mL mark and mix well.
8. Proceed to Transformation of lymphoblasts with Epstein–Barr virus.

If the blood volume is between 2.5 and 3.5mL

1. Transfer the blood to a 15 mL tube.
2. One circle of dry blood spot is made on the blood blotting card. Affix the label on the card. Allow it to dry for at least 30–60 min. before transferring to envelope.
3. Transfer 0.5 mL of blood to a 2 mL Eppendorf tube. Follow the *Processing 0.5 mL whole blood for DNA* protocol for this tube.
4. Spin the tube at 1800 RPM (666g) for 15 min., (or at 4500 RPM, 3300 g for 5 min., program #7).
5. Without disturbing the blood layer, remove plasma layer from the top and discard. Remove the buffy layer (1–1.5 mL) and transfer to a 15-mL tube.
6. Fill the tube with PBS to the 15 mL mark and mix well.
7. Proceed to Transformation of lymphoblasts with Epstein–Barr virus.

Transformation of lymphoblasts with Epstein–Barr virus

Setup

1. Transfer blood into 15 mL centrifuge tube and spin for 15 minutes at 1800 RPM (room temperature). If the amount of blood is less than or equal to 2mL, go to step 2.
 - a. If specimen is for epimerase, remove the plasma and place in a cryotube labeled with patient information and place in freezer (-20 °C). If the specimen is not for epimerase, remove the buffy layer; take ~2 mL total, including some red blood cells (RBC) and transfer to a new 15 mL centrifuge tube.
 - b. If specimen is for epimerase galactosemia testing, label the remaining RBC pellet “pre-Ficoll” and save at 4 °C for biochemical genetics. The specimen must be delivered ASAP.
2. Add warm PBS to the buffy layer of cells to bring the total volume to 6 mL.
3. Gently pipet 6 mL of diluted buffy layer cells into a tube containing 3 mL of pre-warmed Ficoll–Paque.

4. BE GENTLE and pipet VERY SLOWLY so as not to disturb the interface between the Ficoll–Paque, which must stay on the bottom, and the blood, which must stay on the top.
5. Spin at room temperature for 30 minutes at 2000 RPM.
6. After the spin, a distinct white cell layer should be visible in the tube.
 - a. Carefully remove the material above this layer and discard.
 - b. Then remove the white cell layer (~2 mL) and transfer to 15 mL centrifuge tube.
 - c. Remove any leftover material above the RBC pellet and discard.
 - d. If specimen is for epimerase galactosemia testing, label the remaining RBC pellet “post-Ficoll” and save at 4°C for biochemical genetics.
 - e. This specimen and the pre-Ficoll specimen must be delivered to Biochemical Genetics ASAP.
7. Add pre-warmed PBS to white cells to a final volume of 12 mL, and gently mix by pipette.
8. Spin at room temperature for 10 minutes at 1300 RPM.
9. Pour off supernatant and resuspend cell pellet in 12 mL of prewarmed RPMI 1640.
10. Spin at room temperature for 10 minutes at 1300 RPM.
11. Pour off supernatant and resuspend cell pellet in 2 mL pre-warmed RPMI 1640 complete with 20 µL PHA added.
 - a. If the blood volume is less than 100 µL, transfer the entire amount of blood to a T-25 flask. Rinse the blood vial with 2 mL of complete media and place in same T-25. Add 20 µL of PHA and 1 mL of freshly thawed EBV. Place in incubator (37°C, 5.5% CO₂) and leave for 7–10 days before first feeding.
 - b. If blood is clotted, transfer clot to a 100 mm Petri dish. Mince clot very finely by using disposable scalpels and rinse the clot and Petri dish in 3 mL increments. Place cell wash in a 15 mL centrifuge tube (may require more than one centrifuge tube, depending on the number of washes). Centrifuge all wash tubes at 1300 RPM for 10 minutes. After spinning, combine cells from all tubes with buffy layer from step 1 and proceed to Ficoll–Paque separation.
12. Transfer to a T-25 flask and add 1 mL of freshly thawed EBV.
13. Place in incubator (37°C, 5.5% CO₂) and leave for 7–10 days before first feeding.

Culture maintenance

14. The first 7–10 days after setup, leave the flasks in the incubator undisturbed.
15. After 7–10 days, if the culture medium is yellow, feed the culture 0.5 mL of complete media and return to incubator. Use the inverted microscope to see that the cells are transformed (blastic). Blastic cells will appear to be a clump of cells under the microscope.
16. Check flasks every week. If the media is yellow, begin increasing the amount of media by 1 mL. At week 3 and 4, increase media to 2 mL and 4 mL, respectively. Once the culture has reached 10–15 mL, transfer the culture from a T-25 to a T-75 flask. Save the T-25 flask to use for freezing the culture.
17. Once the culture has been transferred to a T-75, feed the cells every 3–4 days as long as the media is yellow. Cells in a T-75 will require at least 10–15 mL of complete media every 3–4 days. If the population is doubling more often, feed the cells 20–25 mL of complete media.
18. DO NOT OVERFEED or keep concentration lower than 2×10^5 cells per mL. If the medium isn't turning yellow, the cells are not hungry. It is ok to skip a media addition until the culture turns yellow.
19. Once you have a healthy culture growing (yellow with lots of cell clumps), cryopreserve 4 vials at no less than 5×10^6 viable cells per mL.

Cell counts

Total counts needed:

For Freeze: at least 5×10^6 viable cells.

1. Do a 1 : 2 dilution of cell suspension: trypan blue stain.
2. Load ~10 µL of the suspension into the hemocytometer. Do not overfill the chamber. Use a phase microscope to count the viable cells (appear bright under scope, dead cells are blue) that are contained in the 4 squares (16 smaller squares are bound by 3 lines creating one larger square) of the hemocytometer.
3. Take the average of the 4 counts and multiply by 2 (dilution factor). Multiply your answer by 10^4 (conversion factor for area). The result is the number of viable cells per mL.

Cryotube preparation

- When cultures are healthy, perform cell counts to determine the concentration of cells per mL.
- Once concentration is determined, aliquot at least 5×10^6 cells in a freezer vial and dilute with freezer media for a final volume of 1.0–1.4 mL.
- Discard the supernatant and resuspend each pellet in 4 mL of freeze media. If you are freezing more than 4 vials per case, remember to use enough media to provide 1.0–1.5 mL of suspension for each vial, at a concentration of at least 5×10^6 viable cells, and place in the appropriate number of centrifuge tubes.
- Centrifuge the cell suspensions at 800–1000 RPM for 10 minutes.
- Dispense 1.0–1.5 mL of cell suspension into an appropriately labeled cryotube. Follow with cryopreservation of cells protocol.

Recovery from cryopreservation

Note: Select the location of removal of LCL vial from liquid nitrogen tank. If you see that there are 2 or less vials in the liquid nitrogen storage, **do NOT remove the vial. Inform cell culture personnel and they will grow LCLs for your needs and will also cryopreserve 4 vials of cells.**

Warm up RPMI 1640 and complete media in 37°C water bath for 15–20 minutes before starting the procedure.

- Fill a 15 mL centrifuge tube filled with 10 mL RPMI 1640.
- Remove one vial of desired cells from the liquid nitrogen tank. Note the vial removal in the database and liquid nitrogen record book.
- Thaw the vial in 37°C water bath.
- Immediately** transfer the cells to a 15 mL centrifuge tube filled with 10 mL RPMI 1640. Rinse the cryovial and collect all the cells in the centrifuge tube. Add RPMI 1640 to fill the tube completely. Mix gently by inverting the tube 2–3 times.
- Centrifuge at 1300 RPM (300 g) for 10 min.
- Re-suspend the cells in 5 mL of complete medium. Set aside 10 µL for cell count and viability determination.
- Transfer the cells in a T-25 flask.
- Determine the cell count and viability according to Protocol 4.18, Cell counting chamber method and Protocol 4.19, Cell viability by dye exclusion.
- Document the original as well as current cell count and viability on the cryo recovery progress report.
- Keep track of culture growth by checking every 2–4 days.
- Feed the cells at regular interval or as needed.

Protocol 4.28 Freezing tissue cultures (cryopreservation)

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

Cells normally die when exposed to freezing temperatures because ice crystals rupture cell membranes. Cells can survive the freezing process if ice crystals are prevented from forming. Solutions of 10% glycerine or 5–10% DMSO can prevent such ice from forming, and make freezing tissue cultured cells possible. Cells also recover from the freezing procedure if they are frozen down gradually (minus 1–1.5°/minute) and kept at very cold temperatures (-198.5°C). At very low temperatures, cultures last indefinitely and can be defrosted and re-grown.

Note: Specimens should not be retained/stored without proper informed consent. Occasionally, cultures for a specimen that is delayed in testing and/or reporting may be frozen down temporarily to insure availability of viable cells. However, once the case is completed, the vials should be discarded, unless there is proper informed consent for storage.

DMSO is an irritant and is flammable. Liquid nitrogen (LN₂) is -195.8 °C and can cause severe burns on contact. Wear laboratory coat, protective eyewear and heavy gloves. Do not use liquid nitrogen with open-toed shoes or bare legs.

II. Materials

See Protocol 4.3, Tissue culture reagents, for prenatal tissue culture reagent protocol.

- Trypsin-EDTA, 1×, shelf life 1 day.
- Hanks' 1× BSS, shelf life 1 month.

3. Ham's F10 or MEM complete medium (see Protocol 4.9, Solid tissue (fibroblast) culturing and harvesting, for fibroblast methods), shelf life 2 weeks.
4. Dimethyl sulfoxide (DMSO) Hybri-Max, Sigma. Store at room temperature. Shelf life indefinite.
5. Corning 2mL cryogenic vials (ampules).
6. Nalgene Cryo Freezing Container.
7. Freezing medium: 0.5–1.0mL of DMSO added to 9mL complete medium in a sterile, screw cap centrifuge tube. Shelf life 1 day.

III. Method

Freeze at least 2 ampules per patient. Each ampule holds cells from one (1) **confluent** T-25 flask. One (1) T-75 can be frozen in 2 ampules.

1. Rinse flask twice with 4mL Hanks' 1× solution, discarding rinses.
2. Trypsinize cells off flask with 1mL trypsin-EDTA 1× solution at 37°C (monitor for detachment on inverted phase microscope).
3. Transfer cell suspension to a sterile, plastic screw-cap centrifuge tube.
4. Rinse flask with 4mL tissue culture medium and add to cell suspension.
5. Centrifuge at 1000 RPM for 10 minutes. Sterilely aspirate fluid.
6. Resuspend pellet in 1–2mL of fresh freezing medium (amount of freezing medium depends upon size of cell pellet).
7. Put cell suspension into freezing ampules, 2mL per ampule.
8. Place ampules in the Nalgene Freezing Container, to which 250mL of isopropyl alcohol has been added (up to line on side of container). This alcohol must be changed every 5th usage of the container.
9. To achieve a -1°C/minute rate of cooling, place the container with the ampules into a -80°C freezer for >4 hours (may be left overnight).
10. Using appropriate eye and hand protection, place into LN₂ freezer.

IMPORTANT!!! When placing ampules in LN₂, it is extremely important to secure the freezing boxes in each rack by replacing the metal pin in the rack when you are finished!

11. Record information about the cells frozen down in Tamtron notes field and LN₂ notebook, including name of patient, GL or other identifying number, date frozen, location in freezer, and diagnosis and cell type.

Defrosting frozen tissue cultures

Safety warning: Ampules are subject to shattering if a leak has allowed liquid nitrogen to enter the vial. Wear protective eyewear.

1. Locate and remove the ampule(s) to be defrosted.
2. **IMPORTANT!!!** When removing ampules from LN₂, it is extremely important to secure the freezing boxes in each rack by replacing the metal pin in the rack when you are finished!
3. Immediately swirl ampules in a 37°C water bath. Avoid getting the top wet.
4. Add cell/freezing medium suspension to a 15mL screw-cap centrifuge tube. Add to the tube 10–12mL of complete medium. Centrifuge at 1000 RPM for 10 minutes.
5. Remove supernatant completely and resuspend in fresh medium for culture in tissue culture flasks. (Optional: rinse pellet with medium before plating to be sure all freezing medium is removed.) Cells should attach within hours of plating.

CHAPTER 5

Prenatal chromosome diagnosis

Kristin M. May¹, Debra F. Saxe² and Jean H. Priest²

¹*Children's Hospital at Erlanger, Chattanooga, TN, USA*

²*Emory University School of Medicine, Atlanta, GA, USA*

5.1 Introduction

Of the numerous medical applications of cytogenetics, prenatal diagnosis by chromosome and FISH analysis to detect fetal abnormalities may be the most critical because a family's decision to continue a pregnancy is often determined by the test result. Thus, laboratory personnel involved in sampling, transport, culture, and analysis of prenatal specimens must appreciate the consequences of technical error. The importance of speed and accuracy in prenatal cytogenetic diagnosis should limit these procedures to laboratories with superior facilities and technical expertise. Strict adherence to established laboratory protocols is critically important. A good background in cell biology, cytogenetics, and cell culture is essential for individuals working in the field of prenatal diagnosis. Laboratories wishing to offer chromosome analysis from amniotic fluid or chorionic villi should first gain experience with the method by successfully performing analyses on at least 50 specimens concurrently with another laboratory [1].

5.2 Amniotic fluid

5.2.1 Amniocentesis

Transabdominal amniocentesis for genetic testing is usually performed at 15–20 weeks of gestation. It can be done earlier but should not be performed earlier than 14 weeks of pregnancy because of the higher risks for complications and pregnancy loss [2–4]. Amniocentesis may also be performed later in pregnancy for various medical needs. With the aid of ultrasound, information is obtained regarding pregnancy age (usually by fetal biparietal diameter or crown–rump length), position of the placenta, size of the amniotic pool, presence of twins, and existence of fetal anomalies.

In multiple gestations the number of samples taken depends upon the zygosity of the fetuses (monochorionic versus dichorionic), location of sac or sacs, position of fetuses and relative risk for each fetus. A single sample may be obtained for mono-chorionic (monozygotic) twins even if two amnion sacs are present. Each fetus is identified, for example, as "A" or "B", at the time of the procedure. That designation is added to the patient name to identify each amniotic fluid sample and must be carefully transferred to all paperwork and culture labels at the laboratory as a mix-up could result in a devastating error.

5.2.2 Amniotic fluid characteristics

Amniotic fluid is contained within the amniotic sac surrounding the fetus. The fluid is produced by transfer of fluid across the placenta, membranes and fetal skin in the first trimester, then by fetal swallowing and urine production in the second and third trimesters. For normal singleton pregnancies the fluid volume increases over the course of the pregnancy. The maximum fluid volume is obtained early in the third trimester, although one study found a peak near term [5,6].



Figure 5.1 Characteristic fern-shaped crystals from amniotic fluid. Courtesy of Dr. Kristin May.

Amniotic fluid consists of a fluid and a cellular component, which are separable by low-speed centrifugation (500 g; about 1000 RPM). The cells form a pellet, leaving fluid that is usually straw-colored. Certain biochemical characteristics, notably protein, pH, and glucose, distinguish amniotic fluid from urine. Commercially available test strips can be used to assess these parameters. Amniotic fluid is alkaline and positive for protein and glucose. Urine is unlikely to have all of these characteristics and usually has none of them. A fern test can also be useful in distinguishing between amniotic fluid and urine. A small amount of the specimen is applied in a thin film on a microscope slide and air dried. Amniotic fluid will generally form fern-like crystals as it dries, whereas urine usually does not (Figure 5.1). The physician obtaining the fluid should be notified immediately if the specimen is initially thought to be urine or another body fluid. The specimen is nevertheless cultured and the outcome reported, as appropriate.

Amniotic supernatant fluid is used for additional studies such as alpha-fetoprotein (AFP) and acetyl cholinesterase assays for open neural tube defects in the fetus. Storage of a frozen aliquot of amniotic fluid allows for a repeat AFP assay or other testing as needed at a later date.

5.2.3 Cells in amniotic fluid

The cell pellet from low-speed centrifugation of whole amniotic fluid can be separated into at least three cell types in culture. These differ in cellular and clonal morphology and in growth potential. A limited number of cells in an amniotic fluid sample are capable of growth into colonies. Estimates of the number of colony-forming cells per milliliter of fluid in the second trimester range from 2 to 16 [7–9]. The following are the three common classes of amniotic fluid cultured cells: fibroblast-like (F) cells, epithelioid (E), cells and amniotic fluid (AF) cells or amniocytes [10] (see Chapter 4, Figure 4.1).

AF cells (amniocytes) predominate in most cultures at the time they are processed for chromosomal diagnosis. E cells usually have the shortest life span, divide slowly, and resist subculture following trypsinization. F cells grow more slowly than AF but have the longest growth potential, 50 or more population doublings. The average life span of serially cultured AF cells is much less and usually does not exceed two or three passages at 1:2 splits.

In addition to the three classes of fetal derived cells just mentioned, approximately 1% of cells in amniotic fluid are pluripotent stem cells that have characteristics of both adult and embryonic stem cells [11,12]. Maternal cells and fetal blood cells may also be present initially in the amniotic fluid as a result of vascular leakage or biopsy of maternal tissue at the time of amniocentesis. Maternal macrophages, if present, attach within hours of setup but fail to divide under usual culture conditions and cannot be subcultured. Rapidly adhering (RA) cells, a mixture of macrophages and cells of neural origin, are often observed in fluid obtained from a fetus with a neural tube defect but may also appear in cultures from normal fetuses. The RA cells attach 24–48 hours after culture initiation but may not divide.

5.2.4 Specimen size

A sample of about 20 mL, or less in the case of an early amniocentesis, is withdrawn. This amount of fluid generally contains sufficient viable cells to establish multiple cultures for analysis. More fluid may be removed if additional cells are needed for molecular or biochemical assays. The volume of fluid lost by sampling is replaced rapidly.

To decrease the chance of maternal cell contamination (MCC), the first several milliliters of specimen should be placed into a separate, labeled container by the physician performing the procedure. This container should be clearly noted as the first draw, should be cultured separately from the remaining specimen, and should be used only if absolutely necessary. **The physician may also choose not to send the first draw to the laboratory.**

Smaller sample volumes may result from an abnormally low amniotic fluid level (oligohydramnios) or difficulties in the procedure. A smaller volume of fluid may necessitate the establishment of fewer cultures, and in the case of an extremely small sample (less than 10 mL), the physician should be notified that a partial analysis may result. Owing to the nature of the sampling procedure and the risk involved to the patient, no specimen should be rejected on the basis of small sample size.

5.2.5 Collection container

Containers for collection of amniotic fluid must be sterile and free of any chemicals known to be toxic to human cells, such as rubber stoppers. Problems in the manufacturing process of collection syringes have been periodically incriminated in poor culture growth, particularly if the amniotic fluid specimen was transported in the syringe [13,14]. Receipt of a specimen in an unusual container should be noted. Prompt analysis to determine the etiology of consecutive culture failures for amniotic fluid cases should include investigation into the type of collection syringe and transport container, if different from the collection syringe.

5.2.6 Specimen transport

Extremes of temperature should be avoided and transport time kept to a minimum. Same-day or next-day setup is recommended. Because components within the amniotic fluid may begin to degrade, specimens should not be subjected to prolonged light exposure. Appropriate biosafety precautions must be used in handling and transporting amniotic fluid. Shipping materials must provide protection for the specimen container from the elements and from damage. In addition, the shipping containers must meet any applicable governmental regulations for safety.

5.3 Culture of amniotic fluid

5.3.1 Open versus closed system for culture

Preferences of the laboratory director or technical personnel will determine whether to grow AF cultures in flasks and/or in situ (on coverslips in Petri dishes or on slide chambers). By definition, Petri dishes are used in an open system. Culture flasks can be used in open or closed culture conditions because the two-position caps allow or block gas exchange. Vented caps for flasks are also available to allow gas exchange. Laboratory staff experienced with both open and closed methods should be able to select the method more appropriate to the procedures used. Petri dishes and slide chambers are maintained in an air flow incubator (open system) that is also humidified in order to avoid evaporation of media.

Most clinical laboratories in the United States use coverslips to grow amniocytes. Experience has shown that these cells grow faster in an open system, particularly in a tri-gas incubator [15] (also see Chapter 4, Temperature, pH, CO₂, and humidity control). A closed culture system may be associated with less contamination and fewer accidental failures than an open system. Some consider closed cultures more troublesome than open systems because each vessel must be gassed to the correct CO₂ and O₂ concentration and closed tightly. A loose cap in a non-air flow incubator can cause culture failure because of the alkaline pH of the medium. On the other hand, flasks in a gas flow incubator may not receive good gas exchange because the caps are not loose enough. These comments assume that a bicarbonate buffer system is used. Medium containing an organic buffer (HEPES) does not depend on gas exchange and CO₂ concentration for control of pH.

Regardless of the type of culture vessel used, cells cultured from amniotic fluid grow best at 37–37.5 °C. If the temperature is lowered because of frequent handling of cultures, the growth rate is slowed. The amount of time that cultures are removed from the incubator when examining them for growth or performing culture maintenance work should be kept to a minimum. Even during brief trips out of the incubator, flasks or Petri dishes should be kept on a 37 °C warming plate when not in immediate use to avoid cooling the cultures.

5.3.2 In situ versus flask growth methods

For the most commonly used in situ method, cells are grown on a sterile square microscope slide coverslip (22 mm × 22 mm) placed inside a small Petri dish. The in situ method refers to the fact that the architecture of cell colonies is maintained throughout the harvest process because the cells are harvested “in place” on the coverslip, and in the flask method, cells are removed from the culture vessel for harvesting.

Preserving the individual colonies in the in situ method provides more information for excluding mosaicism and for distinguishing between pseudomosaicism and true mosaicism [16]. Fewer cells need be analyzed for a case from coverslips than flasks to exclude an equivalent level of mosaicism with the same degree of confidence [17]. All of the factors which influence the quality of any type of chromosome preparation (hypotonic solution and fixative in harvesting; temperature, humidity, and air flow in slide-making) are critically important in coverslip harvesting, because once the coverslip is harvested and dried, there is no opportunity to improve it. Cells growing on coverslips can be dispersed or subcultured using trypsin to seed more vessels, if additional cells or slides are needed.

In contrast, cell preparations from flasks, as for blood or bone marrow harvests, will yield multiple slides, and additional harvests can be performed later from the cells remaining in the flask. This is one reason why many laboratories set up both coverslip *and* flask cultures on *all* specimens. If the technologists are skilled at drying coverslips, the in situ method is usually more efficient than the flask method. A laboratory should, with practice, be able to achieve quality chromosome preparations with both methods.

5.3.3 Media and additives

Amniotic fluid cells will grow in many types of media defined for mammalian cells. For optimal growth, a medium defined specifically for amniotic fluid cell culture is generally the best. Commercially prepared media contain essential vitamins, amino acids, salts, glucose, and a buffering system to maintain appropriate pH (see examples of media in Chapter 4, section 4.2.5, Media). Medium can be classified whether it is “complete” or “basal”, the latter requiring the addition of supplemental component(s) before use. Complete medium, whether purchased as such or prepared in the laboratory, includes the following:

- L-glutamine: an essential amino acid that is unstable at the pH used for cell culture. It should be stored in separate, frozen aliquots and added to medium just before use. Some complete media do not require supplementation as they contain the amino acid in a more stable, dipeptide form.
- Growth factor supplements: included in medium by the manufacturer or added at the time of use. Fetal bovine serum is the most frequently used form of supplement. It should be shipped frozen, thawed once, aliquoted, and stored frozen. Complete medium containing fetal bovine serum is usually stored no longer than 10–14 days at refrigerator temperature. A new lot of serum should be tested for its ability to support growth of test cells prior to use of the serum in medium for new cultures. If the serum is already screened for mycoplasma, routine heat inactivation should not be necessary.
- Antibiotics: used for primary cultures and recommended for setting up amniotic fluid cultures. The reasoning in this situation is that antibiotic treatment can prevent growth of microorganisms present in the primary tissue or acquired when the specimen is obtained. If bacterial contamination is suspected, it is usually a good idea to harvest as soon as possible.

5.3.4 Number of cultures per specimen

At least two cultures, and usually more, are established routinely, and a minimum of two are examined for chromosome analysis, either by the in situ or flask (whole-culture) method. Exceptions include unusually small specimens (less than 5 mL). Many laboratories devise a formula for the number of cultures to establish based upon the amount of fluid received and/or the size of the cell pellet after centrifugation.

Each specimen should be established in at least two parallel growth systems, each receiving different media or different lots of media, and the cultures should be split between two independent incubators. This will help insure that in case of a power or gas supply failure, some cultures are likely to survive.

In addition, each case should be triaged with respect to the referring diagnosis, gestational age, and the number and nature of other tests ordered. An order for a molecular or biochemical test requiring cultured cells indicates that the first priority should be to achieve confluent cultures for that testing. These cultures may need to be more heavily seeded with fewer cultures initiated or with some cultures receiving fewer cells. Chromosome analysis in these cases may take a few additional days for completion on subcultured or in situ cultures that were seeded more lightly. Third trimester specimens may have larger cell pellets but often contain a larger number of nonviable cells. Cultures should be seeded more heavily in these cases as the dead cells will not attach and can be washed off during media changes.

5.3.5 Number of days to first culture check

The time to the first check depends on the method of culture, the method of processing, and the number of cultures set up and maintained. If special culture morphology is being studied, such as the presence of rapidly adhering cells in cases of fetal neural tube defect, culture morphology will need to be monitored almost daily at the onset. Frequent routine examination of the cultures will slow down growth. Waiting too long to examine cultures, however, might cause the examiner to miss contamination or incorrect pH. A quick check for these last two problems may be accomplished best at the time of first medium addition or change. In general, growth should be evident in 4–5 days after setup.

5.3.6 Medium addition or changes

Cells are suspended in a minimum amount of medium and are carefully placed on in situ coverslips in order to maximize their attachment to the coverslip surface rather than the underlying Petri dish. Inoculants, therefore, should contain no more than 0.5 mL to avoid spilling into the Petri dish. Additional medium is added (“flooding”) 1–2 days after culture setup, and medium changes are performed 3–4 days later and every 3–4 days thereafter.

A common plan for flask cultures is to wait at least 3 days before making a half-medium change, and then follow with half-medium changes twice a week. A complete medium change, with a rinse first, may be indicated if debris or red blood cells are present. Cultures for special studies or problem cultures are always exceptions. These may require more frequent feedings.

After completion of a case, all backup cultures should be kept for a minimum of 1 week in a holding incubator before being discarded, in case additional testing is requested. During this time, routine medium changes can be omitted.

5.3.7 Treatment of precipitation in cultures

The cause of precipitation determines the treatment. Therefore, a first step is to diagnose the possible causes. Various possibilities include solid materials in the amniotic fluid itself that come down with the cell pellet and are inoculated into culture; red blood cells that break down in culture; precipitate in culture medium, particularly if alkalinity develops prior to use; precipitate in the fetal bovine serum; unclean culture containers or pipettes; and contamination. If the cause of debris cannot be determined or prevented, the best treatment is to rinse the layer of cells with medium or balanced salt solution, aspirate, and refeed with complete medium.

5.3.8 Treatment of microbial contamination

Cloudiness of the medium in cultures or a change in pH, usually acidic, are indications of microbial contamination. As a general rule, the cultures should be harvested for chromosome analysis as soon as possible or decontaminated and discarded. Several methods for antibiotic treatment may be worth trying if no backup cultures are available. However, a decision to repeat the amniocentesis should be made promptly, along with attempts to find out and correct the cause of contamination. Determination of the type of organism involved is usually unnecessary. The rule to handle only part of the cultures from one specimen at any particular time saves the other cultures if contamination is detected rapidly and was not in the initial specimen. Complete culture failure due to contamination should be a rare occurrence in a prenatal diagnosis laboratory.

5.3.9 Treatment of bloody specimens

Surprisingly, specimens that look like pure blood often grow typical AF cells for successful chromosomal diagnosis. With centrifugation, the red cells will form a layer above the white pellet of amniotic cells and can be removed without jeopardizing the integrity of the lower cell pellet. Once cell attachment is achieved in cultures, the remaining problem is to continue to remove debris from disintegrating red blood cells. Some of this debris can be avoided by irrigating the culture surface after an initial 24 hours. Frequent medium changes may be needed. If the initial cell pellet contains clots, they can be removed, cultured, and analyzed separately on the chance that some viable AF cells may have been trapped in the clot. Blood clots can also be digested by collagenase to release trapped viable cells. Supernatant amniotic fluid, after a low-speed spin (less than 1000 RPM) to pellet the red cells, can also be cultured, usually half-and-half with complete medium. Some viable AF cells frequently remain in suspension and can be cultured, though the yield of primary colonies is low. MCC is always a concern; however, maternal tissues will not grow typical AF cells and careful attention to cell morphology should be practiced.

5.3.10 Treatment of poor growth

An occasional culture grows poorly whereas others treated identically do well. Factors affecting the growth of individual cultures include the number of viable cells per milliliter present initially; the volume of the specimen; conditions of collection and transport; and variations in culture conditions in spite of strict controls. Some slow-growing cultures will speed up even after 8–10 days, allowing a successful diagnosis without repeat amniocentesis. Sudden slow growth in cultures from multiple patients is indicative of a problem with culture technique or conditions. Immediate attention should be focused on identifying the source of the problem.

5.3.11 Notifying the physician about poor growth or no growth

A small number of amniotic fluid cultures grow extremely slowly or do not grow at all. Each laboratory should incorporate a system for daily monitoring of the number of days in culture for each specimen. As part of this system, the lab should establish a cut-off for days in culture at which time a specimen may be considered to be growing slowly or not at all. There is no absolute rule for this decision. It depends on the circumstances of the amniocentesis procedure, reasons for testing, and gestational age. However, the American College of Medical Genetics (ACMG) guidelines indicate that notification of inadequate growth should be provided to the physician within 10 days of the procedure. In addition, the number of test failures per year should not exceed 1 per 100 consecutive samples.

5.4 Analysis of amniotic fluid

5.4.1 Determining harvest times

Processing primary cultures relies on visually assessing when rounded cells (one hopes mitotic) are present in the primary colony. A medium change, usually about 24 hours before processing, can help to induce a wave of cell division. Criteria for harvesting will be determined by the methods of culture and harvest.

In situ processing involves assessment of the number of colonies and of growth parameters of each colony. Smaller and less dense colonies have more actively dividing cells, usually on the periphery. These appear round in shape and form doublets as the cells divide. More than one small colony on a slide or coverslip, and preferably at least five colonies, will make a profitable harvest for analysis of multiple colonies. Lacey appearing colonies of about 50 cells tend to yield the best results. It is important not to wait too long to harvest for in situ analysis. Harvesting flasks by the cell button method may also involve a prior medium change or subculture, or may rely on detection of rapid growth parameters such as a visual increase in cells compared to a prior observation period, the appearance of doublets, and a general appearance suggesting optimal growth for harvesting.

If cells are subcultured from primary flasks onto slide chambers or coverslips for analysis, the primary cultures should be showing rapid growth but should not be confluent. Cells can be left behind to maintain the same culture if further analysis is required. Cells subcultured onto slides or coverslips are processed when doublets appear, usually between 24 and 48 hours after subculture. Avoid harvesting all of the cultures from a patient at once to help ensure that any technical difficulties during harvest do not affect them all.

5.4.2 Steps involved in harvest and slide-making

A *mitotic inhibitor* is added to arrest dividing cells at metaphase during a final incubation period. *N*-deacetyl-*N*-methyl colchicine (Colcemid® or Demecolcine) is most frequently used, at the lowest possible concentration and shortest time to permit longer chromosomes.

Ethidium bromide intercalates within the DNA of chromosomes and interferes with chromatin contraction. It may be added along with Colcemid® to achieve longer chromosomes. Concentrations should be lower than that used for bloods.

Enzymatic dispersal of cells from flask cultures is usually performed with trypsin-EDTA. Purified trypsin is not usually recommended. Primary amniotic fluid cultures (AF type, amniocyte) are easy to suspend from monolayers. Variant morphologic subtypes within the primary culture may resist trypsinization into a suspension, particularly the E (epithelial) types. Trypsin that is two to ten times the normal concentration may be helpful for these cell types. This step is not needed for in situ cultures.

Hypotonic KCl and/or sodium citrate are used most frequently, either at room temperature or warmed to 37–37.5 °C, to achieve swelling of the cells. Generally, the choice of solution used in hypotonic treatment is determined by what works best in a particular laboratory and may need to be modified from time to time. Most in situ methods do not use KCl alone due to

problems with banding quality. Generally, the higher temperature is used to increase metaphase spreading. The time of exposure will depend on cell density and type of specimen, whether on slides or in a cell pellet. The time should be increased up to a maximum of about 45 minutes if more spreading of chromosomes is needed.

A fixative of methanol and acetic acid (usually 3 : 1 ratio respectively) is applied to prepare the chromatin and nuclear membrane for the final steps in slide preparation and staining.

Humidity and temperature play a role in obtaining well spread metaphase preparations. Slide-making chambers are commercially available or can be made to control these factors. Each laboratory must establish what conditions work best for coverslips and slides made from cell suspensions. A humidity level of 40–50% is a good starting point at room temperature. Increasing humidity generally increases spreading.

Slide aging, or thorough drying of slides, is needed prior to banding. G-banding is generally poor on a freshly made slide and is improved if the slides are left overnight, usually at about 60°C. A similar degree of drying can be achieved if the slide or coverslip is placed in an oven at 90°C for about 1 hour.

5.4.3 Number of slides per patient

The number of slides will also be determined by the methods of culture and harvest. In situ processing must involve at least two slides or coverslips to allow examination of more than one culture. Additional slides or coverslips, up to about six, are usually set up for in situ analysis to ensure availability of enough colonies (minimum of 15 total colonies needed). The number of slides possible from a flask harvest will be determined by the size of the cell pellet. Making at least two slides per flask is advisable. If slides are poor, more should be made from the cell pellet or another harvest will need to be done. If mosaicism is suspected, additional cultures and slides need to be analyzed.

5.4.4 Stains performed

G-banding is the method of choice for routine karyotype analysis. C-banding can be a useful tool for further description of a variant or structurally abnormal chromosome. Parental chromosome studies, fluorescence in situ hybridization (FISH) analysis with DNA probes, and/or chromosomal microarray analysis are the most efficient next steps for further interpretation of structural abnormalities of potential clinical significance. The application of other stains, such as NOR (silver staining for nucleolar organizing regions), DAPI, or Q-banding, usually provides little useful information, wastes valuable sample material and time, and while these stains may sometimes be used as an adjunct, they should not be used in place of the studies mentioned above. See Chapter 6 for a more detailed description of staining options.

5.4.5 Analysis of amniotic fluid

For in situ cultures, a minimum of 15 cells from 15 different colonies are counted. The colonies should come from two or more coverslips. Ideally, a majority of colonies will not be analyzed from the same coverslip. A minimum of 20 cells, equally distributed among at least two culture vessels, are counted from flask cultures. For either culture method, at least five cells from five different colonies, preferably from at least two independently established cultures, should be completely analyzed, and at least two karyograms are created and printed. In the case of mosaicism, at least one karyogram is created for each cell line. These analysis practices are based on current ACMG clinical laboratory guidelines.

If there is insufficient material to perform a complete analysis, such as, growth of only a small number of colonies from a small initial sample volume, the physician should be notified. The final report should reflect the fact that the analysis is considered to be incomplete by established clinical standards and that a repeat specimen would be required to complete it. Factors which influence the physician's and the patient's decision to perform or not to perform a repeat amniocentesis include the extent of the analysis obtained (e.g., an analysis based on 10 colonies versus analysis based on two colonies), referring diagnosis, ultrasound findings (abnormal versus normal), gestational age, and psychological comfort level of the patient.

5.4.6 Average time to report cases

The current ACMG guidelines recommend that 90% of cases are completed and reported within 14 calendar days from specimen receipt. This may include a verbal report. Less than 10 days should be the goal of the laboratory. All communication of results should be documented, and all analyses should be recorded accurately and reviewed prior to release of any results.

5.4.7 Maternal cell contamination

Most programs request the doctor obtaining the amniotic fluid discard or send the first few milliliters of fluid separately, on the assumption that this procedure will decrease the chance of growing the maternal cells in the specimen. A specimen obtained through an anteriorly placed placenta, or a bloody or clotted specimen, or the clinical presentation of oligohydramnios are known risk factors for MCC. Examination of cell morphology in culture is a valuable means of assessing growth of fetal rather than maternal cell types. The typical AF cell described earlier in this section does not have a counterpart among maternal cells that can be present in amniotic fluid. Maternal cells should be suspected if fibroblast-like cells grow out of a small piece of tissue that is present in the culture.

The most frequent situation in which MCC is suspected is when a minority of XX cells are observed in otherwise XY cultures. A few of the XX cells should be counted and analyzed; however, a complete analysis should be performed on the male cells if possible. Practices differ among laboratories as to whether all MCC is included in the final report, especially when only a few XX cells are observed. When more than a few XX cells (multiple colonies/multiple sources) are observed, it is generally a good idea to include the XX cells in the final report. A large number of XX colonies or cells in flasks should be investigated further as this may have other clinically significant implications. Confirmation of the fetal sex by ultrasound may be helpful in interpreting the finding. Maternal blood may be required to further resolve some cases.

5.4.8 Mosaicism

The interpretation of mosaicism or potential mosaicism in amniotic fluid cultures is one of the most difficult tasks facing a cytogeneticist providing clinical diagnostic services. Various levels of mosaicism have been defined [18]. True *mosaicism* is defined as the presence of an identical chromosome abnormality in more than one culture, whereas *pseudomosaicism* is present when the aberrant karyotype is restricted to multiple colonies (*in situ*) or multiple cells (flask) in one culture. It must be remembered that a completely normal analysis based on counting and analyzing the recommended number of cells for amniotic fluid *does not exclude* the possibility that another cell line is present in the amniotic fluid or in other tissues from the fetus. Rather, at any given number of cells (flasks) or colonies (*in situ*) analyzed, with a specific degree of confidence, mosaicism greater than or equal to a specific percent has been excluded. It follows that with a greater number of colonies analyzed, a lower percent mosaicism has been excluded. A balance must be maintained, however, between the amount of time spent in analysis for a case and the likelihood that mosaicism is present in a specimen. Pseudomosaicism is found in approximately 1% of amniotic fluid cases and rarely reflects the fetal karyotype, while true mosaicism occurs in approximately 0.2% of cases and frequently reflects the fetal karyotype [19].

How far does one go to investigate what initially appears to be a finding isolated to one colony or culture? To multiple colonies in a culture? In practical terms, a laboratory must have a defined methodology for analysis once an abnormal cell is observed. Hsu and Benn [17] have published guidelines for further investigations to be performed on the basis of the specific abnormality found (e.g., autosomal trisomy versus balanced structural rearrangement) and the number and distribution of abnormal cells (e.g., single colony/single culture versus multiple colonies/single culture). Some cases require investigation of more colonies or cells and cultures than others. The analysis scheme is based upon the clinical impact to the pregnancy should mosaicism truly be present and the frequency, based on the authors' review of many cases, that any given abnormality has been confirmed [20–22].

Clinical correlation with ultrasound findings should always be included in evaluating the significance of mosaicism; however, a normal ultrasound result does not exclude a mosaic finding in the fetus. Further invasive testing for investigation of mosaicism found in amniotic fluid, such as cordocentesis (see section 5.6.4, Fetal blood sampling), should be recommended with caution and should depend on the level of potential clinical impact to the fetus for a chromosome abnormality. Furthermore, not all chromosome abnormalities found in amniotic fluid are found in blood chromosome studies. Confirmation of mosaicism is recommended by analysis of placenta, blood and/or tissue, regardless of the pregnancy outcome.

5.5 Chorionic villus sampling

5.5.1 Amniocentesis versus chorionic villus sampling

Chorionic villus sampling came into clinical practice in the 1980s as a method for obtaining information about the fetus in the first trimester. Although amniocentesis in the second trimester had been in clinical use for years, CVS provided a way to shorten the length of waiting time during pregnancy before genetic diagnosis could be performed. In addition, termination of a first trimester gestation on the basis of an abnormal genetic result could be performed with a greater degree of safety to the mother. More recently, first trimester maternal serum screening, the use of ultrasound measurements of nuchal translucency, and cell-free fetal DNA testing have increased the need for first trimester genetic diagnosis.

CVS, like amniocentesis, provides tissue for cytogenetic, molecular and biochemical diagnostic testing, and can be performed on multiple gestations. CVS cannot, however, provide information regarding risk of a neural tube defect.

5.5.2 Chorionic villus sampling procedures

CVS is performed between 10 and 12 weeks of gestation. At this time the placental site, called the chorion frondosum, is the location for sampling. Also at this stage of pregnancy, the amnion and chorion are separated from each other and are separated from the uterine cavity by a thin layer of maternal decidua. Fusion with decidua of the opposite uterine wall has not yet occurred. Knowing these anatomic considerations is important for understanding why CVS can be performed only at this developmental stage and why MCC may be a problem in cytogenetic analysis.

Two procedures are used for CVS, one by a transcervical approach and the other by a transabdominal. The position of the placenta frequently determines which approach is used, and both procedures are performed under ultrasound guidance. For the transcervical approach, a flexible catheter is inserted into the vagina and cervix for suction biopsy of the chorion frondosum.

CVS is a technically more difficult procedure to perform than amniocentesis. It has been associated with a higher fetal loss rate (1/100) than second trimester amniocentesis (1/200) [23,24]. However, a recent large single-center study of women undergoing CVS or amniocentesis found no statistical difference in safety between the two methods [25]. The change in risk from the earlier studies may be attributed to increase in expertise of the physicians performing the procedures as well as in changes in the procedure. Multiple studies have confirmed that CVS performed in the 10–12-week gestational age period does not carry an increased risk for limb reduction defects; however, sampling prior to 10 weeks is not recommended [3].

5.5.3 Structure and cell types

Chorionic villi at the time of first trimester sampling are composed of multiple layers of cells (Figure 5.2). The innermost cells form the mesodermal or mesenchymal core. Blood vessels supply oxygen and nutrients to the villi through the cores. The core is surrounded by a layer of actively dividing cells called cytotrophoblasts. This layer of cells gives rise to the outermost layer of invasive, multinucleated cells, called the syncytiotrophoblast.

It is important to understand the embryologic derivations of the cell layers in villi. Cells from the outer cell mass of the morula form the cytotrophoblasts and syncytiotrophoblasts of the placental villi. The cytotrophoblasts are mitotically active

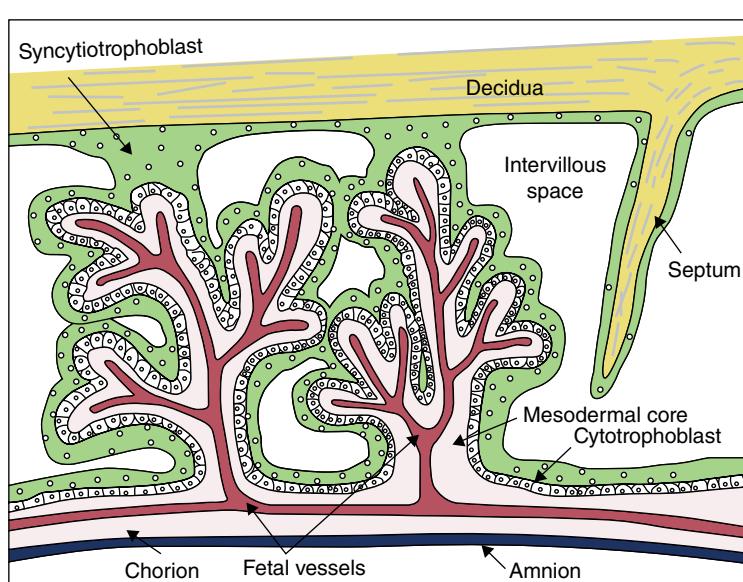


Figure 5.2 Diagram of first trimester chorionic villi within placenta. Diagram shows chorionic villi within the placenta at the time of first trimester prenatal diagnosis. The fetal side of the placenta is at the bottom, and the maternal side is at the top of the diagram. Courtesy of Dr. Kristin May. See insert for color representation of this figure.

and can provide metaphase chromosome preparations in a direct harvest. In contrast, cells of the inner cell mass form the embryo and other extra-embryonic structures such as the placental membranes and core of the villi. Cells of the villus core do not divide as rapidly as the cytotrophoblasts; thus they must be placed in culture to obtain metaphase chromosome preparations.

5.5.4 Specimen transport

Practitioners performing CVS generally perform an initial inspection of the samples they obtain to look for the presence of villi and to estimate the sample size. Laboratories performing CVS genetic analysis should provide sterile media aliquoted into individual transport containers to all referring CVS centers. Labels on the containers should identify the contents, storage conditions (refrigerated), and provide an expiration date. Transport media can be frozen to extend the expiration date; however, the medium should be brought to room temperature prior to placing villi in the medium. It is important that physicians have sufficient supplies of transport media on hand because substitutions of other buffered solutions may quickly compromise the sample. Transport media generally contains cell culture medium supplemented with FBS, antibiotics and L-glutamine. Heparin may also be included to minimize clotting. The addition of HEPES buffer will allow the sample to maintain a proper pH.

Transportation to the laboratory should occur promptly and with avoidance of extreme temperatures. Specimens should reach the laboratory within 24 hours of sampling and should be processed soon after they are accessioned.

5.5.5 Specimen cleaning

The first step in working with CVS material, whether for cytogenetic, biochemical, or molecular diagnostic testing, is to thoroughly remove all maternal tissue and clots from the specimen (see Figure 5.3). This is performed under an inverted or dissecting microscope using aseptic techniques. Healthy villi should be white to clear in appearance (see Figure 5.4) and have many branches and buds with visible vascularization. Less healthy villi will not contain many branches or buds. Maternal decidua is not branched in appearance (see Chapter 4, Figure 4.3). The condition of the villi should be noted on culture records. A visual estimate of the amount of cleaned villi is made by comparing it to established standards created by the laboratory. Usually 20 mg of cleaned healthy villi is sufficient for chromosome analysis (see Figure 5.5). A larger amount is often requested when molecular or biochemical testing is required. Samples in the 5 mg range may yield insufficient cells for a complete analysis. A laboratory must determine a minimum sample size and should, as needed, assist practitioners performing CVS in learning to estimate sample sizes. If molecular testing is requested on an adequate sample, a portion of the cleaned villi (usually about 5 mg) can be sent to the testing laboratory at this time.



Figure 5.3 Removing maternal decidua from villus frond. White amorphous segments of maternal decidua that are attached to the branching villi must be carefully removed before processing or maternal contamination (MCC) may interfere with fetal analysis. Courtesy of Cheryl D. De Mara M.Photog., FP. See insert for color representation of this figure.



Figure 5.4 Cleaned villi. First trimester chorionic villi pieces that have been cleaned of maternal decidua. Note extensive branching. Although not shown in this figure, blood vessels may be visible within the villi. Courtesy of Dr. Kristin May.

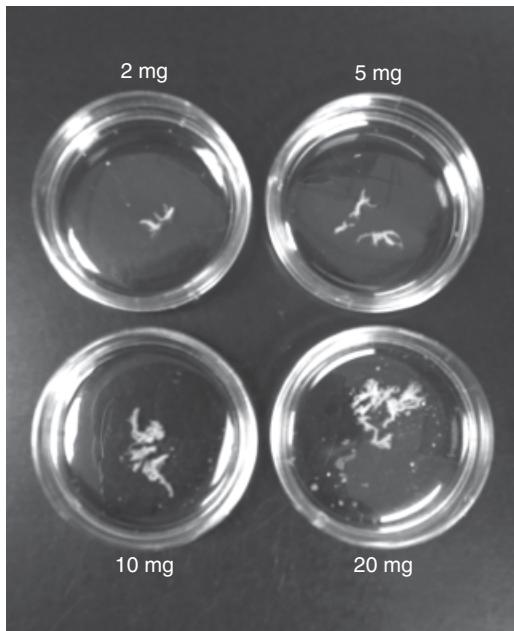


Figure 5.5 Determining CVS weight in milligrams (mg). Visual estimation of villi volume is critical not just to the technologist setting up the culture, but to the physician performing the procedure. Having a visual guide can help both parties more accurately estimate the success of the sampling. Petri dishes used in this photo are 60 mm in diameter. Courtesy of Dr. Kristin May.

Table 5.1 Comparison of direct preparations and cultured cells from CVS for chromosome analysis

CVS Chromosome Analysis Method	Advantages	Disadvantages
Direct preparation Source: cytotrophoblast cells	Results available within 12 hours or less. Maternal cell contamination is not a concern. FISH or chromosome analysis may be used to confirm normal female finding in cultured cells or as an additional source for mosaicism in cultured cells.	Results may not accurately reflect fetal karyotype due to abnormalities existing only in the placenta. Band resolution is often lower than that obtained in cultured cells.
Cultured cells Source: mesodermal core cells	Embryologic derivation is closer to that of the fetus and thus more often reflects the true fetal status more than direct preparations. Band resolution is generally higher than in a direct preparation.	Maternal cell contamination may occur. Takes longer than direct; final report available in ~5–14 days.

5.5.6 Direct harvest

Cytotrophoblast cells are actively dividing and can be processed without culturing for metaphase chromosome preparations. This is referred to as a direct harvest, and there are several different techniques available for preparation of the cytotrophoblast cells. An advantage for performing a direct harvest is confirmation of a female karyotype, either by FISH or chromosome analysis, as maternal cells would not be dividing in these cultures (see Table 5.1).

A common harvest method involves incubation of the villi to be used in medium with Colcemid®, digestion with trypsin to remove the trophoblast layer of cells, and then treating the loosened cells with a hypotonic solution and fixative. The remaining villi can then be used for establishing cultures.

5.5.7 Villus culture

Analysis of cells from cultured villi is recommended, regardless of whether analysis from a direct preparation is performed or not. Experience has shown that there are fewer discrepancies between the CVS result and the true fetal karyotype when cultured cells are examined.

The first step in establishing cultures from CVS is to remove the outer trophoblast layer. This is performed by trypsin digestion, and as noted above, the loosened cells may be used for direct analysis or FISH. The villi are then treated with collagenase to prepare a cell suspension that is then plated onto multiple coverslips or flasks similar to amniotic fluid. Healthy villus cells will grow quickly and should be checked daily after setup. As with amniotic fluid, cultures for a patient should be established at least in duplicate, receive different media or different lots of media, and be split between two incubators. Avoid harvesting all of the cultures from a patient at the same time in case of technical difficulties; this will ensure that a backup is still available.

If biochemical testing is requested or the specimen was too small to send villi for molecular studies, extra cultures should be set. A backup culture should always be kept until results on the additional testing are completed. These cells may be needed to complete or confirm their results.

5.6 Analysis of chorionic villi

5.6.1 Routine chromosome analysis

Some laboratories perform chromosome analysis from both a direct harvest and from cultured villi and report both results. Others do not prepare a direct harvest at all and perform chromosome analysis only from the cultured cells (see Table 5.1). A third approach is to perform chromosome analysis from cultured cells and use cells from a direct harvest for FISH analysis to confirm findings from the cultures or as an additional source if needed for a mosaic result. Many laboratories perform aneuploidy FISH screening on an aliquot of the single cell suspension rather than direct chromosome analysis. Chromosome analysis based only on cells from direct preparations is not recommended [1]. The advantages and disadvantages of analysis from direct harvest and cultured cells are given in Table 5.1.

Guidelines for chromosome analysis of chorionic villus from the ACMG (2009) recommend counting a minimum of 20 cells, selected as equally as possible from at least two independent cultures, with full analysis of five cells, of which at least

two are karyotyped. If analysis is performed on both direct harvest and cultured cells, at least 10 cells should be counted from cultured preparations and at least four of the five required analyses should be on cultured cells.

As determined in two large collaborative studies of chromosome analysis from CVS, one in the United States and the other in the United Kingdom, nonmosaic trisomies of 13, 18, and 21, triploidy, and sex chromosome aneuploidies are highly diagnostic of the fetal status [26,27]. Less likely to be diagnostic of fetal abnormality are rare trisomies, tetraploidy and mosaic results (1–3% of cases). When mosaicism is detected in cells from direct preparation, it is less likely to be confirmed in subsequent amniotic fluid or fetal tissue studies than mosaicism detected in cultured villus cells (discussed later).

Initially, approximately 1% of women having CVS performed required follow-up testing, such as amniocentesis or fetal blood sampling, for further interpretation of CVS test results. However, improvements in the sampling technique, as well as knowledge gained from laboratories' experience in working with CVS, have resulted in fewer women requiring a second invasive procedure [28].

Some of the chromosome abnormalities observed in direct and/or cultured CVS preparations are not generally observed in amniotic fluid. This may reflect differences in fetal viability at different gestational ages. In addition, an abnormality may be present in the trophoblast layer but not in cultured cells because of differences in selective pressures between cells in the developing embryo and the placenta. Situations that provide challenges in interpretation of results, such as mosaicism and MCC, are discussed later.

5.6.2 Confined placental mosaicism and uniparental disomy

Mosaicism occurs in 1–3% of CVS chromosome studies. It may take the form of two cell lines present in the direct and/or cultured cells or in completely discrepant results between the direct harvest preparations and cultured villi. The majority of these cases, 80–90%, represent cases in which the abnormality is **not** found in the fetus [27,29,30]. For the remainder of the cases the mosaic finding is present in both fetal and placental cells. When a chromosome abnormality exists only in cells from the placenta (cytotrophoblasts *or* stromal cells), it is termed confined placental mosaicism (CPM). CPM can arise when a chromosome abnormality develops during somatic divisions after fertilization, in cells that are destined for the placenta.

Conversely, CPM may occur when a mitotic error occurs in a cell of a chromosomally abnormal conceptus and yields a chromosomally normal cell as in the following example. In a trisomic situation, loss of one of the trisomic chromosomes "corrects" the chromosome number back to disomy and is referred to as "trisomy rescue" because it results in gain of chromosomally normal cells. Somatic changes occurring very early in embryogenesis will affect a larger number of descendant cells and tissues than changes that occur days later in development. A normal fetal karyotype and an abnormal placental karyotype will result if the disomic (normal) cells primarily form the fetus and the trisomic cells primarily form the placenta.

The difficulty in interpretation of mosaicism in CVS lies in determining whether the mosaic finding is confined to the placenta or not. Because the chromosome pattern of cultured villi more frequently mirrors the karyotype of the fetus, mosaicism detected in cultured cells is more often confirmed in follow-up testing of fetal cells than mosaicism detected in direct preparations. High-resolution ultrasound evaluation and amniocentesis are frequently recommended to confirm unexpected abnormal results on CVS. Analysis of fetal blood may also be recommended under certain circumstances. A normal result on amniotic fluid or blood generally supports, but does not prove, that the CVS result represents CPM. In addition, as discussed earlier, certain abnormalities are more likely to be diagnostic of the fetus than others. This information is provided to couples for consideration along with ultrasound and follow-up testing results. However, the presence of a chromosome abnormality in the placenta but not in the fetus may still have clinical consequences to the pregnancy. The placenta may become compromised with resultant growth restriction in the fetus [31–33].

The fetus may also be adversely affected by another possible consequence of trisomy rescue. Consider the example above of a trisomic cell that is changed to a disomic by loss of one of the trisomic chromosomes. If the remaining two chromosomes of the original three are derived from the same parent, the result is termed uniparental disomy (UPD) (see Chapter 10, Genomic imprinting). A potential for genetic imbalance in UPD occurs only when activity of a gene or genes on the chromosome is dependent upon the chromosome's parental origin. In somatic cells, one chromosome of a pair is contributed by the father and one by the mother. Thus, if a gene is actively transcribed only from the maternal chromosome, there will be little or no gene activity in a cell containing two *paternally* derived chromosomes. Clinical abnormalities have been described with UPD for several chromosomes [34–36]. Detection of UPD requires molecular analysis of fetal and parental DNA. Testing is available for chromosomes that are associated with clinically significant UPD phenotypes. For example, mosaicism for either trisomy 14 or trisomy 15 in CVS should be followed up with genetic counseling and a recommendation for molecular diagnostic testing to test for UPD in the fetus [37,38]. For a more in depth discussion on UPD, see Chapter 10.

For all of the reasons listed above, CPM is a significant risk factor for a pregnancy. Thus a finding of mosaicism in CVS, while not common, should be investigated thoroughly.

5.6.3 Maternal cell contamination

Maternal decidua is often obtained along with villi during the placental sampling process. Thorough cleaning of the sample, including removal of all clots and decidua from the villi, is a critical step in minimizing contaminating maternal cell growth in CVS cultures. Cleaning of villi should be performed by experienced technologists. Regular monitoring for the presence of XX cells among XY cultures is recommended as an important quality indicator of the sample setup process. However, even large laboratories that process many CVS specimens have reported a 2–4% MCC rate.

Confirmation that a 46,XX result from cultured cells truly represents a female fetus can be obtained by chromosome or FISH analysis using probes for the sex chromosomes on cells processed directly from the outer villus cells. An alternative is to compare DNA markers in the villi with DNA from a maternal blood specimen.

5.6.4 Fetal blood sampling

Cordocentesis or percutaneous umbilical blood sampling (PUBS) is performed after 17 weeks of gestation for diagnosis of fetal blood conditions or infection or for chromosome analysis to evaluate mosaicism detected in CVS or amniotic fluid [39–41]. The procedure involves insertion of a needle into the umbilical cord near its insertion with the placenta and removal of a small amount of fetal blood. As a follow-up test, it must be reserved for cases that cannot be resolved by the other methods already discussed in this chapter due to the risk of pregnancy loss or infection. Whenever possible, two cultures should be initiated for 48-hour and 72-hour harvests.

References

1. American College of Medical Genetics, Standards and Guidelines for Clinical Genetics Laboratories, E. Clinical Cytogenetics, 2009 edition, http://www.acmg.net/StaticContent/SGs/Section_E_2010.pdf
2. Canadian early and mid-trimester amniocentesis trial (CEMAT) group. Randomized trial to assess safety and fetal outcome of early and midtrimester amniocentesis. *Lancet* 1998; 351: 242–247.
3. Philip J, Silver RK, Wilson RD, Thom EA, Zachary JM, Mohide P, Mahoney MJ, Simpson JL, Platt LD, Pergament E, Hershey D, Filkins K, Johnson A, Shulman LP, Bang J, MacGregor S, Smith JR, Shaw D, Wapner RJ, Jackson LG, NICHD EATA Trial Group. Late first-trimester invasive prenatal diagnosis: Results of an international randomized trial. *Am J Obstet Gynecol* 2004; 187: 1164–1173.
4. Cederholm M, Haglund B, Axelsson O. Infant morbidity following amniocentesis and chorionic villus sampling for prenatal karyotyping. *Br J Obstet Gynecol* 2005; 112: 394–402.
5. Brace RA, Wolf EJ. Normal amniotic fluid volume changes throughout pregnancy. *Am J Obstet Gynecol* 1989; 151: 382–388.
6. Magann EF, Bass JD, Chauhan SP, Young RA, Whitworth NS, Morrison JC. Amniotic fluid volume in normal singleton pregnancies. *Obstet Gynecol* 1997; 90(4 Pt 1): 524–528.
7. Sandstrom MM, Beauchesne MT, Gustashaw KM, Latt SA. Prenatal cytogenetic diagnosis. In: Latt SA, Darlington GJ, eds., *Methods in Cell Biology*, vol 26. London: Academic Press, 1982.
8. Richkind KE, Risch NJ. Sensitivity of chromosomal mosaicism detection by different tissue culture methods. *Prenat Diagn* 1990; 10: 519–527.
9. Sikkema-Raddatz B, van Echten J, van der Vlag J, Buys CHCM, te Meerman GJ. *Prenat Diagn* 2002; 22:164–165.
10. Van Dyke DL. Amniotic fluid cell culture. In: Milunsky A, ed., *Genetic Disorders and the Fetus*, 5th ed. Baltimore, MD: Johns Hopkins University Press, 2004.
11. De Coppi P, Bartsch G Jr, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EY, Yoo JJ, Furth ME, Soker S, Atala A. *Nature Biotech* 2007; 25: 100–106.
12. Bossolasco P, Montemurro T, Cova L, Zangrossi S, Calzarossa C, Buiatiotis S, Soligo D, Bosari S, Silani V, Lambertenghi Delilars G, Rebulla P, Lazzari L. Molecular and phenotypic characterization of human amniotic fluid cells and their differentiation potential. *Cell Res* 2006; 16: 329–336.
13. Burles JV, Huxley MP, Kennedy TS. Syringe toxicity in amniotic fluid cell cultures. *Lancet* 1983; 1: 1336–1337.
14. Howell RT, McDermott A, Gregson NM. Syringe toxicity in amniotic fluid cell cultures. *Lancet* 1983; 1: 1099–1100.

15. Held KR, Sonnichsen S. The effect of oxygen tension on colony formation and cell proliferation of amniotic fluid cells in vitro. *Prenat Diagn* 1984; 4: 171–179.
16. Featherstone T, Cheung SW, Spitznagel E, Peakman D. Exclusion of chromosomal mosaicism in amniotic fluid cultures: determination of number of colonies needed for accurate analysis. *Prenat Diagn* 1994; 14: 1009–1017.
17. Hsu LYF, Benn PA. Revised guidelines for the diagnosis of mosaicism in amniocytes. *Prenat Diagn* 1999; 11: 1081–1082.
18. Worton RG, Stern RA. A Canadian collaborative study of mosaicism in amniotic fluid cell cultures. *Prenat Diagn* 1984; 4: 131–144.
19. Gardner RJM, Sutherland GR. *Chromosome Abnormalities and Genetic Counseling*, 3rd ed. New York: Oxford University Press, 2004.
20. Hsu LYF, Yu M-T, Richkind KE, Van Dyke DL, Crandall DF, Saxe DF, Khodr GS, Mennuti M, Stetten G, Miller WA, Priest JH. Incidence and significance of chromosome mosaicism involving an autosomal structural abnormality diagnosed prenatally through amniocentesis: A collaborative study. *Prenat Diagn* 1996; 16: 1–28.
21. Hsu LYF, Yu M-T, Neu RL, Van Dyke DL, Benn PA, Bradshaw CL, Shaffer LG, Higgins RR, Khodr GS, Morton CC, Wang H, Brothman AR, Chadwick D, Disteche CM, Jenkins LS, Kalousek DK, Pantzar TJ, Wyatt P. Rare trisomy mosaicism diagnosed in amniocytes, involving an autosome other than chromosomes 13,18,20 and 21: Karyotype/phenotype correlations. *Prenat Diagn* 1997; 17(3): 201–242.
22. Wallerstein R, Ming-Tsung Y, New RL, Benn P, Bowen CL, Crandall B, Disteche C, Donahue R, Harrison B, Hershey D, Higgins RR, Jenkins LS, Jackson-Cook C, Keitges E, Khodr G, Lin CC, Luthardt FW, Meisner L, Mengden G, Patil Sr, Rodriguez M, Sciorra LJ, Shaffer LG, Stetten G, VanDyke DL, Wang H, Williams F, Zaslav A, Hsu LYF. Common trisomy mosaicism diagnosed in amniocytes involving chromosomes 13, 18, 20 and 21: karyotype–phenotype correlations. *Prenat Diagn* 2000; 20(2): 103–122.
23. Canadian Collaborative CVS-Amniocentesis Clinical Trial Group. Multicentre randomized clinical trial of chorion villus sampling and amniocentesis. First report. Canadian Collaborative CVS-Amniocentesis Clinical Trial Group. *Lancet* 1989; 1: 1–6.
24. Borrell A, Fortuny A, Lazaro L, Costa D, Seres A, Pappa S, Soler A. First-trimester transcervical chorionic villus sampling by biopsy forceps versus mid-trimester amniocentesis: a randomized controlled trial project. *Prenat Diagn* 1999; 19 :1138–1142.
25. Caughey AB, Hopkins LM, Norton ME. Chorionic villus sampling compared with amniocentesis and the difference in the rate of pregnancy loss. *Obstet Gynecol* 2006; 108: 612–616.
26. Ledbetter DH, Zachary JM, Simpson JL, Golbus MS, Pergament E, Jackson L, Mahoney MJ, Desnick RJ, Schulman J, Copeland KL, Verlinsky Y, Yang-Feng T, Schonberg SA, Babu A, Tharapel A, Dorfman A, Lubs HA, Rhoads GG, Fowler SE, De La Cruz F. Cytogenetic results from the U.S. Collaborative Study on CVS. *Prenat Diagn* 1992; 12: 317–345.
27. Smith K, Lowther G, Maher E, Hourihan T, Wilkinson T, Wolstenholme J. The predictive value of findings of the common aneuploidies, trisomies 13, 18 and 21, and numerical sex chromosome abnormalities at CVS: Experience from the ACC U.K. Collaborative Study. *Prenat Diagn* 1999; 19: 817–826.
28. Wapner RJ. Invasive prenatal diagnostic techniques. *Seminars in Perinatology* 2005; 29: 401–404.
29. Hahnemann JM, Vejerslev LO. Accuracy of cytogenetic findings on chorionic villus sampling (CVS)-diagnostic consequences of CVS mosaicism and non-mosaic discrepancy in centers contributing to EUCROMIC 1986–1992. *Prenat Diagn* 1997; 11: 133–136.
30. Stetten G, Escallon CS, South ST, McMichael JL, Saul DO, Blakemore KJ. Reevaluating confined placental mosaicism. *Am J Med Genet* 2004; 131A: 232–239.
31. Kalousek DK, Dill FJ. Chromosomal mosaicism confined to the placenta in human conceptions. *Science* 1983; 221: 665–667.
32. Robinson WP, Barrett IJ, Bernard L, Telenius A, Bernasconi F, Wilson RD, Best RG, Howard-Peebles PN, Langlois S, Kalousek DK. Meiotic origin of trisomy in confined placental mosaicism is correlated with presence of fetal uniparental disomy, high levels of trisomy in trophoblast, and increased risk of fetal intrauterine growth restriction. *Am J Hum Genet* 1997; 60(4): 917–927.

33. Stipoljev F, Latain V, Cos M, Miskovic B, Kurjak A. Correlation of confined placental mosaicism with fetal intrauterine growth retardation. A case control study of placentas at delivery. *Fetal Diagn Ther* 2001; 16: 4–9.
34. Roberts E, Dunlop J, Davis GS, Churchill D, Davison EV. A further case of confined placental mosaicism for trisomy 2 associated with adverse pregnancy outcome. *Prenat Diagn* 2003; 23(7): 564–565.
35. Grati FR, Grimi B, Frascoli G, Di Meco AM, Liuti R, Milani S, Trotta A, Dulcetti F, Grossi E, Miozzo M, Maggi F, Simoni G. Confirmation of mosaicism and uniparental disomy in amniocytes, after detection of mosaic chromosome abnormalities in chorionic villi. *Eur J Hum Genet* 2006; 4(3): 282–288.
36. Kotzot D. Prenatal testing for uniparental disomy: indications and clinical relevance. *Ultrasound Obstet Gynecol* 2008; 31(1): 100–105.
37. Kotzot D. Maternal uniparental disomy 14 dissection of the phenotype with respect to rare autosomal recessively inherited traits, trisomy mosaicism, and genomic imprinting. *Ann Genet* 2004; 47(3): 251–260.
38. Christian SL, Smith AC, Macha M, Black SH, Elder FF, Johnson JM, Resta RG, Surti U, Suslak L, Verp MS, Ledbetter DH. Prenatal diagnosis of uniparental disomy 15 following trisomy 15 mosaicism. *Prenat Diagn* 1996; 16(4): 323–332.
39. Costa D, Borrell A, Soler A, Carrio A, Margarit E, Ballesta F, Puerto B, Caballin MR, Fortuny A. Cytogenetic studies in fetal blood. *Fetal Diagn Ther* 1998; 13(3): 169–175.
40. Gosden C, Nicolaides KH, Rodeck CH. Fetal blood sampling in investigation of chromosome mosaicism in amniotic fluid cell culture. *Lancet* 1988; 1: 613–617.
41. Hahnemann N. Possibility of culturing foetal cells at early stages of pregnancy. *Clin Genet* 1972; 3(4): 286–293.

Contributed protocols section

IMPORTANT: No protocol included in this manual should be used clinically unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements of all chemicals used in the laboratory.

Protocol 5.1 Amniotic fluid culture setup and routine maintenance

Contributed by Genetic Diagnostic Laboratory, Children's Hospital at Erlanger, Chattanooga, TN

I. Principle

Transabdominal amniocentesis is usually performed at 15–16 weeks of gestation, although it can be performed as early as 11–14 weeks. Generally 20–30 mL of amniotic fluid is obtained for prenatal diagnostic studies. Amniotic fluid consists of a fluid component and a cellular component which are separable by low speed centrifugation. Upon centrifugation, the cells form a pellet leaving a straw-colored supernatant. The supernatant fluid is used for studies such as alpha-fetoprotein (AFP) and acetylcholinesterase (AChE) to detect open neural tube defects. The cell pellet is cultured on coverslips to obtain a sufficient number of cells for prenatal chromosome studies. Speed and accuracy are essential in prenatal cytogenetic diagnosis.

Specimen

- Amniotic Fluid, usually 20–30 mL, collected under sterile conditions. Smaller volumes are acceptable.
- Generally received in collection syringes; however, sterile collection bottles or tubes are also acceptable.
- Specimen must not have been frozen.

II. Materials

Reagents

1. AmnioMAX-C100 Complete Media – Use two different lot numbers for setting up duplicate cultures. 15 mL supplement added to bottle of 90 mL basal medium. Basal stored in refrigerator at 2–8 °C; supplement stored in freezer at –5 to –20 °C. Store completed media in refrigerator at 2–8 °C. Keep out of light. Discard after 10 days.

Lab supplies

2. Petri dish/coverslip (MatTek Corporation, Coverslip Kit #CSG-K/F)
3. Sterile disposable pipettes (assorted sizes)
4. 15 mL sterile centrifuge tubes, polystyrene
5. LabStix (Bayer #2181)
6. Sterile Pasteur pipettes, 5 ¾ in
7. T-25 tissue culture flasks
8. Sterile 8 mL round-bottom tubes with caps, polystyrene
9. Two cell culture incubators, 5% CO₂ or tri-gas

III. Method

Quality control

1. *Sterility Check for Complete Media:* AmnioMAX-C100 medium is completed by adding thawed supplement (contains antibiotic) to the basal medium. Place 2 mL in sterile tube. Place sterile tube in incubator 72 hours for sterility testing. If the medium remains clear, it can be considered sterile; if turbidity develops, media is contaminated and should be discarded. Record results in media log book.
2. *Growth Check for Complete Media:* Each new lot of completed media is assigned a Laboratory Media Number. This is recorded in the media log and includes the lot number of each media component. Each new media number must be tested for ability to support growth before using it for clinical testing. (See Protocol 4.2A, Transport medium A (without Fungizone), Note 1.)
3. Use of two different media numbers: Duplicate cultures for each patient are established with different lot numbers of media.
4. Only one specimen at a time is worked within the hood.
5. This is an aseptic procedure. All precautions must be taken to ensure the protection of specimen, technologist, and any reagents that must remain sterile for further use. Protective clothing must be worn at all times when handling specimens.

Initiating cultures

1. Log in the amniotic fluid specimen and turn on amniotic fluid hood fan and light. Collect any necessary reagents and equipment for set up. Record the amount of fluid and its color (clear, cloudy, yellow, brown, blood).
2. Check to see what testing is ordered (chromosome, AFP, molecular testing requiring unspun or spun fluid or cultured cells). It is important to know what the requirements are for such testing – how much fluid or how many flasks will be needed? If the volume of the sample received is not sufficient for the requested test AND chromosome analysis, consult with a supervisor or director. Fewer coverslips for chromosome analysis may need to be set up in order to accommodate needs for additional testing.
3. For testing which requires unspun fluid (some molecular genetic tests and AneuVysion FISH testing), remove the required amount (2–5 mL for AneuVysion), place in pre-labeled sterile tubes, and set aside.
4. If amniotic fluid arrives in syringes, fluid must first be transferred into 15 mL sterile centrifuge tubes prior to centrifuging. This will yield 1, 2 or more centrifuge tubes per patient. All must be labeled with the patient name and laboratory number. If some of the fluid is bloody, do not combine with fluid from other tubes. Put caps on tubes, sealing tightly, and centrifuge amniotic fluid 1000 RPM for 10 minutes.
5. Remove as much supernatant as possible without disturbing the cell pellet. If AFP testing is ordered, put about 5 mL of the supernatant into each of two 8 mL round bottom tubes. Label with patient's name, laboratory number and date.
6. Place a few drops of the supernatant onto a LabStix strip. Note the pH and protein levels on the lab sheet. The pH readings should be in the 7–8 range for amniotic fluid and have protein present. If the pH is in the acidic range (5–6), especially if there is no protein, this should be a signal that the specimen may be urine and a **FERNING TEST** should be performed. (See Note 1, Ferning test.)
7. **For cultures needed for chromosome analysis only (no additional testing requested):** Determine the number of coverslips and flasks are to be set up. A minimum of 2 coverslips is set up for all samples. For samples for which there are two or more centrifuge tubes, set up the following number of coverslips per pellet: 1 for a very small pellet (invisible or barely coats bottom of tube), 2 for small or medium pellets, 3–4 for large pellets. This results in 2 to 6 coverslips being set up per patient. Even though they may be quite large, pellets from late gestational pregnancies (~28 weeks or older) generally

contain a high proportion of nonviable cells and should not be placed on more than 3 coverslips. One flask is established unless the cell pellets are very small. In that case no flask is initiated. Label each dish or flask with the patient's name, medium used, lab number, and consecutive number of culture (1,2,3...). Petri dishes are labeled on top, flasks on side. For cases that also need to have flask cultures initiated for specialized testing, see step 10.

8. Resuspend one of the pellets in a volume of complete medium equal to 0.5 mL for each coverslip to be set up from that pellet. Pipet 0.5 mL of the suspension onto a 22 mm square coverslip in each of the 35-mm tissue culture dishes to be established from that pellet. Take care to confine the suspension to the coverslip. Using another media lot number, repeat the process with the remaining pellet or pellets. Do not discard the centrifuge tubes.
9. Carefully transfer the dishes into the incubator.
10. **For very small pellets:** Place 0.5 mL of complete medium into one of the centrifuge tubes and mix to wash the cells. Transfer this medium and cell mixture to each of the remaining centrifuge tubes to collect all of the cells. Place the medium/cell mixture on a new coverslip and label as "Wash Out" culture. Carefully transfer to incubator.
For cases which require flask cultures for specialized testing (including establishing a back-up culture for possible microarray testing) and chromosome analysis: Usually a larger sample volume is sent to the laboratory for these cases. The amount of cell pellet that will go into flasks is at least twice as much as what is normally applied to a coverslip (roughly the equivalent of a small to medium cell pellet). The total amount of medium in the flask is 2.5 to 3 mL.
11. Clean hood and turn off fan and light if no other cases need to be set up. Dispose of syringes in biological hazard receptacle.

Culture maintenance

1. After 24 hours flood each dish with 1.5 mL medium, being very careful to slowly add media away from center of coverslip where the cells are adhering. Cultures should then be placed on trays in incubator at 37 °C. Cultures may wait up to 72 hours if necessary before flooding, as long as the incubator humidity level is properly maintained to prevent the cultures from drying out.
2. After 3–4 days, cultures should be checked for growth. Cultures should be fed once growth has been observed. Feed cultures by gently tilting the Petri dish and removing the medium from near the edge of the Petri (do not discard). Gently add 2 mL of fresh AmnioMAX-C100 media (same lot number as originally set up in). The media and floating cells from the coverslips can be used to establish a back-up flask culture. This is termed the CSPO or coverslip pour-off culture. This is established by placing all media taken from primary coverslips during the process of the first media change into a single T-25 flask. It is marked as "CSPO". This culture is monitored for growth as a normal flask and more media is added when growth is noted. This culture is used as a back-up culture and is most useful when a small sample is received.
3. All cultures should be routinely fed every 3 to 4 days to insure adequate media changes. Routine feeding is done twice weekly, *usually* on Monday and Thursday. To obtain maximum number of metaphases, cultures should be fed 12–22 hours before harvest. Therefore, adhering strictly to a Monday to Thursday feeding schedule will not insure maximum number of metaphases from harvests.
4. For cultures that are grossly bloody or full of debris (dead cells, meconium, etc.), Petris should be washed down at the first media change. Aspiration of old medium is performed. Draw up 1–2 mL of Hanks' Balanced Salt Solution in transfer pipette and rinse down the coverslip by holding Petri at 45° angle and squirting down coverslip, re-aspirate Hanks' and wash down Petri several more times. Discard final aspirate and replace with 2 mL fresh medium.
For bloody specimens that have blood beneath the coverslip, the coverslip must be transferred to a fresh Petri. Prepare a fresh Petri with 2 mL warm medium. Aseptically remove the coverslip from the original Petri and place it in the fresh Petri. Save the original Petri as there may be growth around the edges.
5. Cultures should be monitored daily after first growth to determine when to harvest.

IV. Notes

1. Ferning test

Materials: Microscope slides, sterile Pasteur pipettes

Procedure: Place a drop of suspected fluid on a slide and prepare a thin film by drawing the edge of a second slide through the drop and across the slide. Label the slide with the patient's last name & first initial, lab number, and date. Prepare a slide of a known amniotic fluid specimen for comparison. Dry the slides on a slide warmer. View the slides under phase.

Amniotic fluid will dry along the edges in a feathering pattern which resembles the fronds of a fern (see Figure 5.1). A urine sample will not feather, but will appear to have a blunt edge and exhibit a lot of cellular debris. Notify the laboratory director if result is negative for amniotic fluid.

V. Additional reading

Priest JH, Rao KW. Prenatal chromosomal diagnosis. In: Barch MJ, Knutsen T, Spurbeck JL, eds. *AGT Cytogenetics Laboratory Manual*, 3rd ed. 1997; Philadelphia:Lippincott- Raven Publishers,199–258.

Protocol 5.2 Coverslip (*in situ*) harvest procedure for chromosome preparations from amniotic fluid, CVS, or tissues (manual method)

Contributed by authors Kristin May and Debra Saxe

I. Principle

The harvest procedure prepares the specimen for microscopic analysis. Metaphases are obtained by the use of a mitotic inhibitor which accumulates metaphases. The addition of a hypotonic solution causes the cells to swell and the chromosomes to spread. The fixative hardens the cells in the expanded state and when the cell membrane flattens out, the chromosomes attach to the surface of the coverslip in a dispersed arrangement with few overlaps.

II. Materials

Lab supplies

1. Sterile disposable serological pipettes (assorted sizes)
2. Permount (Fisher Healthcare) or similar mounting medium
3. Pre-cleaned microscope slides with frosted end
4. 200-µL sterile pipette tips
5. Wooden applicator sticks
6. Forceps
7. Pasteur pipettes, 5 ¾”
8. Rubber pipette bulb

Equipment

9. Biological safety cabinet (BSC), Class 2 with laminar flow
10. CO₂ or tri-gas (low oxygen) cell culture incubator
11. Micropipettor, 5–100 µL size
12. Glass Pasteur pipette
13. Drying oven, set at 90 °C
14. Thermotron slide drying chamber (optional)

Note: Drying chamber should be on for at least 1 hour prior to use, set to 25 °C and 42% humidity (these settings may be adjusted as needed; increase or decrease humidity by 2-degree increments to increase or decrease spreading respectively).

Reagents

15. Ethidium bromide (EB) solution, 500 µg/mL aqueous solution (Sigma Aldrich)
16. Colcemid – 10 µg/mL KaryoMAX Colcemid® solution in PBS (Invitrogen)
17. Hypotonic working solution: 50% 0.075 M KCl (0.56 g KCl in 100 mL deionized H₂O) and 50% sodium citrate 0.8% solution (0.80 g sodium citrate in 100 mL deionized H₂O). Determine the amount of hypotonic solution needed for harvest: 3 mL per coverslip. Always prepare more than you think you need. Mix equal volumes of KCl and sodium citrate solutions together in clean glass bottle. Label bottle and place in 37 °C water bath.
18. Fixative: 3 parts methanol to 1 part glacial acetic acid, room temperature

Quality assurance measures

1. If possible, do not harvest more than 2 cultures for a patient in one batch.
2. *Never* harvest all cultures for a patient in one batch.
3. Assess the quality of metaphases obtained from a harvest before beginning another harvest. In that way, if there was a problem with any of the harvest variables (reagent quality, hypotonic time, drying conditions), the problem can be addressed before starting another harvest.

III. Method

1. *Determining when to harvest:* Primary cultures should be checked daily for harvest after first feeding. They are ready for harvest when several small colonies of 50 to 200 cells are present and dividing cells are seen. Selecting the cultures to be harvested the afternoon before harvesting insures that the cells have time to recover after removal from the incubator.
2. After choosing all the cultures to be harvested, place the cultures back in the incubator for an additional 30 minutes to re-warm. (This step is not necessary if the harvest is chosen the afternoon before as the cultures will already have been in the incubator).
3. Remove culture dishes from incubator and carefully add 40 µL of the EB solution using a sterile tip and microliter pipette. Gently swirl the dishes. Return the cultures to the incubator for 10 minutes.

Note:

Ethidium bromide is toxic – see SDS for proper handling and precautions.

After each of the following steps through step 9, the lids to the Petri dishes are always replaced.

4. Carefully add 10 µL of Colcemid® solution using a micropipettor. Replace lid and gently swirl Petri dishes.
5. Place dishes in 37 °C incubator for 50 minutes.
6. At this point, the cultures can be harvested on a robotic harvest or manually as follows. Aspirate supernatant. Add 3 mL of hypotonic solution to each dish. Allow to stand at room temperature for 40 minutes (lid on). Be very gentle with cultures after this point so as not to disturb metaphase spreads.
Note: Do not move cultures around once hypotonic solution has been added. When removing supernatant and adding solutions to dishes, be very careful not to disturb metaphase cells as this may reduce the number of analyzable metaphase spreads.
7. Add 2 mL fixative to the hypotonic solution in each dish. This should be done carefully at the edge of the dish. After fixative has been added to the last dish in the harvest, let stand at room temperature for 2 minutes.
8. Remove the hypotonic/fix solution with Pasteur pipette and add 2 mL of fixative carefully at the edge of the dish. Allow to stand at room temperature for 10 minutes.
9. Repeat the 10 minutes fixing step 2 more times.
10. Using a metal tray to transfer, place dishes in Thermotron (go to step 14 below, if a drying chamber is not available) and remove all fixative using a glass pipette. Remove lids. Put the lid under the dish. Keep lid in this position until last step as it identifies the coverslip.
11. Remove dishes from Thermotron when completely dry (should take several minutes).
12. Label microscope slides with two patient identifiers and culture number. Pick up coverslip with forceps and invert Petri dish so that the identifiers can be read from the lid. Place the coverslip on the corresponding Petri dish with the *cell side up*. Place a very small amount of Permount onto the appropriate labeled slide with a wooden applicator stick. Using the forceps put the coverslip with the *cell side up* onto the Permount. Press gently to seal.
13. Age coverslips for 30 minutes in a 90 °C oven before staining.
14. *If a slide drying chamber is not available*, work with one culture at a time to complete slide preparation. The goal is to allow the coverslips to dry at the appropriate rate. In general, increasing humidity and temperature will slow drying time and increase spreading and vice versa.
Remove the coverslip from the fixative with forceps and blot off excess fixative from the back of the coverslip. Keep the lid of the coverslip with patient name and culture number close by and work with one culture at a time. Allow to dry at room temperature or use one of the following:
 - a. Using an alcohol burner, carefully pass coverslip (cell side toward flame) over flame from a distance of 8–10 inches until coverslip is dry.
 - b. Blow air gently across the surface with mouth or small fan.
 - c. Using a hot plate at a temperature of approximately 50 degrees, directly place coverslip (cell side up) on the hot plate until dry.

- d. Create a steam bath by heating a beaker of water on a hot plate. Place the coverslip near the steam to increase the amount of humidity and temperature.
- e. Note that methods may have to be changed daily or more than once a day to account for fluctuations in temperature, humidity and air flow.

After drying, go back to steps 12–13.

Protocol 5.3 Harvest of flask amniocyte cultures

Genetic Diagnostic Laboratory, Children's Hospital at Erlanger, Chattanooga, TN

I. Principle

Under typical circumstances harvests can be performed within 6–10 days. During the harvest procedure, Colcemid® is added to act as a mitotic inhibitor. This results in an accumulation of metaphases by preventing the formation of the spindle apparatus. With the addition of the hypotonic solution, water rushes into the cell due to the concentration gradient shift, causing the cells to swell. When fixative is added, the cell is preserved in its swollen shape. The outer layer hardens slightly at this point. As the fixative quickly evaporates during the drying procedure, the cells will flatten completely, thus forcing the chromosomes to spread out. Spreads selected for analysis should have clearly banded chromosomes with few overlaps.

Specimen: Flask amniotic fluid cultures

II. Materials

Lab supplies

1. Sterile disposable serological pipettes (assorted sizes)
2. Glass Pasteur pipettes, 5 ¾ in
3. Permount mounting medium (Fisher Healthcare)
4. Microscope slides
5. Sterile pipette tips

Reagents

1. AmnioMAX-C100 Complete Media
15 mL AmnioMAX-C100 Supplement added to bottle of 90 mL AmnioMAX-C100 Basal Medium. Basal is stored at 2–8 °C; supplement is stored at –5 to –20 °C. Storage conditions for complete media: Store at 2–8 °C; keep out of light. Discard after 10 days.
2. TrypLE Express Trypsin with phenol red, 1× (Invitrogen); stored at 2–8 °C.
3. Acetic acid, glacial
4. Absolute methanol
5. KaryoMAX Colcemid® 10 µg/mL in PBS (Invitrogen)
6. Ethidium bromide solution, 500 µg/mL in H₂O (Sigma)
7. Hanks' balanced salt solution without Ca and Mg salts, warmed to 37 °C
8. **0.8% Sodium Citrate Solution** Dissolve 0.80 g of sodium citrate in 100 mL of deionized H₂O in a clean glass bottle and label 0.80% sodium citrate and date. Storage: Refrigerate 2–8 °C. Discard after 30 days.
9. **0.075 M KCl** Dissolve 0.56 g KCl in 100 mL deionized H₂O and label 0.075 M KCl and date. Store in refrigerator at 2–8 °C. Discard after 30 days.
10. **Hypotonic working solution** of 50% 0.075 M KCl and 50% sodium citrate 0.8% solution. Determine the amount of hypotonic solution needed for harvest and always prepare MORE than you think you need. Mix equal volumes of KCl and sodium citrate solutions together in clean glass bottle. Label bottle and *place in 37 °C water bath*. Any remaining hypotonic solution may be stored at 2 to 8 °C for up to 30 days.
11. **Fixative**, 3 parts absolute methanol to 1 part glacial acetic acid.

Equipment

12. Biological safety cabinet (hood)
13. Micropipettor, 5–100 µL size

14. Electric Pipet-Aid
15. Warming tray at 37 °C
16. Cell culture incubator, 37 °C, 5% CO₂

Quality control

- A limited number of cultures should be removed from the incubator at a time in order to minimize the amount of time that they are exposed to room temperature.
- NEVER HARVEST ALL CULTURES FROM A PATIENT AT THE SAME TIME.
- Work with one patient's cultures under the hood at a time!

III. Method

Determining when to harvest

- Cultures should be checked daily for harvest after first feeding (see Protocol 5.1 for routine feeding procedure).
- Each culture is viewed with an inverted microscope and evaluated as follows:
They are ready for harvest when several small colonies of 50 to 200 cells are present and dividing cells are seen. Record the number of colonies on the culture sheet.
- Pick out harvest the afternoon before harvesting. This ensures that the cells have time to recover after removal from the incubator.
- A flask is ready for harvest when there are many “rounded up” cells which indicate active cell division.
- *If harvest is picked out in the morning, return cultures to incubator for 30 minutes before harvesting.*

After choosing harvest

- Place the flasks in the incubator for an additional 30 minutes to re-warm. (This step is not necessary if the harvest is chosen the afternoon before.)
- Make sure that biological safety cabinet (hood) is ready for use (i.e., fan has been running for at least 10 minutes and all necessary reagents and supplies are ready for use).
- Pre-label centrifuge tubes with stickers from patient's chart and enter the culture identifier on the sticker.
- Record the date of harvest on the culture sheet in the patient chart.

To begin harvest

1. Remove flasks from incubator and carefully add 90 µL of the ethidium bromide solution using a calibrated microliter pipette. Gently swirl the flasks. Return the cultures to the incubator for 30 minutes. *Note: Ethidium bromide is toxic. Wear suitable protective clothing, gloves and eye/face protection.*
2. In the hood, carefully add 50 µL of Colcemid® solution. Re-cap the flasks.
Gently rock the flasks from side to side.
3. Put flasks in 37 °C incubator for 20 minutes (lying down).
4. Remove flasks from the incubator. Pour or pipet off contents of each flask into a correspondingly labeled 15 mL conical centrifuge tube. If the flasks are to be maintained, sterile technique is very important at this step and all subsequent steps until the flasks are returned to the incubator.
5. Using sterile technique, pour or pipet approximately 2 mL of prewarmed 37 °C Hanks' balanced salt solution into each flask. Replace cap. Holding the flask in a horizontal position, gently swirl so that the cells are “washed” by the Hanks'. You can tap the flask to help loosen the cells.
6. Pour the “wash” into the labeled centrifuge tube.
7. Repeat steps 5 and 6.
8. Pipet 2 mL of TrypLE Express into each flask using a sterile 5-mL serological pipette and put cap on.
9. Place the flasks on a 37 °C warming tray. Cells should be starting to release after 15 seconds.
10. Check flask with inverted scope to see if many cells are floating free (tapping flask sharply against a solid surface may help dislodge some cells). If there are not many free cells, return the flask to the warming tray for a few more seconds.
11. When there are sufficient unattached cells, pour or pipet into the proper centrifuge tube containing previous washes from that same flask.

12. Rinse the flask with 1 or 2 mL of Hanks' BSS to recover as many cells as possible. A second wash with Hanks' BSS may be used here.
13. Centrifuge the tubes for 8 to 9 minutes at 1000 RPM.
14. Feed the original flask with the same media with which it was originally established. Incubate for further growth or as a back-up culture.
15. After tubes have been centrifuged, remove the supernatant preserving only the cell pellet and approximately 0.5 mL of supernatant.
16. Resuspend the cells by gently tapping the bottom of the tube.
17. Add approximately 7 to 8 mL of prepared, prewarmed hypotonic solution to each tube. Incubate tubes in water bath for 12 minutes. This incubation time should begin at the time the hypotonic solution is added to the tube.
18. After 12 minutes, 1 mL of fixative (3 : 1 methanol–acetic acid) should be added to each tube to stop the action of the hypotonic solution. Gently invert the tube to mix.
19. Centrifuge for 8 to 9 minutes at 1000 RPM.
20. Remove supernatant, retaining approximately 0.5 mL of fluid above the cell pellet.
21. Resuspend the cells by tapping on bottom of the centrifuge tube.
22. Add 4 mL of fixative, then centrifuge for 8 minutes at 1000 RPM.
23. Repeat steps 20 to 22.
24. Repeat steps 20 and 21. Add fixative according to size of cell pellet for 2–3 slides to be made. Usually this will be about 0.2 to 0.5 mL.
25. Make slides and band according to established procedures.
26. Clean up all work areas, including glassware. Wipe hood with 70% ethanol.

IV. Additional reading

Priest J. General cell culture principles and fibroblast culture. In Barch MJ, Knutsen T, Spurbeck J, eds. *AGT Cytogenetics Laboratory Manual*, 3rd ed. 1997. Philadelphia: Lippincott-Raven Publishers; 173–197.

Protocol 5.4 Amniotic fluid culturing, subculturing, and harvesting (flask method)

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

This procedure provides a method for initiating, maintaining and harvesting amniocyte cultures for prenatal cytogenetic study of fetuses following early amniocentesis (11–13.9 weeks gestation)* and routine amniocentesis (14 weeks of gestation and greater). ***Early amniocentesis is not recommended due to increased risk to the fetus of birth defects and paucity of amniocytes which delays culture growth.**

Flasks containing primarily open AF colonies of sufficient size and number are harvested directly without subculturing (“passage”) and shall be referred to as “*direct harvests*.“ Flasks containing colonies which are packed, and not open and active, which have sufficient cell numbers, are prepared for harvest according to the “*subculture flask*” method.

II. Materials

For setup and culture

1. 25 mm² sterile Corning tissue culture flasks
2. 12.5 mm² sterile Falcon tissue culture flasks
3. 5 mL and 10 mL sterile graduated pipettes
4. Sterile cotton-plugged Pasteur pipettes
5. AFP tubes – 5 mL snap-cap sterile tubes
6. Cylinder of 5% O₂, 5% CO₂, 90% N₂ with regulator
7. Warming plate
8. Discard bottles
9. Chang C medium with Chang supplement, 1% L-glutamine, and 0.1% gentamicin (shelf life 10 days)
10. AmnioMAX medium, Invitrogen (shelf life 14 days)

For subculture and harvest

11. Polypropylene centrifuge tubes with screw caps
12. Test tube rack
13. 1, 5 and 10 mL pipettes
14. Centrifuge
15. Micropipettors with sterile tips
16. Colcemid®, Gibco®, 10 mg/mL
17. Hanks' 1× saline, sterile (10× Hanks' diluted 1 part to 9 parts double distilled sterile water)
18. Hanks' trypsin-EDTA (2 mL of 10× Gibco® trypsin-EDTA diluted to 30 mL of Hanks' 1× saline)
19. Harvest medium (Ham's F-10 supplemented with 10% fetal bovine serum)
20. 0.075 M KCl (0.559% in distilled water)
21. Hypotonic solution
Stock hypotonic: 1 : 4 fetal bovine serum: sterile double distilled water
Working solution: 1 : 3 stock hypotonic/0.075 M KCl
22. Fixative, 1:3 acetic acid: methanol, freshly made

III. Method

Safety warnings

All tissue specimens should be handled as biohazardous, using Universal Precautions. Use the laminar flow hood for all steps up to harvest spin. Wear a laboratory coat and protective gloves for all steps. Avoid spills and contact of any biological materials with skin. Clean up spills immediately with fresh Sanimaster 4, or 70% ethanol. Dispose of Pasteur pipettes in sharps container. Wash hands thoroughly after removing gloves.

Colcemid® is mutagenic, tumorigenic, embryotoxic, and teratogenic with acute overexposure. Wear gloves and lab coat at all times when handling. Avoid contact with skin or inhalation, flush for 15 minutes if accidental contact.

Flask setup and culture

1. The specimen is drawn any time from 11 weeks gestational age to term. It is usually delivered to the laboratory in two or three aliquots, in 15 mL centrifuge tubes, 6–10 mL in each tube (for early amnios, approximately 1 mL per gestational week is received).
2. Each fluid is assigned a case number, entered into the log book, entered on the tissue culture flow sheet and in the computer with pertinent patient information. If sample is bloody, clotted, discolored, etc., note on amniotic fluid flow sheet and on the worksheet.
3. Centrifuge amniotic fluid tubes at 1000 RPM for 10 minutes.
4. Carry out set-ups and tissue culture in laminar flow hood. Decant fluid with sterile pipette. Save a 3 mL aliquot of supernatant in a sterile 5 mL tube for AFP (alpha-fetoprotein) testing (see AFP notes which follow) and another 3 mL aliquot in a second tube if AChE (acetylcholinesterase) is required. Save 3 mL of supernatant for a backup. Leave 1 mL amniotic fluid for each 6–8 mL of original volume.
5. Label Corning T-25 and T-12.5 flasks with GL#, name, date, and flask number. Number of flasks depends upon volume of sample and gestational age of patient, but the usual is to set up one T-25 and two T-12.5 flasks/patient. If in doubt, consult with the Lead Technologist. Label only one patient's flasks at a time. Work with only one patient at a time in the hood. Using sterile pipette, for each tube, resuspend cells and transfer cell suspension to a flask containing 3 mL medium for T-25 flask, and 2 mL medium for T-12.5 flasks. (The first and third flasks are labeled A and C and have complete Chang medium; the second flask is labeled B and contains AmnioMAX medium.)
6. Gas all flasks for 3–10 seconds with 5% CO₂, 5% O₂, and 90% N₂, capping tightly and incubate in 37 °C water-jacketed CO₂ incubator. Flasks A and B are incubated in incubator #1 and flask C in incubator #2.
7. Incubate and leave undisturbed until first feeding, which usually takes place on first Monday following set-up. Note: If on Monday they are less than 5 days old, cultures may be fed later in the week.
8. Feed twice weekly with 2 mL (T-12.5 flasks) or 3 mL (T-25 flasks) aliquots of fresh medium per flask until ready to subculture or harvest.

Flask subculture

9. Reagents should be at room temperature. Rinse culture two times with 2 mL of prewarmed Hanks' 1×.
10. Trypsinize with 1 mL of the Hanks'-trypsin. Shake or tap vessel to loosen cells, if necessary.
11. Depending on the number of cells, transfer 0.1–0.9 mL of this cell solution to another T-25 flask. In cases where growth is light, it may be necessary to transfer all of the material from the primary to the subculture, or to simply disperse and feed original flask without transferring cells.
12. Add 2–3 mL medium (T-12.5 and T-25 flasks, respectively) to subculture flask and 2–3 mL medium to primary flask.
13. Gas flasks with 5% CO₂, 5% O₂ and 90% N₂ and incubate in a 37 °C water-jacketed CO₂ incubator.
14. Cells are normally ready for harvest 1–4 days following subculture.

Flask harvest

REMINDER: Wear protective gloves and coat. Use universal precaution until cells are in fixative.

15. Add 3 µL of stock solution Colcemid® per mL of culture medium (e.g., for 3 mL, add 9 µL of Colcemid®) to each flask. Incubate at 37 °C for approximately 2.5 to 3 hours.
16. Decant medium and place in 15 mL centrifuge tube.
17. Add 2 mL Ca/Mg-free Hanks' BSS to culture (Hanks' without trypsin), rinse, decant fluid into same tube. Repeat, pipet into tube (4 mL total rinse).
18. Add 1 mL Hanks'-trypsin-EDTA to culture vessel. Place on warming tray for 1–5 minutes. Shake vessel to loosen cells, if necessary.
19. Add cell suspension to the same tube.
20. Rinse flask with 4 mL harvest medium and add to tube.
21. Centrifuge at 1000 RPM for 10 minutes. Aspirate supernatant down to approximately 1 mL.
22. Resuspend cells by flicking tube with index finger.
23. Start timer for 15 minutes. Add 3 drops of warm hypotonic, mix, then add up to 3 mL and incubate at 37 °C for 15 minutes.
24. Add 5 to 10 drops of fixative into each tube; mix well.
25. Centrifuge at 1000 RPM for 10 minutes. Aspirate to 1 mL.
26. Resuspend gently but as thoroughly as possible before addition of fixative.
27. Add 3 drops of fixative (1:3, acetic acid: methanol), mix, add to 1/2 mL. Mix, add to 2 mL for subculture and 1.5 mL for direct. Let stand 20 minutes.
28. Centrifuge as before and aspirate to 1 mL.
29. Add approximately 1 mL fixative, judging by pellet size, resuspend and make slides.

IV. Notes

1. This method for tissue culture of amniotic fluid cells employs closed flasks, which improves the length of the chromosomes and the quality of the study, and controls or eliminates contamination problems encountered with an open system, and allows additional material for follow-up special staining or FISH.
2. Sufficient growth is judged on colony size, cell type, and number of colonies. Amount transferred requires a total cell number such that subcultures will yield a T-25 close to confluence in 3–4 days (usually 4 or more colonies per flask which fill the microscope field). Cultures are generally subcultured 6–9 days following initiation.

V. Additional readings

1. Bergsma D, ed. Intrauterine diagnosis. In: Birth Defects- Original Article Series VII/5. White Plains: National Foundation - March of Dimes (USA), Apr 1971.
2. Hoehn H, Bryant EM, Karp LE, Martin GN. Cultivated cells from diagnostic amniocentesis in second trimester pregnancies. I. Clonal morphology and growth potential. *Pediatric Res* 1974;8:746–754.

Protocol 5.5 Criteria for interpreting mosaic amniotic fluid cultures

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

Categories	Harvest third culture	Extend only in 2 orig. cultures	Add'l scores only	Add'l counts	FISH
I. True mosaics: Two or more distinct karyotypes, each present in more than one cell, existing in more than one independently harvested culture flask. [1]	X				X
II. Suspected mosaics: two or more distinct karyotypes, each present in more than one cell, found in one culture flask with the exception of the predominant karyotype. FINAL REPORT WILL READ: Normal—true mosaicism is unlikely.	See specific types 1–5				
1. Two or more cells with a gain (“up”) of a chromosome associated with a syndrome compatible with life	X				X
2. Two or more cells up a marker chromosome	X				X
3. Two or more XO cells	X				X
4. Two or more cells with deletion or duplication	X				X
5. Two or more cells with the same rearrangement	X				X
III. Cultural Artifact: two or more distinct karyotypes, one predominant and the other(s) present in one cell. FINAL REPORT WILL READ: Normal—likely cultural artifact.	See specific types 1–11				
1. One cell up an autosome associated with a common viable mosaic syndrome (#8, #9, #13, #17, #18, #20, #21, #22)	X				X
2. One cell up a sex chromosome	X				X
3. One cell with deletion associated with a common syndrome (del 4p, del 5p, del 9p, del 10p ter, del 11p (interstit.), del 12p, del 13q, del 18p		X			X
4. One cell with del (15q11–13) as with Prader-Willi/Angelman, del(17p), or del (22q11.2) as in DiGeorge/Velocardiofacial syndrome		X			X
5. One cell up an autosome rarely associated with viable mosaic syndrome (#3, #10, #12, #14, #15)		X			X
6. One XO cell	X				
7. One cell up a marker chromosome		X			

Categories	Harvest third culture	Extend only in 2 orig. cultures	Add'l scores only	Add'l counts	FISH
8. Two or more cells up an autosome not associated with a syndrome compatible with life. (Conclusions drawn from 6,969 amniotic fluid specimens indicated actual fetal mosaicism is a problem only when the aneuploidy karyotype is one known to be compatible with life and associated with known phenotype.) [2]	N/A	N/A	N/A	N/A	N/A
9. *** One cell up an autosome not associated with a syndrome compatible with life	N/A	N/A	N/A	N/A	N/A
10. ***One cell with rearrangement	N/A	N/A	N/A	N/A	N/A
11. ***One cell with deletion or duplication	N/A	N/A	N/A	N/A	N/A

***FINAL REPORT will not mention observed abnormalities in these cases.

Summary

Routinely two flasks are harvested on each patient and 10 cells from each culture examined for a total of 20. A third flask is harvested and 20 cells examined from each flask for a total of 60 cells in cases fitting criteria for Categories I and II and Numbers 1 and 2 of Category III. In cases fitting criteria for Numbers 3, 4, 5, 6, and 7 of Category III, an additional 20 cells are examined from the two original flasks for a total of 40, while no additional cells are examined in cases fitting criteria for Numbers 8, 9, 10 and 11 of Category III. Additional cells examined for hypermodal cells require cell *counts*, while cells examined for rearrangements, deletions, duplications, and missing sex chromosomes need only be *scored* for the specific abnormality. Number 4 of Category III requires FISH with region-specific probe and identifier.

References

1. Golbus MS, Loughman WD, Epstein CJ, Halbasch G, Stephens J, Hall BD. Prenatal diagnosis in 3,000 amniocenteses. *New Engl J Med* 1979;300(4):157–163.
2. Summitt RL, Breg WR, Hook EB, Magenis RE, Palmer CG, Pasztor LM, Wilson RT. Spurious cells in amniotic cell cultures from multiple laboratories. 31st Annual Meeting, Am. Society Human Genetics, 1980:90A.

Protocol 5.6 Chorionic villi sampling – setup, direct harvest, and culture

Contributed by the UW Cytogenetic Services Laboratory, Madison, WI

I. Principle

Cytogenetic analysis of cells from chorionic villi can yield important prenatal diagnostic information. The chorionic villi contain cells from the trophoblast (outer layer of the villi) and mesoderm (mesenchymal core). The trophoblast is actively dividing and is used for the direct preparation; the mesoderm is used for the long-term culture. In situ harvest of cultures maintains the integrity of the colonies that were formed; this allows analysis of the chromosomes and provides information regarding clonality. Chorionic villus sampling for chromosome analysis is generally performed between the 10th and 12th weeks of gestation, but may be performed up to the 35th week.

Safety: Appropriate personal protective equipment (PPE) must be used whenever handling biological specimens. Universal precautions for blood borne pathogens must be followed at all times. Cultures must be set up in a Biological Safety Hood; the work surface must be wiped down with fresh 10% bleach solution at the beginning and end of each work session. Counters must be wiped down with fresh 10% bleach solution at the end of each work session.

II. Materials

Reagents

1. 10% bleach solution, prepared fresh
2. Chang Medium D, liquid: Irvine Scientific, 100 mL, store at -20 °C
3. AF/ CVS media: Chang Medium D. Label with "A or B", date, and initials of preparer, store at 4–8 °C, expiration of two weeks.
4. Collagenase-Dispase Powder: Roche, 100 mg lyophilized lots
5. Collagenase-Dispase working solution: 100 mg collagenase-dispase in 100 mL Hanks' (Ca/Mg free), filter (0.2 µ) 3.0 mL aliquots into 15 mL tubes, store at -20 °C.
6. Hanks' balanced salt solution, with phenol red (1×) (Ca/Mg free): Invitrogen Corp, 500 mL lots
7. Trypsin-EDTA (1×), liquid: Invitrogen, 500 mL lots, aliquot into 5 mL and store at -20 °C
8. Acetic acid, glacial: Fisher Scientific, 2.5 L lots

General supplies/equipment

9. Biological Safety Hood, Laminar Flow; Baker Co.
 - a. SterilGARD® III Advance
 - b. SterilGARD® II Type A/B3
10. PPE including lab coats and gloves
11. Autoclave: Harvey SterileMax™, Fisher Scientific
12. Centrifuge set to 1000 RPM
 - a. DuPont Sorvall T 6000D, Fisher Scientific
 - b. IEC HN-SII, Fisher Scientific
13. Centrifuge tubes
 - a. Polystyrene conical (15 mL): Sarstedt, 50 tubes
 - b. Polypropylene disposable (15 mL): Sarstedt
14. Coverslips: 22 × 22 mm Gold Seal: Fisher Scientific, #2 thickness, 10 ounce lots, sterilize by autoclave before use
15. Exam gloves: Fisher Scientific
 - a. Powder-free Nitrile: qty 100
 - b. Microflex: qty 100
16. Forceps: Fisher Scientific, Jeweler Micro forceps, straight, sterilize by autoclave before use
17. Incubators: All at 37 °C, 5% CO₂
18. Inverted Phase microscopes (x3): Olympus CK2 ULWDC.30 CH-B145-2
19. Lab coats: Allegiance, Kimberly-Clark White Universal Precautions Lab Jackets
20. Petri dish: Fisher Scientific
 - a. 35 × 10 mm: 20/bag
 - b. 60 × 15 mm: 20/bag
 - c. 100 × 15 mm: 20/bag
 - d. 150 × 15 mm: 10/bag
21. Pipettes
 - a. 5 mL: Sarstedt, sterile, 25/bag
 - b. 10 mL: Sarstedt, sterile, 25/bag
22. Pipettes (Transfer)
 - a. Sterile, Fine-tip: Fisher Scientific, 500/box
 - b. Fine-tip: Fisher Scientific, 500/box
 - c. Wide-tip: Sarstedt, 3.5 mL disposable, 500/box
 - d. Glass Pasteur: Fisher Scientific, 250/box
23. Pipet Aide: Drummond Scientific Pipet-aid® S/N P-66122, Fisher Scientific
24. Refrigerator/Freezer Combo: GE General Electric
25. Scale: Mettler Toledo, Model BD202, Fisher Scientific
26. Scissors: Fisher Scientific, #08-951-20, sterilize by autoclave before use

27. Super Sani-Cloth: Owens-Minor
28. Tissue Culture Flasks: Fisher Scientific T-25, 25 cm²: canted neck, sterile, phenolic style cap

III. Method

A. Setup procedure: Day 1

1. Accession the specimen.
2. If more than one CVS specimen is received in a day, the labels for each case are color-coded using the highlighters available.
3. Move the inverted phase microscope into the Biological Safety Hood so that the eyepieces remain outside of the hood when the sash is down*. *Note: This may not be possible with some microscopes, may disrupt the air flow in the hood and is also an uncomfortable working position for some technologists. Alternatively, cleaning of the villi can be done outside of the Biological Safety Hood, as long as measures are taken to avoid specimen contamination.
4. Put one 100 × 15 mm and one 60 × 15 mm Petri dish into the hood. Pipet 5 mL sterile Hanks' solution into the bottom of the 100 mm dish and pipet 5 mL AF/CVS media into the 60 mm dish. Place a printed case label on the 60 mm Petri dish.
5. Verify that the name and case number on the specimen flask matches the name and case number on the 60 mm dish before continuing with the procedure.
6. Transfer (pour) the CVS specimen into the top of the 100 mm Petri dish and examine the specimen using the inverted microscope.
7. Use sterile forceps to transfer the pieces of villi to the dish containing Hanks' solution.
8. Using a 22 gauge needle with a 1 mL syringe as a handle to manipulate the tissue, carefully examine each piece of villus using the inverted phase microscope. Hold the villus in place with the sterile forceps and remove all attached maternal decidua (soft and homogenous-looking tissue), blood clots, and mucus using the needle. Transfer the villi into the 60 mm Petri dish with AF/CVS media using the forceps and needle.
9. After all of the material has been transferred, the specimen is examined by a second technologist (each technologist will initial the case folder). A second technologist MUST check the specimen to verify that no decidua is present.
10. **After use, the syringe and needle must be disposed of in a sharps container following universal guidelines for bloodborne pathogens. It must be disposed of as a single unit. Do NOT attempt to remove the needle from the syringe!**
11. Estimate the milligram amount of villi present and document it on the front of the case folder and in the computer. If no villi or less than 10 mg of villi are present, notify the supervisor, laboratory director, or an advanced technologist who will then notify the referring clinician.
12. Place the specimen in the incubator at 37–38 °C with 5% CO₂ until the next morning.

B. Setup procedure: Day 2

Note: If the sample is less than 10 milligrams proceed to step 15. Do not process for a direct harvest. Inform an Advanced Technologist or Supervisor who will then notify the ordering physician that a direct harvest will not be performed.

1. Verify that the patient name and lab number on the specimen dish matches the patient name and lab number on all other accompanying material before continuing with the procedure.
2. Take a tube of collagenase-dispase (1 mg/mL) and a vial of Trypsin-EDTA (1×) from the freezer. Place the tube and vial into the incubator at 37–38 °C to thaw and pre-warm. Before use, label the tube and the vial with a patient label, the date and “LT”.
3. Put a printed case label on one 15 mL polystyrene centrifuge tube and label with the date and “Direct”.

The remainder of the day 2 setup must be done aseptically inside the Biological Safety Hood.

4. Use a sterile Pasteur pipette to add three drops of STERILE Colcemid® (10 µg/mL) to the 60 mm dish containing the specimen. Return the dish to the incubator for 45–60 minutes.
5. Near the end of the incubation period, put one 60 × 15 mm and one 35 × 10 mm Petri dish into the hood. Pipet the pre-warmed 1× EDTA trypsin and 2 drops of Colcemid® solution into the 60 mm dish and pipet 3–4 mL Hanks' solution into the 35 mm dish. Place a printed case label on the 60 mm Petri dish.

6. Use sterile forceps to transfer the villi from the specimen dish to the 35 mm dish with Hanks' solution.
7. Swirl the dish for several seconds to rinse the medium off the villi, and then use the forceps to swish each piece of villus briskly and transfer it to the labeled dish containing trypsin + Colcemid®.
8. Incubate the villi at 37–38 °C with 5% CO₂ for 5 minutes. Using a sterile transfer pipette, pipet the solution with the villi up and down 8–10 times to cause the outer cells to slough off (avoid air bubbles).
9. Repeat step 8.
10. Examine villi under scope; if outer cells are not sloughing off, return to incubator for an additional 2–5 minutes. (NOTE: Villi should not be left in the trypsin solution for more than 15 minutes; they will become too sticky to handle.)
11. If cells are coming off, use the forceps to swish each piece of villus briskly and transfer it back to the 35 mm dish containing 5 mL Hanks' solution.
12. Use a sterile Pasteur pipette to add two drops of FBS to inactivate the trypsin solution left in the 60 mm dish.
13. Using a sterile *transfer* pipette, pipet the Hanks' solution with the villi up and down 8–10 times.
14. Invert the lid of the 35 mm dish to use as a working dish; pour about 1.5 mL of the pre-warmed collagenase-dispase working solution into the lid dish. Use sterile forceps to transfer the villi cores to the lid dish. Use scissors to thoroughly mince the villi cores. Using a sterile transfer pipette, transfer the minced material to the labeled tube (rinse dish with remaining 1.5 mL of collagenase-dispase working solution). Incubate the tube 37–38 °C with 5% CO₂ (with the cap loosened for gas exchange) for 3–4 hours, mixing periodically during the incubation. Incubation time may be shortened to ~2.5 hours on weekends and holidays.
15. Using a sterile transfer pipette, transfer the cells in the inactivated trypsin and Hanks' solution dishes to the pre-labeled 15 mL polystyrene centrifuge tube. Rinse the dishes using the medium from the specimen dish and transfer to the centrifuge tube. These cells will be used for the direct preparation. See step E (Direct harvest procedure).
16. When collagenase-dispase incubation is complete, tightly cap the tube and centrifuge at 1000 RPM for 8–10 minutes.
17. Write the processing day's date on three labels; use one to label a T-25 flask, one for a 150 × 15 mm Petri dish, one for a cardboard slide box. Using the remaining labels, label the appropriate number of slides, generally 6. Refer to Table 5.2 in the IV. Notes section of this protocol for guidelines to variation in the number of cultures to set for small specimens.
18. Label the lids of the same number of 35 × 10 mm Petri dishes as slides using the Lumocolor permanent marker with the patient's last name, the last three digits of the case number and 1, 2, etc. Label the side of the bottom part of the dish with the last three digits of the case number.
19. Place the 35 × 10 mm Petri dishes into the 150 × 15 mm Petri dish.
20. Flame forceps tips to sterilize. Use forceps to remove sterile coverslips one at a time from storage container, flame each side of the coverslips and place one into each of the 35 × 10 mm Petri dishes.
21. Verify that the patient name and case number on the specimen tube matches the patient name and case number on the culture dishes before continuing with the procedure.
22. Remove the supernatant from the centrifuged tube using a sterile pipette. With a 5 mL sterile plastic pipette, add the appropriate volume of AF/CVS medium to the tube and resuspend the pellet by gently pipetting up and down; avoid creating bubbles in the cell suspension. Typically 3.5 mL of medium is used unless fewer coverslips will be set due to small sample size.
23. Pipet 0.5 mL of the cell suspension onto each of the coverslips (use the tip of the pipette to spread the suspension evenly across the coverslip, but do NOT allow it to go over the edge) and pipet the remaining suspension into the T-25 flask. With a 5 mL sterile plastic pipette, add an additional 3 mL of AF/CVS medium to the tube, rinse by pipetting up and down, and transfer the medium to the flask.
24. Place the cultures (flask and Petri dishes with coverslip cultures) in the incubators at 37–38 °C with 5% CO₂. Make sure the cap on the flask is loose for CO₂ exchange and is cultured flat on its back in the back incubator. The coverslips cultures are placed in the "A" incubator and the flask culture is placed in the "B" incubator.

C. Culture procedure

The culture procedures must be done aseptically inside the Biological Safety Hood.

1. Two days after set up, add 1 mL of fresh AF/CVS Chang D media to each coverslip culture dish: **A** media to the **1, 2, and 3** coverslips and **B** media to the **4, 5, and 6** coverslips. Cultures set on Friday will have the fresh media added on Monday. This step is referred to as "flooding."

- a. If there is a lot of cellular debris, blood, or protein on the coverslip cultures, pipet the culture media gently across the coverslips to loosen the debris before removing the media.
2. Return the cultures to the incubator.
3. After the media has been changed, the coverslip cultures are checked daily for growth. In the afternoon, cultures are examined using the inverted phase microscope to select coverslips to be harvested the following morning. Coverslips that are ready for harvest should have five to fifteen mid-size colonies (50–100 cells per colony) and show signs of active cell division (cells that have rounded up, dumbbell-shaped cells that are in anaphase).

D. Reagent addition for harvest of long term cultures

1. Add 30 µL EtBr and 30 µL Colcemid® (1 HR cultures only) working solution to each dish, swirl very gently to disperse the reagent (avoid dislodging cells from the colony they belong to). Return cultures to the incubator for 60 minutes. Add 30 µL of EtBr only and return the cultures to the incubator for 60 minutes.
NOTE: If cultures appear to have a low mitotic index, ONC (overnight Colcemid®) incubation may be designated on the top of the dish; 4 µL Colcemid® is added at the end of the day before the harvest.
2. Cultures are ready to be harvested using the TECAN robotic harvester or manually (see Protocol 5.2 for the manual (*in situ*) harvest procedure).

E. Direct harvest procedure

1. Centrifuge cells from step B.15 at 1000 RPM for 8–10 minutes.
2. After centrifuging, use a fine tip transfer pipette to remove the supernatant and *gently* tap the tip of the tube to resuspend the cell pellet. Add 2–3 mL pre-warmed hypotonic solution (0.075 M KCl). Cap tightly, mix gently, and incubate at 37–38 °C for 20 minutes.
3. Use a wide tip transfer pipette to add 8–10 drops of 3 : 1 methanol–glacial acetic acid fixative, gently mix and let stand at room temperature for ~5 minutes.
4. Centrifuge the tube at 1000 RPM for 8 minutes. Use a fine tip transfer pipette to remove the supernatant to within 0.5 mL of cell pellet. Resuspend the cell pellet in 2–3 mL of fresh fixative, running the fixative along the side of the tube. Place in freezer for 25–30 minutes.
5. Centrifuge the tube at 1000 RPM for 8 minutes. Use a fine tip transfer pipette to remove the supernatant to within 0.5 mL of cell pellet. Resuspend the cell pellet in 2–3 mL of fresh fixative.
6. Repeat step 5 (resuspend in fresh fix a total of 3 times).
7. Procedure for preparing slides is described in Chapter 2, section 2.4, Slide-making. Check for appropriate cell density and chromosome spreading with phase microscopy.
8. Make desired dilution changes and make remaining slides (usually 2 slides per culture).
9. Bake the slides in the oven at 90 °C for 30–60 minutes, sometimes longer (based on drying conditions, i.e., humidity).
10. The slides are now ready for G-banding (see Protocol 5.8, *G-banding with Leishman's stain*) or special stain methodologies in Chapter 6.
11. Follow Chromosome analysis guidelines (see Protocol 5.5, Criteria for interpreting mosaic amniotic fluid cultures) when examining the slides.

IV. Notes

1. Variation in number of cultures to set for small specimens is outlined in Table 5.2.
2. If, when setting up coverslips, the cell suspension does not stay confined to the coverslip, transfer the cell suspension to a new coverslip in a new 35 mm Petri dish, being careful to confine the suspension to the surface of coverslip.
3. Media that “wicks” out of dish and onto the lid and the rim of dish is a potential cause for contamination.
 - a. If this happens at any time before the fourth day of culture, with a sterile 5 mL pipette, transfer all of the culture media to a 15 mL centrifuge tube and centrifuge at 1000 RPM for 8–10 minutes. Remove the coverslip from the dish and place into a new, labeled, culture dish. Resuspend the cell pellet in 1.5 mL fresh AF/CVS medium and pipet the cell suspension onto the coverslip in the new culture dish.
 - b. If this happens at any time after the fourth day of culture, remove the coverslip from the dish and place into a new, labeled, culture dish. With a sterile 5 mL pipette, add 1.5 mL of fresh AF/CVS medium to the culture dish.

Table 5.2 Determining culture number based on specimen size

Size of sample	Direct harvest?	Long term cultures to set		Sample distribution
		# Flask	# in situ	
≤5 mg	No	0	2	Divide equally between both culture types
6–10 mg	No	1	4–6	Each in situ culture is seeded with 1–2 mg villi and remaining amount is added to flask
≥10 mg	Yes	1–2	6	Each in situ culture is seeded with 1–2 mg villi and remaining amount is added to flask(s)

4. Save one coverslip in reserve for possible subculturing. It also may be necessary to trypsinize or “spread out” slow growing cultures (record the number of colonies present on the dish before trypsinizing). Follow the protocol for subculturing on coverslips under Protocol 4.11, Routine subculture of fibroblast cultures in Chapter 4.
5. A common source of error is mislabeling. To avoid this error:
 - a. Label and set up only one patient specimen at a time.
 - b. Only handle cultures from one case at a time.
 - c. All slides must be labeled as soon as the cell suspension has dried and before proceeding to the next culture tube.

Protocol 5.7 Chorionic villus sampling

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

This is a method for obtaining fetal chromosome preparations from samples of chorionic villi at 10–12 weeks gestational age. This relatively early timing may provide chromosomal results in the first trimester. Samples must be well-cleaned of maternal decidua to prevent maternal cell contamination. It is well-documented that chromosomal mosaicism may be confined to the placenta supporting a chromosomally normal fetus. Approximately 2% of CVS samples exhibit mosaicism, requiring follow-up amniotic fluid studies to assess the significance of the mosaicism to the fetus.

Safety warnings

1. All tissue specimens should be handled as biohazardous, using Universal Precautions.
2. Use the laminar flow hood for all steps up to harvest spin.
3. Wear a laboratory coat and protective gloves for all steps through slide-making.
4. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with Sanimaster 4 (made weekly) or 70% ethanol.
5. Cover cuts with protective bandages even when gloves are worn.
6. Dispose of glass Pasteur pipettes, needles and syringes in sharps container.
7. Wash hands thoroughly after removing gloves.
8. Colcemid is mutagenic, tumorigenic, embryotoxic and teratogenic with acute overexposure. Wear gloves and lab coat at all times when handling. Avoid contact with skin or inhalation, flush for 15 minutes if accidental contact.
9. *Do not bend, recap, break, or remove needles from disposable syringes. Dispose of all needles in a labeled sharps container.*

II. Materials

A. For setup and culturing

See Chapter 4, Protocol 4.3 Tissue culture reagents.

1. 60 mm² sterile plastic Petri dishes
2. 1 mL TB syringes
3. Sterile forceps
4. Sterile, plastic, screw-cap centrifuge tubes
5. Sterile 25 mm² tissue culture flasks (Corning)

6. 1 mL, 5 mL and 10 mL sterile graduated pipettes
7. Sterile cotton-plugged Pasteur pipettes
8. Discard bottle
9. Cylinder of 5% O₂, 5% CO₂, 90% N₂ with regulator
10. Warming plate
11. Chang C medium with Chang supplement, 1% L-glutamine and 0.1% gentamicin
12. Trypsin 0.25% (Invitrogen) already aliquoted, 5 mL in test tubes, frozen (same as banding trypsin)
13. Collagenase
 - a. Sigma type IV-S, 536 units/mg, reconstituted with 10 mL sterile Hanks' saline or incomplete media. Use 0.20-0.25 mL in 5 mL incomplete media.
 - b. Worthington Type I Collagenase, 210 units/mg. Dilute with incomplete medium to a final concentration of about 1 unit per mL, rounding up to the closest multiple of 10 (e.g., for 142 U/mg use 150 mL medium). Filter through a vacuum filtration unit with 0.2 µm pore size. Aliquot into 4 mL snap cap sterile tubes and freeze. Shelf life 1 year.

B. For subculture in preparation for harvest

See Prenatal tissue culture reagents under 5.4 Amniotic fluid culturing, subculturing, and harvesting (flask method).

1. Test tube rack
2. Sterile polypropylene centrifuge tubes with screw caps
3. 1, 5, and 10 mL pipettes
4. Micropipettor with sterile tips
5. Colcemid®, Invitrogen, 10 mg/mL
6. Hanks' 1× saline, sterile
7. Hanks'-trypsin EDTA (2 mL of 10× Invitrogen trypsin in EDTA diluted to 30 mL of Hanks' 1× saline), sterile
8. Harvest medium: Ham's F-10 supplemented with 10% fetal bovine serum
9. 0.075 M KCl (0.559% in distilled water)
10. Stock hypotonic: 1 : 4 fetal bovine serum–sterile water
11. Working hypotonic solution: 1 : 3 stock hypotonic/0.075 M KCl

C. For direct harvest

1. Trypsin 0.25% (Invitrogen) already aliquoted, 5 mL in test tubes, frozen (same as banding trypsin)
2. Colcemid®, Invitrogen, 10 mg/mL
3. Polypropylene centrifuge tubes with screw caps
4. Working hypotonic solution: 1:3 stock hypotonic/0.075 M KCl
5. 0.075 M KCl (0.559% in distilled water)
6. Stock hypotonic: 1 : 4 fetal bovine serum–sterile water
7. 3 : 1 methanol–acetic acid fixative

D. For direct harvest (on uncultured cells)

1. Trypsin 0.25% (Invitrogen) already aliquoted, 5 mL in test tubes, frozen (same as banding trypsin)
2. Colcemid® (Invitrogen), 10 mg/mL
3. Polypropylene centrifuge tubes with screw caps
4. Working hypotonic solution: 1 : 3 stock hypotonic/0.075 M KCl
5. 0.075 M KCl (0.559% in distilled water)
6. Stock hypotonic: 1 : 4 fetal bovine serum–sterile water
7. 3 : 1 methanol–acetic acid fixative

III. Method

A. Setup and culture

1. Pour contents of transport vial into sterile plastic 60 mm Petri dish. Transfer villi with sterile forceps to 5 mL fresh Chang in sterile 60 mm Petri dish.
2. Under dissecting microscope, give villi initial cleaning using needles of two TB syringes, and choose best villi, stripped of maternal decidua, for cultures and transfer to a new dish with 5 mL Chang. A second technologist should check and re-clean the villi.

3. Add 5 mL tube of 0.25% banding trypsin into a sterile 60 mm Petri dish. Remove villi from Chang with sterile forceps, and place in dish with trypsin. Incubate in trypsin one hour at 37 °C, 5% CO₂ in air. (If sample is >20 mg, set up two Petri dishes for trypsin incubation.)
4. At the end of one hour, evaluate villi using inverted microscope. The outer layer of cells should be coming off, and single cells will be observed floating in trypsin. If not, add 1–2 mL fresh trypsin and incubate 30 minutes. **Note: If sample size is ≤10 mg, adjust (lessen) time in trypsin and/or collagenase accordingly.**
5. Pick up each piece with sterile forceps, shake in trypsin to dislodge loose cells, and transfer tissue to a sterile 60-mm Petri dish containing 5 mL of collagenase solution (4 mL defrosted Worthington).
6. Incubate the collagenase-cell suspension for 2 hours. (If more than one Petri was used for trypsin incubation, use an equal number for collagenase treatment.) Meanwhile, if a *direct harvest for interphase or metaphase cells* is desired, transfer the trypsin solution to a 15-mL centrifuge tube and follow the Direct Harvest protocol below (III.E). Otherwise, trypsin solution can be discarded.
7. After 2 hours, transfer the collagenase-cell suspension to a sterile centrifuge tube with a sterile Pasteur pipette. Spin at 1000 RPM for 10 minutes.
8. Aspirate supernatant by hand with Pasteur pipette and discard. Resuspend pellet in 8 mL of Chang medium. Spin at 1000 RPM for 10 minutes.
9. Resuspend cell pellet in 3 mL of Chang. Divide medium and villi between 2 or 3 T-25 flasks according to the sample size. Gas with 5% CO₂, 5% O₂, 90% N₂ and incubate at 37 °C.
10. Cell growth should be observed after 2–3 days at which time 2 mL of Chang medium is added. When colonies are established, cells are fed with 3 mL of Chang.

B. Subculturing flask in preparation for harvest

Caution: Wear protective gloves and coat. Use universal precaution until cells are in fixative.

1. Cultures can be subcultured for harvest when they have reached sufficient growth, as determined by colony size, cell type, and number of colonies, so that a T-25 flask will reach confluence in 3–4 days (usually 4 or more colonies per flask which fill the microscope field), generally 6–9 days following initiation.
2. Reagents should be at room temperature. Rinse culture two times with 2 mL of pre-warmed Hanks' 1×.
3. Trypsinize with 1 mL of the Hanks'-trypsin. Shake or tap vessel to loosen cells, if necessary. Depending on the number of cells, transfer 0.1–0.9 mL of this cell solution to another T-25 flask.
4. Add 3 mL of medium to subculture flask and 3 mL of medium to primary flask.
5. Gas flasks with 5% CO₂, 5% O₂ and 90% N₂ and incubate in a 37 °C water-jacketed CO₂ incubator.
6. Cells are normally ready for harvest 1–2 days following subculture.

C. Harvest of cultured chorionic villi

1. Using stock Colcemid® solution, add 3 µL/mL of medium from micropipettor to each flask (9 µL for 3 mL of medium). Incubate at 37 °C for approximately 2.5 hours.
2. Decant medium and place in 15-mL centrifuge tube.
3. Add 2 mL of Ca/Mg-free Hanks' BSS to culture (Hanks' without trypsin), rinse, and decant fluid into the same tube. Repeat and pipet into tube (4 mL total rinse).
4. Add 1 mL of Hanks'-trypsin-EDTA to culture vessel. Place on warming tray for 1–5 minutes. Shake vessel to loosen cells, if necessary.
5. Add cell suspension to the same tube.
6. Rinse flask with 4 mL of harvest medium and add to tube.
7. Centrifuge at 1000 RPM for 10 minutes. Aspirate supernatant down to approximately 1 mL.
8. Resuspend cells by flicking tube with index finger.
9. Start timer for 15 minutes. Add 3 drops of hypotonic (room temperature), mix, add up to 1/2 mL. Mix, add to 3 mL for subculture and 2.5 mL for direct. Incubate at 37 °C for remainder of the 15 minutes.
10. Add 5–10 drops of fixative to each tube. Mix well.
11. Centrifuge at 1000 RPM for 10 minutes. Aspirate to 1 mL.
12. Resuspend gently but as thoroughly as possible before addition of fixative.
13. Add 3 drops of fix (1:3, acetic acid: methanol), mix, add to 1/2 mL. Mix, add to 2 mL for subculture and 1.5 mL for direct. Let stand 20 minutes.

14. Centrifuge as before and aspirate to 1 mL.
15. Add approximately 1 mL of fix, judging by pellet size, resuspend and make slides.
16. See Trypsin G-banding procedure (Protocol 6.12 in Chapter 6, Chromosome stains) for slide-making technique.

D. Direct harvest for FISH using interphase cells

1. During setup and culturing (III.A, step 6), instead of discarding the trypsin solution, it is used as a source of interphase cells. If insufficient cells are present, a little of the collagenase-cell suspension may be added to it after digestion has proceeded sufficiently. Pipet cell-trypsin suspension into a 15-mL conical centrifuge tube and centrifuge for 10 minutes at 1000 RPM.
2. Remove supernatant down to about ¼ mL above the pellet. Resuspend cells by flicking tube. Add 1–2 mL working hypotonic solution at 37 °C and incubate 10–15 minutes.
3. Add 5–10 drops of freshly made 3 : 1 fixative to the tube and mix gently. Centrifuge for 10 minutes at 1000 RPM.
4. Remove supernatant down to about ¼ mL above the pellet. Resuspend cells by flicking tube. Gently add 1–2 mL fresh 3 : 1 fixative. Let stand about 15 minutes or place in freezer for 15–20 minutes.
5. Centrifuge tube for 10 minutes at 1000 RPM. Remove supernatant, add fresh fixative, and make slides for FISH according to environmental humidity and temperature.

E. Direct harvest for metaphase chromosomes

During setup and culturing (III.A, step 6), instead of discarding the trypsin solution, it is transferred to a 15-mL centrifuge tube and used as a source of metaphase cells.

1. Add 15 µL of Colcemid® to the 5 mL of trypsin-cell suspension. Incubate 1–4 hours at 37 °C. Colcemid® may be added to the trypsin solution during the trypsin digestion step or it may be added after the villi have been removed to the collagenase solution.
2. Pipet cell-trypsin suspension into a 15-mL conical centrifuge tube and centrifuge for 10 minutes at 1000 RPM.
3. Remove supernatant down to about ¼ mL above the pellet. Resuspend cells by flicking tube. Add 1–2 mL of working hypotonic solution at 37 °C and incubate 10–15 minutes.
4. Add 5–10 drops of freshly made 3 : 1 fixative to the tube and mix gently. Centrifuge for 10 minutes at 1000 RPM.
5. Remove supernatant down to about ¼ mL above the pellet. Resuspend cells by flicking tube. Gently add 1–2 mL of fresh 3 : 1 fixative. Let stand about 15 minutes or place in freezer for 15–20 minutes.
6. Centrifuge tube for 10 minutes at 1000 RPM. Remove supernatant, add fresh fixative, and make slides for FISH according to environmental humidity and temperature.

F. Maternal cell contamination

46,XX samples suspected of being contaminated by maternal cells may be studied further using quinacrine heteromorphism origin studies to compare CVS and maternal variants. This requires a fresh heparinized blood sample from the mother, processed according to the Blood Specimen protocol. Quinacrine staining can be found in Chapter 6, Chromosome stains, under staining protocols.

IV. Notes

1. Problems encountered with CVS cytogenetic studies that are not usually encountered with amniocentesis cytogenetic studies are presence in or overgrowth of maternal (decidua) cells in cultures [1] and confined placental mosaicism (abnormal karyotype in placenta that is not present in the fetus) [2]. Villus samples must be well cleaned of maternal decidua to prevent maternal cell contamination. It is well-documented [3] that chromosomal mosaicism may be confined to the placenta supporting a chromosomally normal fetus. It is also possible for certain chromosomally abnormal placentas to affect the growth of a chromosomally normal fetus, especially when the abnormality affects placental function or when it indicates uniparental disomy resulting from a trisomic embryo rescue. Approximately 2% of CVS samples exhibit mosaicism, requiring follow-up amniotic fluid studies (which do not usually contain cells from the placenta and better reflect the karyotype of the fetus) to assess the significance of the mosaicism to the fetus.
2. Maternal deciduae are distinguished from fetal villi by morphology. Villi are whitish-pink in color with tiny capillaries sometimes running along the branches, which may be either attached together at a common point or detached from each other in single branches. Branches may be very thin and threadlike, or enlarged and balloon-like. Maternal decidua appears more white in color, and amorphous or cotton-like. It may be in separate pieces from the villi or it may be attached to the

villi. The villi are embedded in the decidua at their nonjoined tips *in vivo*, so look for bits of decidua attached to the tips of the villi looking like cotton or shredded Kleenex. It is important to remove as much of the nonvillous material as possible, including blood clots.

3. Chorionic villus samples are solid tissues requiring enzymatic digestion into small groups of cells to allow attachment and growth. This is accomplished using trypsin to remove the outer cytotrophoblast cell layer, and then collagenase to digest apart the villi. The trypsin portion can be discarded, as it does not attach and grow very well. However, it has spontaneously dividing cells in it that can be harvested immediately (direct harvest) and used for quick karyotyping results. The quality of the chromosomes is generally poor compared to cultured cell harvests and there is a small chance that the resulting karyograms do not represent the fetal genotype. For best results and to confirm the direct harvest results, the cells should be cultured and harvested after several days.

V. References

1. Bergsma D, ed. Intrauterine diagnosis. In: Birth Defects- Original Article Series VII/5. White Plains: National Foundation - March of Dimes (USA), Apr 1971.
2. Hoehn H, Bryant EM, Karp LE, Martin GN. Cultivated cells from diagnostic amniocentesis in second trimester pregnancies. I. Clonal morphology and growth potential. *Pediatric Res* 1974;8:746-754.
3. Kalousek DK, Dill FJ. Chromosomal mosaicism confined to the placenta in human conceptions. *Science* Aug 1983;221(4611):665-667.

Protocol 5.8 G-Banding with Leishman's stain (GTL)

Contributed by Genetic Diagnostic Laboratory, Children's Hospital at Erlanger, Chattanooga, TN

Editors' Note: for additional banding procedures, see Chapter 6: Chromosome Stains

I. Principle

G-banding techniques are the most widely used for banding in the cytogenetics laboratory. Slide preparation is important for optimum G-banding. Chromosomes should be well spread and medium gray in contrast under the phase microscope. Slides should be aged in order to achieve sharp banding patterns.

Specimen

Slides prepared from a cell suspension or prepared with a mounted coverslip from *in situ* culture, and aged 30 minutes in a 90 °C oven.

II. Materials

**CAUTION: REFER TO SAFETY DATA SHEET (SDS) FOR INFORMATION REGARDING USE AND HANDLING OF CHEMICALS.

Reagents

1. Buffer Working Solution
Preparation: Dissolve one pH 7.0 buffer tablet (pHydrion Buffer Tablets, pH 7.0., Micro Essential Laboratory, Inc.) in one liter deionized water. Storage is stable for 3 weeks at room temperature.
2. Hanks' Balanced Salt Solution (1×) with Ca and Mg, without phenol red; without sodium bicarbonate
3. Sodium Phosphate Dibasic Solution
Preparation: Dissolve 0.946 g of sodium phosphate dibasic (Na_2HPO_4) in 100 mL deionized water. Store at room temperature on stain table; shelf life, 1 year.
4. Leishman's Stock Stain
Preparation: Dissolve 0.8 g Leishman's stain in 500 mL of methanol. Add magnet and mix on magnetic stirring plate for 10–30 minutes. Incubate at 37 °C for 12–16 hours. Filter through Whatman paper into dark bottle. Store at room temperature; use within 1 month.

5. Leishman's working solution

Preparation: Make a cone out of a round sheet of Whatman paper, pour 10 mL of Leishman's Stock Stain through the paper into Coplin jar, add 30 mL of pHydriion solution and mix. Shelf life: Prepare fresh daily.

6. Trypsin EDTA Stock Solution (10×), stored frozen. Source: Gibco®, 100 mL

- a. Thaw.
- b. Aliquot 2 mL into fifty (50) 8-mL round bottom tubes.
- c. Freeze and use within 2 years.

7. Trypsin-EDTA Working Solution

- a. Add 2 mL of sodium phosphate solution to 36 mL of Hanks' balanced salt solution in Coplin jar.
- b. Add 2 mL of trypsin EDTA stock (10×) solution (thawed aliquot).
- c. Prepare fresh daily.

8. Deionized water (DI)

General lab supplies

1. Whatman #1 filter paper
2. Coplin staining jars
3. Paper towels

III. Method

1. Prepare two Coplin jars of working solutions listed above and place on staining table.

First jar: Trypsin working solution

Second jar: Leishman's working solution

2. Prepare 500-mL beaker with DI water.

3. Place slide in trypsin solution for desired time. Times vary greatly (15 seconds to over 2 minutes) for tissue type and depending on the temperature and humidity in the laboratory. A good starting point is 30 seconds.

4. Blot edge of slide on paper towel and place in stain solution for 2 minutes.

5. Rinse in beaker of water.

6. Drain water from slide completely and air dry or use air jet.

7. Oil is applied directly to the slide without a coverslip for analysis.

IV. Quality assurance measures

- Always stain a test slide on each patient to check the banding and the stain. You may need to adjust the trypsin and the staining times to get optimum G-bands.
- Overtrypsinized chromosomes appear bloated and will not take up stain well.
- Undertrypsinized chromosomes will not be well-banded.
- Overly dark chromosomes have been in stain too long.
- ALWAYS check with someone else if they have banded a slide before you have on the same day. Their trypsin time will give you a starting point.

V. Additional reading

Gustashaw KM. Chromosome stains. In: Barch MJ, Knutsen T, Spurbeck JL, eds. *AGT Cytogenetics Laboratory Manual*, 3rd ed. 1997. Philadelphia:Lippincott-Raven Publishers; 259–324.

Protocol 5.9 Cystic hygroma fluid protocol

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

Serous fluid from cystic hygromas is a plentiful source of lymphocytes which can be utilized to obtain a rapid karyotype for prenatal diagnosis. There are also cells present which can attach and grow in culture, as do amniocytes. Lymphocytes are stimulated by the mitogen phytohemagglutinin (PHA) to rapidly grow and divide such that two to three days of culturing is

sufficient to yield material of acceptable quantity. Suspended cells in the culture are likely to contain the stimulated lymphocytes, while the attached cells will be fibroblastic in nature, and will take longer to begin to divide rapidly enough to harvest. Therefore, the suspended cells are removed and harvested at two to three days, and the attached cells are re-fed and grown as a backup in case there are insufficient cells in the stimulated cell culture. If there are enough cells present in the hygroma fluid, three cultures are set up, an “A”, “B”, and “C”, with the A and B cultures unstimulated and the C culture stimulated with PHA.

Specimen: 10–20 mL cystic hygroma fluid collected by trans-abdominal needle aspiration and heparinized, if bloody.

II. Materials

1. Micropipettors with sterile tips
2. 15-mL Corning polypropylene tube (Lab Stores)
3. Pasteur pipette, 5 3/4" (Lab Stores)
4. Pipette bulbs, 2 mL (Lab Stores)
5. 5-mL sterile pipettes (Falcon)
6. 10-mL sterile pipettes (Falcon)
7. 25-mL sterile pipettes (Falcon)
8. 25-cm² sterile Corning tissue culture flasks
9. 12.5-cm² sterile Falcon tissue culture flasks
10. 5-mL and 10-mL sterile graduated pipettes
11. Sterile cotton-plugged Pasteur pipettes
12. Cylinder of 5% O₂, 5% CO₂, 90% N₂ with regulator
13. Warming plate
14. Discard bottles
15. Complete blood culture medium
 - a. Formula 1, add together:
 - 446.5 mL of RPMI 1640 (Gibco[®])
 - 50 mL of Fetal Bovine Serum (Irvine Scientific)
 - 10 mL of L-Glutamine 200 mm (Gibco[®])
 - 0.5 mL of Gentamycin 50 mg/mL (BioWhittaker)
 - 1.0 mL of Phytohemagglutinin M-Form (Gibco[®])
 - b. Formula 2
Chang complete medium (see Protocol 4.3, Tissue culture reagents)

III. Method

Flask setup

1. If there is more than 10 mL of fluid, separate into 2 centrifuge tubes and centrifuge fluid at 1000 RPM for 10 minutes.
2. Aspirate and discard supernatant.
3. Set up one suspension culture flask (“C”) by resuspending the cells in 5 mL of complete RPMI with PHA and adding to a T-25 flask, gassing flask.
4. Set up any remaining tubes in the same manner as an attached amnio “A” and “B” culture flask. See Protocol 5.4, Amniotic fluid culturing, ring, subculturing, and harvesting (flask method).
5. Incubate at 37 °C for 48 hours for STAT samples, or 72 hours for mitotic index.

Suspension culture harvest

1. After appropriate incubation with PHA, add 15 µL of Colcemid[®] to the 5 mL of “C” culture for 2 hours. Add 3 µL of Colcemid[®]/mL of culture medium for other flasks.
2. Transfer the fluid into a centrifuge tube. Re-feed and gas the culture flask to grow any attached cells for future additional harvests.
3. Harvest as an amniotic fluid culture (see Protocol 5.4, Amniotic fluid culturing, subculturing, and harvesting).

IV. Additional readings

1. Costa D, Borrell A, Margarit E, Carrió A, Soler A, Balmes I, Estivill X, Fortuny A. Rapid fetal karyotyping from cystic hygroma and pleural effusion. *Prenat Diagn* 1995;15:141–148.
2. Gole LA, Anandakumar C, Bongso A, Chua TM, Wong YC, Ratnam SS. Analysis of cystic hygroma, ascitic and pleural fluids by conventional lymphocyte culture and fluorescent in situ hybridization. *Prenat Diagn* 1997;17:1151–1157.

CHAPTER 6

Chromosome stains

Helen J. Lawce

Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA

6.1 Introduction

Chromosomes in their natural state are colorless, and can only be seen under a light microscope that is equipped with phase contrast capability (Chapter 14). For this reason, many staining methods have been introduced to help visualize them. There are stains that show the chromosomes as solid structures, termed “solid stains” (Figure 6.1), and those which exhibit differential staining with obvious domains, patterns, or bands, the locations of which vary with the method used. These methods usually work with acid-alcohol fixed chromosomes that have been placed onto a glass slide (see Chapter 2).

The first chromosome stains used in the cytogenetics laboratory produced uniformly stained chromosomes. Since there were no banding patterns to identify each homologue, the karyotype technologist grouped these solid-stained chromosomes by size, shape, and arm ratios only on the karyogram, and placement of the chromosomes was therefore not always accurate. Chromosomes within their groupings were difficult to tell apart, and when a chromosome changed size or shape due to a rearrangement, it was often impossible to tell what the rearrangement was (Figure 6.1). These limitations were mostly eliminated in the early 1970s, when the first banding methods were introduced that allowed for each chromosome and arm to be positively identified (Figure 6.2).

Banding techniques can be divided into two principal groups: those that produce thin, alternating bands along the length of the entire chromosomes (G, Q, and R), and those that stain only a specific band or region of some or all chromosomes (C, T, NOR, DA-DAPI). Other techniques require certain substances to be added to growing cultures in order to investigate patterns associated with DNA replication.

The first group of banding methods mentioned above (G, Q, and R) produces unique patterns on each homologous set that allow for their identification. The three most common methods for producing these band patterns are (1) G-banding, which stains the more dense DNA segments with Giemsa; (2) Q-banding, which uses quinacrine to produce bands that resemble G-bands; and (3) R-banding, which shows the reverse pattern of G-bands. These methods provide unambiguous identification for each normal human chromosome by producing patterns that, in turn, reflect the unique, inner core of each homologous set (see Chapter 7, Human chromosomes identification and variation). Using these patterns, many chromosome abnormalities can be ascertained for clinical and research purposes.

The second type of banding pattern is sometimes called selective staining, because it targets specific parts of the chromosomes, such as constitutive heterochromatin (C-bands); telomeres (T-bands); subgroups of heterochromatin, for example, those on the short arm of chromosome 15 and the Y long arm (DA-DAPI); and nucleolus organizing regions (NOR or N-bands). They do not, however, provide the ability to unambiguously identify every chromosome. These stains are used primarily as adjuncts to the analytical process, for example, when questions arise about the nature of a possible abnormal/variant chromosome segment.

Staining patterns that require adding chemicals to growing cultures, such as replication bands and sister chromatid exchange, depend upon DNA substitution with agents that will affect the dye binding and/or fluorescence intensities. These methods are used to examine the inactive (late labeling) X sex chromosome and to characterize certain instabilities, such as

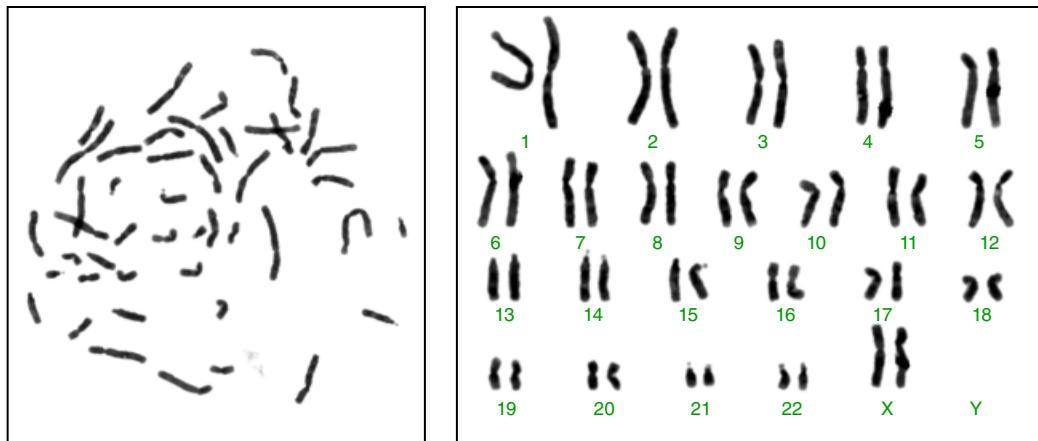


Figure 6.1 Solid staining with Wright stain. A metaphase spread and karyogram using Wright stain to visualize chromosomes. Note that it does not differentiate all of the chromosomes. They are placed by size and arm ratio. If there is a structural abnormality, it will be difficult to observe using this type of solid stain.

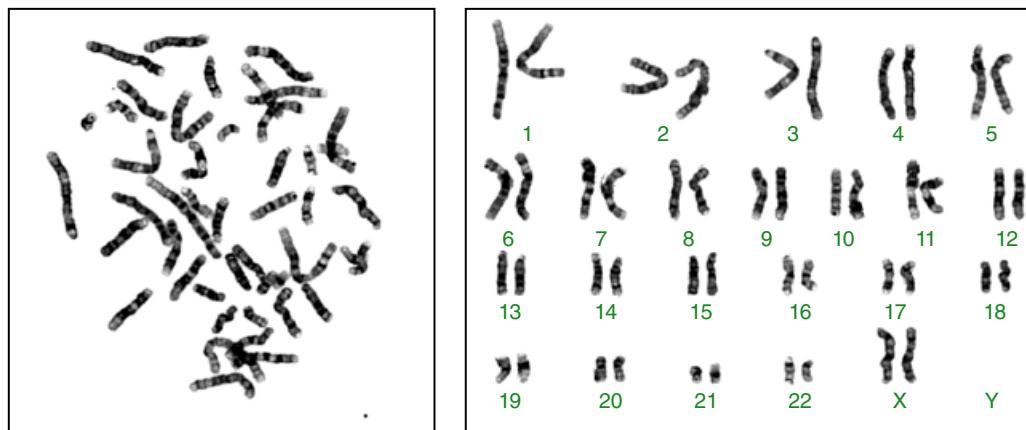


Figure 6.2 G-banded karyogram using Wright stain. A metaphase spread and karyogram that have been G-banded using trypsin and Wright stain. This procedure allows positive identification of each chromosome and each arm so that abnormalities are much easier to identify.

high rates of sister chromatid exchanges in patients with Bloom syndrome. In addition, the various banding methods provide knowledge about chromosome and chromatin structure, nuclear architecture, gene location and mapping, replication physics, and other valuable data.

Human chromosomes can be seen at their upper limit to contain about 2000 distinguishable bands per haploid set, which coalesce into fewer and fewer bands as the chromosomes condense into metaphase (see Chapter 7). Chromosomes obtained from some sources (peripheral blood) are capable of reaching very extended lengths in the early stages of mitosis, which in turn will provide a high band level (700–850) for analysis; whereas, those from other tissue types (some tumors) may not provide that same level of detail due to innate packaging differences. In fact, the architecture of these microscopic chromosomes are so extraordinarily compact that at the 550-band level, there are about 10^7 base pairs of DNA per band, each of which could contain hundreds of genes.

Some staining procedures, such as NOR (silver) staining, DA-DAPI, and C-bands, have been improved with fluorescence *in situ* hybridization (FISH) methods. The original silver stain, for example, was only able to detect active NORs, while FISH probes bind to the NOR DNA, whether it is expressing or not. DA-DAPI and C-bands, which were used to study certain

types of heterochromatin, have been mostly replaced with pericentromeric heterochromatic DNA probes specific to each chromosome. DAPI counterstain can also identify the additional heterochromatic regions of the 15 short arm and the Y long arm. Most of these special stains complement a G-banded chromosome study; therefore, there may be occasions when using one or all of these special stains could be beneficial. A competent cytogeneticist knows about all of them.

In spite of the fact that chromosome-banding techniques have been in use for more than 45 years, the mechanisms of chromosome bands are still not completely understood. It is known that both DNA content and the proteins of the chromosome are important to producing them. However they work, banding techniques are indispensable tools in all cytogenetics laboratories.

6.1.1 Definitions of banding patterns and reference tables

According to the Paris Conference (1971), a chromosome band is part of a chromosome that can be distinguished from adjacent segments by appearing darker or lighter by one or more techniques. Chromosomes should be visualized as consisting of a continuous series of light and dark bands, with no interbands [1]. The *International System for Human Cytogenomics Nomenclature (ISCN 2016)* summarizes these banding patterns and supersedes all previous guides to nomenclature [2]. This handbook sets the universal standard for cytogenetic nomenclature and is thus an essential reference for everyone in the field.

Q-bands are naturally produced by quinacrine staining, but require a fluorescent microscope for analysis; most brightfield methods, on the other hand, require a pretreatment before staining with Giemsa or its substitute. For example, G-bands are those generally produced by a proteolytic enzyme; R-bands are generally produced by high heat; C-bands are blocks of constitutive heterochromatin revealed by denaturing methods; and NOR-bands are produced by silver staining methods. The choice of banding methods ultimately depends upon what is being investigated and which method is available to provide the best answer. For use as the stain of choice in the diagnostic cytogenetics laboratory, G-band methods have an advantage over Q-banding because they are permanent, while Q-bands will quench in the presence of light, and will fade over time. G-band methods also show an advantage over Q- and R-banding because they also are the most sensitive for showing large numbers of sub-band definition for high-resolution analysis, although R-banding has the advantage of staining the less dense, more critical regions of each chromosome.

The *Paris Conference (1971) Supplement (1975)* [3] introduced a three-letter code to describe the various banding techniques (see Box 6.1). The first letter denotes the type of banding (G, Q, R, C, T); the second letter denotes the general technique (H, heating; F, fluorescence; T, trypsin; B, barium hydroxide); and the third letter denotes the stain used (G, Giemsa; Q, quinacrine; A, acridine orange; and so forth). Not all designations, however, fall into this triplet code. As previously mentioned, the NOR triplet actually stands for the *nucleolus organizing regions*, usually observed with silver staining techniques. Methods that utilize two or more dyes are described as dye pairs (e.g., DA-DAPI); these are further discussed in another section. Any new triplet should be defined when first introduced in the literature.

Table 6.1 shows the variation in staining individual chromosomes with different techniques [2]. This table may be useful in selecting a particular technique for highlighting or defining an unusual or abnormal chromosome. Tables 6.2 and 6.3 give spectral characteristics and microscope filter combinations for several fluorescent dyes that are commonly used for cytogenetic studies. In general, fluorescent dyes are light sensitive and should be protected from light. Slides stained with these dyes should also be protected from light, and exposure to ultraviolet light should be kept to a minimum.

6.1.2 Slide mounting

The practice of mounting slides with coverslips has advantages and disadvantages, and laboratories are divided on the issue. For routine cytogenetic analysis, covering slides will protect the cells from being scratched or scraped off during analysis or storage, and will prevent immersion oil from destaining and degrading the chromosome banding [4, 5]. If not covered, prompt removal of the oil with xylene or xylene substitute is a necessity, unless nondegrading immersion oil is used.

There are also advantages to using slides without coverslips. For example, the mounting medium for coverslipping slides may also cause some stain degradation over time. Second, if multiple staining techniques are to be performed on the same slide, it is easier and less time-consuming to destain and restain a slide that has not been coverslipped. Also, old slides on which the stain has faded or crystallized can often be refreshed following destaining and restaining. There are nondegrading immersion oils available.

If one prefers to cover slides, the choice of coverslip is important. Thickness should be 0.17 mm or less for an optimum image. Coverslips of no. 1 (0.15 mm) thickness are recommended for standard brightfield photomicroscopy; those of no. 0 thickness may be more advantageous in fluorescence microscopy, but they are thin and fragile. A 24 × 50 mm coverslip is ideal for general cytogenetic use, because it allows access to almost the entire slide for analysis.

BOX 6.1 CODE TO DESCRIBE BANDING TECHNIQUES^{a,b}

Q	=	Q-bands ^c
QF	=	Q-bands by fluorescence
QFQ	=	Q-bands by fluorescence using quinacrine
QFH	=	Q-bands by fluorescence using Hoechst 33258
G	=	G-bands ^c
GT	=	G-bands by trypsin
GTG	=	G-bands by trypsin using Giemsa
GTL	=	G-bands using Leishman
GTW	=	G-bands using Wright stain
GPG	=	G-bands using pancreatin and Giemsa stain
GUG	=	G-bands using urea and Giemsa stain
GAG	=	G-bands by acetic saline using Giemsa
C	=	C-bands ^c
CB	=	C-bands by barium hydroxide
CBG	=	C-bands by barium hydroxide using Giemsa
R	=	R-bands ^c
RF	=	R-bands by fluorescence
RFA	=	R-bands by fluorescence using Acridine orange
RH	=	R-bands by heating
RHG	=	R-bands by heating using Giemsa
RB	=	R-bands by BrdU
RBG	=	R-bands by BrdU using Giemsa
RBA	=	R-bands by BrdU using Acridine orange
T	=	T-bands ^d
TH	=	T-bands by heating
THG	=	T bands by heating with Giemsa
THA	=	T-bands by heating with Acridine orange

^a Adapted from Paris Conference (1971). Supplement 1975) (ref. 3), with thanks to The National Foundation-March of Dimes, White Plains, New York.

^b In this three-letter code the first letter denotes the type of banding, the second letter the general technique, and the third letter the stain. Any new triplet should be defined in the text of the publication in which it is first used.

^c Paris Conference (1971) (ref. 1).

^d ref. 325.

For staining procedures that require sequential steps with small quantities of stain or fluorochrome-conjugated antibodies, plastic coverslips are ideal. These can be purchased or cut from Parafilm or thick plastic bags. After the stain or antibody is applied to the slide, the plastic coverslip is gently lowered onto it at an angle. The flexibility of the plastic helps eliminate air bubbles. The coverslip can easily be removed for the next step in the procedure and replaced with a clean plastic coverslip.

6.1.3 Mounting media

Two types of slide/coverslip mounting procedures, wet mounts and permanent mounts, are used for various staining methods. A wet mount, which is primarily used in fluorescence microscopy, is prepared by placing a few drops of water, buffer, or glycerin on the slide and covering it with a coverslip. Excess liquid should be blotted out to minimize spherical aberration under the microscope.

To prevent evaporation of the mounting medium, the edges of the coverslip can be sealed with Kronig's cement, rubber cement, dental wax, or clear fingernail polish. Sealing the edges is not absolutely necessary if one works quickly or is careful to replenish the aqueous mounting medium as it evaporates. (Air bubbles prevent focusing at the microscope.) Glycerin does not evaporate and thus does not require sealing the coverslip.

Table 6.1 Examples of variation in staining of specific bands with various techniques^a

		Chromosome Number																						
Technique	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
C	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
Qhv	qhv	—	cv	—	q11	—	p11	—	qhv	q11	—	—	pv	pv	qhv	—	p11	—	q11	pv	pv	—	q12v	q12v
G11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R or T	p36	q37	—	p16	p15	—	p22	q24	q34	q26	p15	p13	p12v	p12v	p13	q25	—	p13	q13	p12v	p12v	q22	q11	q13
NOR	—	—	—	—	—	—	—	—	—	—	—	—	p12v	p12v	p12v	—	—	—	—	—	p12v	p12v	—	—
Q ^b	—	—	cv	—	cv	—	—	—	—	—	—	—	p11v	p11v	p11v	—	—	—	—	—	p11v	p11v	—	q12v
DA-DAPI ^c	qhv	—	—	—	—	—	—	—	qhv	—	—	—	—	—	—	p11v	qhv	—	—	—	—	—	—	q12v

^a c = cen = centromere; h = secondary constriction; v = var = variable; p = short arm; q = long arm.^b only the brilliant and variable Q-bands have been considered.^c DA-DAPI = Distamycin A and 4,6-diamidino-2-phenylindole.

Table 6.2 Spectral characteristics of commonly used fluorescent dyes when complexed with calf thymus DNA

Dye	Maximum absorbance	Maximum fluorescence	References
Quinacrine	455 nm	495 nm	23, 28, 40
Chromomycin A3	430 nm	570 nm	233–235
Acridine orange	500 nm	530 nm	215–217
DAPI	355 nm	450 nm	240
Hoechst 33258	356 nm	465 nm	311

The choice of filters to visualize fluorescent dyes depends upon the excitation or absorbance wavelength and the emission wavelength of the dye as complexed with chromatin. Trouble shooting dim fluorescence includes making sure that the microscope filters are designed to complement these variables for the stain used.

Table 6.3 Suggested filter combinations for fluorescent microscopy^a

Dye	Excitation color	Excitation wavelengths	Dichroic mirror (nm)	Suppression wavelengths (nm)	Fluorescence color
Quinacrine	Violet/blue	BP 390–490	RKP 510	LP 515	Yellow-green
	Blue	BP 436/7	RKP 455	LP 490	Green
Chromomycin A3	Violet/blue	BP 390–490	RKP 510	LP 515	Yellow-orange
Acridine Orange	Blue	BP 450–490	RKP 510	LP 515	Yellow-green, Red-orange
Hoechst 33258; DAPI	Ultraviolet	BP 340–380	RKP 400	LP 430	Aqua

^a These filter combinations represent Leitz filter cubes. Other manufacturers produce similar filter combinations.

Some cytogeneticists believe that storing slides at 4 °C in a moist chamber helps to reduce evaporation. Others mount slides in a 6% sucrose solution, which also retards evaporation so that sealing is unnecessary. To prevent fading of fluorescent specimens, an antifade solution (such as *n*-propyl gallate or *p*-phenylenediamine) can be used as a mounting medium [6–8].

Once a wet-mounted or temporarily mounted slide has been scanned, the coverslip should be removed before the mounting medium has dried out. A few drops of buffer or water applied to the edge of the coverslip may ease its release. Gentle action with a scalpel or a pair of forceps under one corner is all that is required to flip off the coverslip. Sliding the coverslip across the slide may damage the cells if done too quickly.

Permanent mounts require the use of a natural or synthetic resin in a solvent, followed by careful covering with a coverslip. The important factors to keep in mind when choosing a mounting medium are its refractive index (preferably no higher than 1.52) and composition (it should not react with the stain). Most mounting media are soluble in xylene, toluene, aromatic hydrocarbons, or chlorinated hydrocarbons; the manufacturer usually recommends the solvent of choice for its particular product. These products should be used in a fume hood due to inhalation safety issues.

Drying may take place at room temperature or in a low-temperature (37 °C) dry incubator. This drying period is important because mounting medium and/or solvent might get on the lens of the microscope objective. In addition, any residual mounting medium on the underside of the slide or top of the coverslip should be removed with solvent so that it will not interfere with the ability to focus the image at the microscope.

6.1.4 Slide aging for chromosome staining methods

Before banding was developed, chromosomes were stained with one of the commonly used Romanowsky dyes (see 6.1.5, Conventional staining methods); no special pretreatments were needed after making the slide, because the stain always worked well. However, when the first Giemsa banding methods were published, many laboratories struggled with obtaining bands until it was discovered that slides that had been sitting at room temperature for a few days banded better than fresh slides. It then became the

Table 6.4 Slide-aging methods. Aging slides will improve banding quality for most methods by partially removing water from the chromatin.

Method	Temperature	Time
Natural aging: Use desiccants if humidity is high	20 °C (room temperature)	3–5 days
Heat on warming plate or on an aluminum tray in an oven	65 °C	24–48 hours. Usually overnight is sufficient unless humidity is high
Heat on slide warmer	75 °C	1–2 hours
Heat on metal tray in oven	90–95 °C	20–60 minutes
Prestain with Giemsa	20 °C (room temperature)	2 days; destain and band
Expose to hydrogen peroxide, 15% w/v	20 °C (room temperature)	7 minutes. Rinse in distilled water, proceed to banding.
Microwave slides	Varies	Varies, brief times depend upon wattage of microwave

Aging of slides is performed to improve the banding patterns for most methods and depends upon the partial removal of water from the chromatin. There are many different permutations of chromosome aging, depending upon the preference of the laboratory and possibly the local climate conditions.

norm to use 3-day “aged” slides for banding, but this was not practical for STAT cases or good turnaround times. This led to the development of artificial aging methods, which are still used today, that involve heating a slide to 60–95 °C for 20 minutes to overnight (Table 6.4). This gave results that were comparable to naturally aged slides. It was also discovered that slides that were older than a few weeks did not band well either, indicating that there is an optimal window of time for obtaining the best G-banded chromosomes. Fluorescent stains, however, are not generally dependent upon aging for good results.

The connection between slide aging and good banding has now been determined to work by partial removal of the water in chromatin. Excess water keeps the chromatin from collapsing into the dark and light staining regions, and removal of too much water from the chromatin causes complete collapse of both the light and dark bands. Aging may be reversed to some degree by running slides through a hydration series, starting with 95% alcohol and going through 80–85%, 70%, and distilled water stages to rehydrate the chromatin.

6.1.5 Conventional (solid) staining methods

Prior to the use of Giemsa stain for chromosome preparations, conventional cytological stains, such as aceto-orcein, acetocarmine, gentian violet, hematoxylin, and Feulgen stains, were used to stain chromosomes. These methods uniformly stained the chromosomes and left the centromeres constricted and colorless, enabling measurements of chromosome length, centromeric position, and arm ratio. Giemsa, a Romanowsky stain, has supplanted these stains for chromosome analysis, because it can be used for both banded and unbanded chromosome staining. Other stains had drawbacks; for example, aceto-orcein, a stain derived from lichen, is unsuitable for banding and is difficult to remove from the chromatin once stained.

Romanowsky staining, a type of nuclear stain from which many modern stains descended, was invented in 1891 by the Russian physician, Dmitri Leonidovich Romanowsky. It was unique in that it used a mixture of acidic and basic dyes that interacted to produce a beautiful purple color. Modifications to the method resulted in a number of useful stains, including Giemsa, Leishman, and Wright stains, which are now used for conventional staining and banding. Giemsa stain, however, is the most popular stain for chromosome analysis (Figures 6.1 and 6.2). Some cytogeneticists even use conventional Giemsa staining as a pretreatment for artificially aging their slides (see 6.1.4, Slide aging for chromosome staining methods). Others have reported that a dilute concentration of Giemsa or Wright stain will induce spontaneous chromosome banding.

Historically, conventional (solid) Giemsa staining provided the ability to visualize the centromere's location, measure short/long arm ratio, and detect numerical and relatively large structural abnormalities. The only chromosomes, however, that could be absolutely identified by size and morphology alone were 1, 2, 3, 16, and Y (if they were normal). It was not until the introduction of Q-banding (see 6.2.1, Quinacrine banding) that all chromosomes became unequivocally identified.

Although conventional staining may seem outdated, its value should not be overlooked. For example, it can be very useful in studying certain structural abnormalities, such as, satellite presence (single or multiple), dicentric chromosomes, ring

chromosomes, fragments, double minutes, fragile sites, breaks and gaps. Most banding techniques cause chromosomes to swell, thus obscuring primary constrictions (centromeres), secondary constrictions (including NORs), fragile sites, and so on. Conventional staining, however, does not induce these changes; therefore, it is the preferred staining method for diagnosing breakage syndromes (see Chapter 13, Chromosome instability syndromes). Conventional Giemsa staining can also be used before performing FISH in order to pre-locate small populations of specific types of interphase cells, such as plasma cells in bone marrow samples from patients with multiple myeloma, so that these cells can be evaluated during FISH analysis.

6.2 Chromosome banding methods

6.2.1 Quinacrine banding (QFQ)

Historical and theoretical perspectives

Q-banding is the first banding method ever reported. In 1968, Caspersson et al. [9] introduced the use of quinacrine mustard (QM) (Figure 6.3) for differentially staining plant chromosomes. A rapid succession of studies followed, using QM and quinacrine dihydrochloride on human as well as plant chromosomes [10–15]. In 1971 Caspersson and his colleagues published the culmination of their studies – banding patterns that uniquely identified each of the 24 human metaphase chromosomes [16]. Their work, and the work of many other researchers, is outlined in the following discussion. Figure 6.3 shows a normal male metaphase stained with quinacrine dye.

Quinacrine mustard and quinacrine dihydrochloride are members of the acridine family of dyes that have a core structure of three flat aromatic rings (Figure 6.4). The former contains a terminal mustard configuration in the molecular side chain. It was well known that alkylating groups of the mustard variety have an affinity for the N-7 atom of guanine [17–19]; therefore, it was assumed that quinacrine mustard either intercalated its tricyclic acridine nucleus into the double helix and ionically bound its diaminoalkane side chain to the phosphate groups [20, 21], or it alkylated the DNA bases, particularly at the N-7 atom of guanine, thereby displaying GC specificity.

Vosa observed that quinacrine alone, without the mustard moiety, possessed the same staining specificity and produced comparable banding patterns [22]. Quinacrine itself was therefore determined to be the important component of the dye.

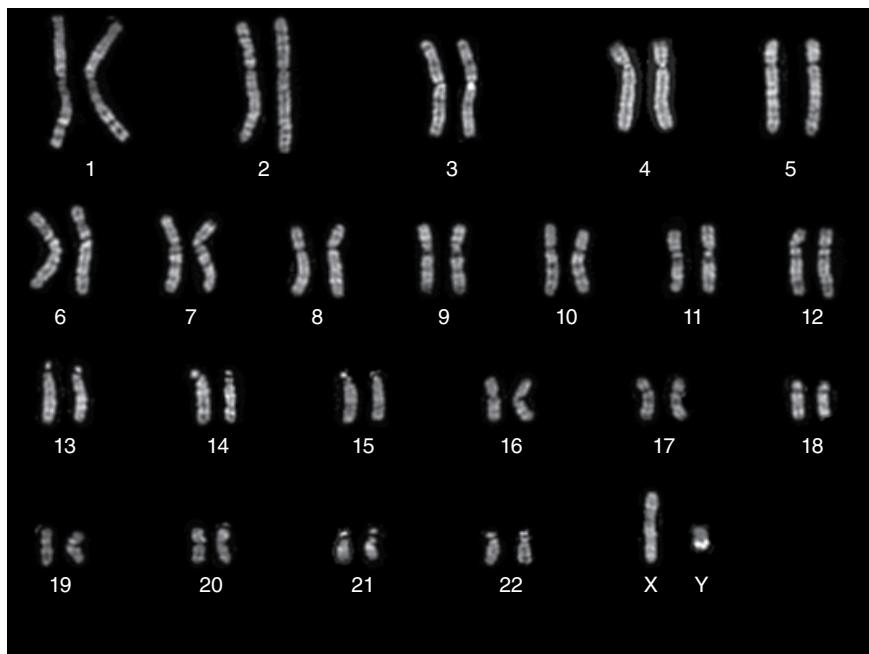


Figure 6.3 Quinacrine banding of a normal male cell. Q-banding patterns are similar to G-banding patterns with the Q-band bright bands corresponding to the G-band dark bands. The exceptions are that the heterochromatin regions of chromosome 16 and Y are bright with Q-banding but do not exhibit dark G-bands.

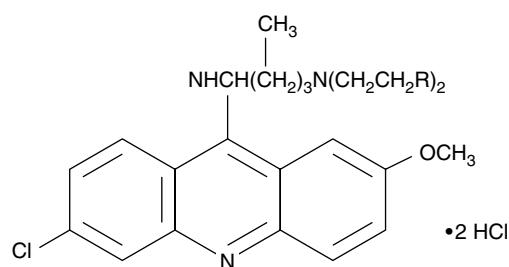


Figure 6.4 Structure of quinacrine. Note that quinacrine dihydrochloride differs from quinacrine mustard by the substitution of a hydrogen molecule for a chlorine molecule in the R position. Lin 1975 [17]. Reproduced with permission of Springer.

Either the mustard or the dihydrochloride compound can be used successfully. Although Caspersson et al. [15] pointed out several advantages of QM over quinacrine dihydrochloride, the latter is more commonly used in routine cytogenetic studies.

Caspersson et al. hypothesized that the fluorescent pattern of chromosomes was determined by the amount of DNA in different chromosomal regions, as well as by the qualitative differences in the QM binding capacity of the DNA in different chromosomal regions. A certain correlation seemed to exist between a strong capacity for QM binding and the distribution of heterochromatin as defined by thymidine labeling techniques or cold treatment (according to Darlington and LaCour) [12, 13].

With regard to base composition, Caspersson's suggestion that fluorescent bands were related to GC content was later found to be incorrect. Weisblum and de Haseth [23] observed a marked enhancement of fluorescence in the presence of bihelical polynucleotides, such as poly(dA-dT), poly(dA)-poly(dT), and poly(rA)-poly(rU), but not in the presence of single-stranded nucleotides. They also observed that poly(dG) and poly(rG) quenched fluorescence proportionally in native and denatured DNA. In summary, their data suggested that base pairs composed of AT residues were responsible for the variations observed with quinacrine fluorescence, which supported the proposal of Ellison and Barr [24] that the highly localized quinacrine fluorescence in cytological preparations reflected DNA with a high (A-T)/(G-C) ratio. Other studies also confirmed these findings [25–30].

The experiments of Dev et al. [31] and Schreck et al. [32] support the importance of base composition to the banding process. By applying fluorescent-labeled antibodies to the various denatured base pairs, they showed that anti(A) and anti(T) antibodies produced typical quinacrine (and Giemsa) banding patterns. This finding is consistent with the fact that positive Q bands usually represent late replicating, usually AT-rich regions. In contrast, however, mouse constitutive heterochromatin fluoresces poorly with quinacrine, even though it is composed of AT-rich satellite DNA [33, 34]. Therefore, base composition is apparently not the only explanation for the banding phenomenon.

Protein–DNA interactions presumably also play an important role. Comings and colleagues suggested that histones have little effect on the ability of DNA to quench fluorescence, while nonhistone proteins inhibit dye interaction and quinacrine fluorescence [25]. These authors concluded (a) that quinacrine binds to chromatin by intercalation of the three planar rings with the large group at position 9 lying in the small groove of DNA; (b) that most pale-staining regions are due to a decreased binding of quinacrine; and (c) that this inhibition of binding is predominantly due to nonhistone proteins.

By using fluorescent conjugates of polylysine, a competitor of quinacrine, Latt and Gerald [35] demonstrated that the distribution of chromatin along the length of the chromosomes was not sufficiently different to account for the observed banding patterns. Sumner's research supported this observation [36]. Since then, Sumner demonstrated by X-ray microanalysis that (a) quinacrine binds uniformly along the length of the chromosomes; (b) bands result from differential quenching of the fluorescence; and (c) an intact double-stranded DNA structure is an essential requirement for normal quinacrine fluorescence of nuclei and chromosomes [37, 38].

Although AT regions preferentially show brighter fluorescence in many circumstances, base composition is apparently not the only factor involved in the production of banding patterns. Protein modifications do not appear to influence Q-banding. A review of the literature since Caspersson and his colleagues' initial introduction of quinacrine banding shows that despite extensive research, the exact molecular mechanism of fluorescent Q-banding is still not completely understood.

Clinical significance

Although quinacrine banding is not routinely performed in most cytogenetics laboratories, it can be a useful adjunct to other staining techniques. Since quinacrine staining does not depend upon pretreatment of chromosomes to manifest bands, the chromosomes are not altered, and slides that have been stained with quinacrine can be destained and sequentially stained by

other methods, including FISH. From a clinical standpoint, quinacrine banding is a reliable technique that permits the identification of specific chromosomes and structural rearrangements. It is especially useful in distinguishing the Y chromosome from the D, E, and G group chromosomes. Quinacrine heteromorphisms (variable regions of the centromeres and satellites of certain chromosomes—see Chapter 7) can sometimes be useful in the identification of maternal versus fetal cells, donor versus recipient cells (if donor and recipient are opposite sexes, the brightly fluorescent Yq can also be used to score bone marrow transplant engraftment), paternal/maternal chromosome origin, and inherited chromosomal variants, although they may be uninformative in some cases and should not be relied on exclusively [39].

Technical considerations

For fluorescence microscopy, filter selection is important. Quinacrine (complexed with calf thymus DNA) has an absorbance (excitation) maximum at 455 nm and a fluorescence (emission) maximum at 495 nm [40]. A wide-band excitation filter may give brighter fluorescence but results in photochemical changes (quenching) that prevent good-quality sequential staining [41]. A single band excitation filter, such as a 436-nm filter, gives slightly reduced, but certainly adequate fluorescence and allows better quality sequential staining of the ultraviolet-exposed metaphase cells. (For detailed discussions of filter selections, readers should consult manufacturers of fluorescence microscopes and optical filters.) For laboratories that lack a fluorescence-equipped microscope, Nakagome et al. [42] reported adequate fluorescent banding with the use of a quartz iodide lamp, a darkfield condenser, and the appropriate filters on a standard clinical laboratory microscope.

The basic technique for quinacrine banding is the same in most laboratories. Variations in dye concentration and staining time do not appear to be critical, although overstaining will enhance background cell components and increase flare, and over rinsing may wash out the stain, making it more difficult to locate metaphase spreads under 10 \times magnification. Buffer that is too hypotonic may cause the chromosomes to swell, and an incorrect pH may result in decreased contrast between bands. Optimal conditions for Q-banding were given by Lin et al. [43], who rinsed with buffer and double-distilled deionized water and mounted in pH 4.5–5.5 in very low ionic strength. According to Lin, increasing the pH to 5.5 increases brightness, and decreasing the pH to 4.5 increases band contrast. Old slides may therefore require pH 4.3 [44]. Some other important technical considerations concerning mounting include avoiding air bubbles, blotting excess buffer, and rinsing off or removing the coverslip soon after microscopic examination.

Quinacrine-banded slides can be destained with 3 : 1 fixative and restained by other banding/staining techniques (Chromomycin A3/Methyl Green, GTG, C, NOR). Any trace of oil must be removed with xylene prior to destaining. Slides should be dried completely on a slide warmer (50 °C) before proceeding with the next staining technique.

Q-bands correspond to G-bands for the most part, with dark G-bands appearing Q-band bright. Exceptions are the 1qh and 16qh regions, which appear dim on Q-banding, while they are dark with G-bands. See the Contributed Protocols section of this chapter for examples of Q-band techniques.

Caution should be exercised when using quinacrine dyes. Because quinacrine is an intercalating agent, it is a potential mutagen/carcinogen. Users should wear gloves and avoid inhaling the powder. Eyes should be protected from direct exposure to ultraviolet light by using appropriate filters on the microscope.

6.2.2 Giemsa banding (GTG, GTW, GAG, GTL)

Q-banding represented a tremendous breakthrough in chromosome identification and clinical diagnosis. Historically, the next type of chromosome differential staining method to be discovered was C-banding, which was an extension of Pardue and Gall's [45] *in situ* hybridization technique, and allowed the study of certain chromosome polymorphisms. C-banding is discussed later in this chapter.

The introduction of Giemsa banding (G-bands) in 1971 by Sumner et al. [46] was another major advance in the field of cytogenetics. It eliminated the need for an expensive fluorescence photomicroscope and provided permanently stained slides with very high-resolution bands. Today, Giemsa banding (or similar Romanowsky dyes) is the most widely utilized staining technique for chromosome analysis.

Historical and theoretical perspectives

Sumner and colleagues introduced the ASG (or GAG) method of banding (acetic acid fixation/saline/Giemsa) as an extension of the method that was used to obtain C-banding. The method involved the incubation of cytological preparations in 2 \times SSC at 60 °C, with subsequent staining in 2% Giemsa. The resulting banding pattern was very similar to QFQ banding, with the Q-band bright bands now seen as dark bands with the Giemsa stain (see Figure 6.5) [46, 47].

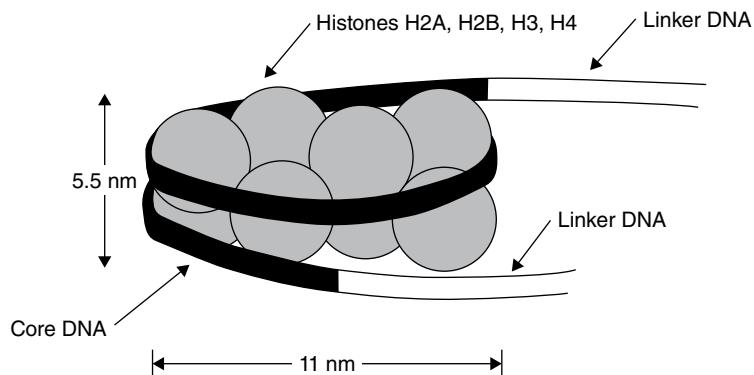


Figure 6.5 Schematic diagram of a nucleosome core particle. The DNA molecule is wound 1-3/4 turns around a histone octamer (two molecules each of histones H2A, H2B, H3, and H4). Histone H1 (not shown) is bound to the linker DNA. Note that the two linker units point in the same direction. Adapted from Freifelder D. *Molecular Biology*. Boston: Jones and Bartlett, 1987.

Other techniques for G-banding were simultaneously and subsequently described. Patil et al. described a method that involved staining the slides in Giemsa at pH 9.0 rather than the usual pH 6.8; no pretreatment was required [48]. Drets and Shaw [49] described a rather time-consuming method that began with incubation in 0.07 N NaOH followed by up to 3 days of incubation in 12× SSC 65 °C, with subsequent alcohol dehydration and staining. Schnedl described a similar wavelengths and reassociation technique [50]. Seabright [51] introduced a rapid method of banding using a trypsin pretreatment, as did Wang and Federoff [52]. This method was later called the GTG technique, and is the gold standard trypsin G-band method performed every day in laboratories all over the world.

Many other pretreatments were used to induce similar or identical G-banding patterns, some of which will be discussed in more detail in this chapter. In fact, Kato and Moriwaki [53] tested 73 reagents for their band-producing ability. The observation that a wide variety of chemicals produced G-bands prompted a great deal of interest in the nature and mechanisms of Giemsa banding. Several areas in particular were targeted for study: the role of DNA, with or without proteins; the role of proteins; the ionic strengths of the incubation solution and staining solution; and the structure and binding properties of Giemsa and its component dyes and other related dyes. These factors will be discussed in detail.

Electron, light, and atomic force microscopy indicate that the natural condensation or organization of chromosomes correlates with G-band and Q-band patterns [54–58]. G-banding itself is an enhancement of the basic chromomere pattern of meiotic pachytene chromosomes [59, 60]. Comings proposed that G-banding occurs because there is a basic chromomere structure in the metaphase chromosome waiting to be enhanced [61].

Electron microscopy has also been useful in studying the collapse of chromosomes, particularly after treatment in hot saline [62–64]. Subsequent Giemsa staining showed re-swelling of the chromosomes with transversely swollen ridges in the areas of positive G-bands. Negative bands remain collapsed. Destaining removed the ridges, indicating that these ridges were an accumulation of dye molecules in specific regions of the chromosomes. The role of this collapse in banding, however, is still not currently understood.

The original G-banding methods were explained, at least in part, as denaturation and renaturation of moderately repetitive DNA sequences [46, 49, 50]. However, other evidence and subsequent reports of chemical agents that produced identical banding patterns changed this belief. When trypsin and other proteases were shown to be effective pretreatments, there was a shift to concentrate on the role of proteins in chromosome banding. Banding techniques could conceivably produce many different effects on chromosomal proteins, including extraction, solubilization, denaturation, disruption of peptide bonds, or redistribution of proteins along the chromosomes.

Wang and Federoff [52] suggested that trypsin hydrolyzed the protein component of the chromatin, thereby allowing the Giemsa dye to react with the exposed DNA. Hsu later suggested that trypsin and other agents might act by chelation rather than by protein digestion [65]. Kato and Yoshida [66] studied band production by protein extraction. Lee et al. [67] studied the band-inducing ability of several protein denaturants: urea, sodium lauryl sulfate and sodium deoxycholate (both strongly anionic sulfate detergents), and 7X (a nonionic detergent). Although they did not disrupt peptide bonds, these agents gave a banding pattern that was identical to those that were produced by proteolytic enzyme treatment. The authors suggested that banding patterns probably reflected relatively gross changes in chromosome organization rather than biochemical composition.

Utakoji [68] studied the modification of pyrimidine residues of DNA with potassium permanganate. Permanganate was known to degrade DNA chemically by preferential oxidation of pyrimidine residues (especially thymine residues). During the study, it was noted that if cytological preparations were treated with buffered Giemsa following permanganate treatment, the result was a banding pattern similar to other described patterns. This pattern did not appear to be a result of oxidation of thymine residues. A follow-up study involving the blocking of permanganate-induced chromosome banding by organic mercurials [69] suggested that the differential staining by permanganate was closely associated with sulfhydryls and disulfides, which might be unevenly exposed or distributed along the chromosomes.

Sumner also observed that reagents that attacked protein disulfide or sulfhydryl groups had a pronounced effect on banding; he suggested that the proteins in dark G-bands were relatively rich in disulfides, whereas those in weakly stained bands were relatively rich in sulfhydryls [70]. At that time, his hypothesis could not be tested directly. Several years later, however, the availability of sensitive fluorochromes specific for –SH groups made this feasible [71]. All of the fluorescent reagents that were tested gave essentially similar results and led to the conclusion that there appeared to be no relationship between banding patterns and disulfide/free sulfhydryl (–SS/–SH) distribution along the chromosomes.

Role of proteins

An understanding of protein involvement requires a basic understanding of chromosome structure [72–74]. Because of its size, DNA must be packaged into a compact structure. This is accomplished with two classes of DNA-binding proteins, which in eukaryotes include the histones and the nonhistones. The protein–DNA complex is known as chromatin.

Histones are present in extremely high concentrations in the cell nucleus; in fact, their total mass is approximately the same as that of the DNA. The histones are basic proteins with a strong positive charge at neutral pH. Because they contain a high proportion of the positively charged amino acids lysine and arginine, histones bind tightly to negatively charged DNA. There are five types of histones: the H1 histones, and the four nucleosomal histones, designated H2A, H2B, H3, and H4.

Nonhistone proteins are a heterogeneous group of acidic proteins present in much smaller quantities than the histones in the cell nucleus. The functions of the nonhistone chromatin-associated proteins concern higher order structure and gene expression. Some examples of these proteins are the high-mobility group (HMG) proteins, RNA polymerase, and DNA polymerase.

Native chromatin consists of a double helix of DNA wrapped around the outside of a protein core. The core consists of two copies of each of the nucleosomal histones – H2A, H2B, H3, and H4 – arranged in a disk-shaped octamer. Approximately 200 base pairs of DNA wrap around this histone core to form a nucleosome (Figure 6.6). In undigested chromatin, the DNA exists as a continuous double helix from nucleosome to nucleosome, connected by linker DNA that varies in length. This “beads-on-a-string” form of chromatin is also known as the 10-nm fiber and does not require the presence of histone H1. The 10-nm fiber subsequently coils to form a solenoid structure, the 30-nm fiber, whose formation does require histone H1. H1 binds to the nucleosome at a ratio of one molecule per nucleosome. The 30-nm fiber is the basic constituent of interphase chromatin and mitotic chromosomes.

In human chromosomes, the 30-nm fiber of DNA forms loops of approximately 75–100 kb, which are tethered or anchored at their bases to form what is known as a chromosome scaffold. This structure can be seen by removing most histones and nonhistones from the chromosomes and surface-spreading them appropriately for electron microscopy. The residual structure appears as a scaffold, with numerous and extensive loops of DNA radiating from a coarsely fibrous structure that resembles a metaphase chromosome [75–78]. The scaffold presumably forms a nonhistone protein backbone that is responsible for maintaining the basic shape of the chromosome and for serving as an anchor for DNA as it loops in and out along the chromosome [78]. One of the major scaffold proteins is Sc1 [79], also known as topoisomerase II [80, 81]. AT regions of the DNA, known as scaffold-associated regions (SARs) or matrix-associated regions (MARs), may define the base of the chromatin loops. A number of proteins (topoisomerase II, H1 histone, HMG-I/Y) selectively bind SARs, and the protein–DNA interaction appears to be mediated by the non-B structural features of DNA. The organization of the scaffold may actually contribute to the banded appearance of metaphase chromosomes [82]. The final packaging of the 30-nm fiber of DNA into chromosomes results in a 10,000-fold reduction in DNA length (Figure 6.7).

Fixation in 3 : 1 methanol-acetic acid is an important preliminary step for G-banding. Fixation extracts a portion of all histones, especially H1, and a group of nonhistones in the 50,000- to 70,000-dalton (Da) range, but it is far from a complete removal of proteins. Considerable amounts of each type of protein remain in the fixed chromosomes [83–86]. Hancock and Sumner found that partial extraction of all histones, plus a group of nonhistones with molecular weights between 50,000 and 70,000, was necessary for G-banding to occur [86]. More extensive removal of proteins inhibited all band production. Protein–protein and protein–DNA crosslinking inhibited some or all types of banding, with the amount of banding being roughly proportional to the degree (but not the type) of crosslinking. The crosslinking data indicated that the conformation of chromosomal proteins in relation to DNA strongly influences banding.

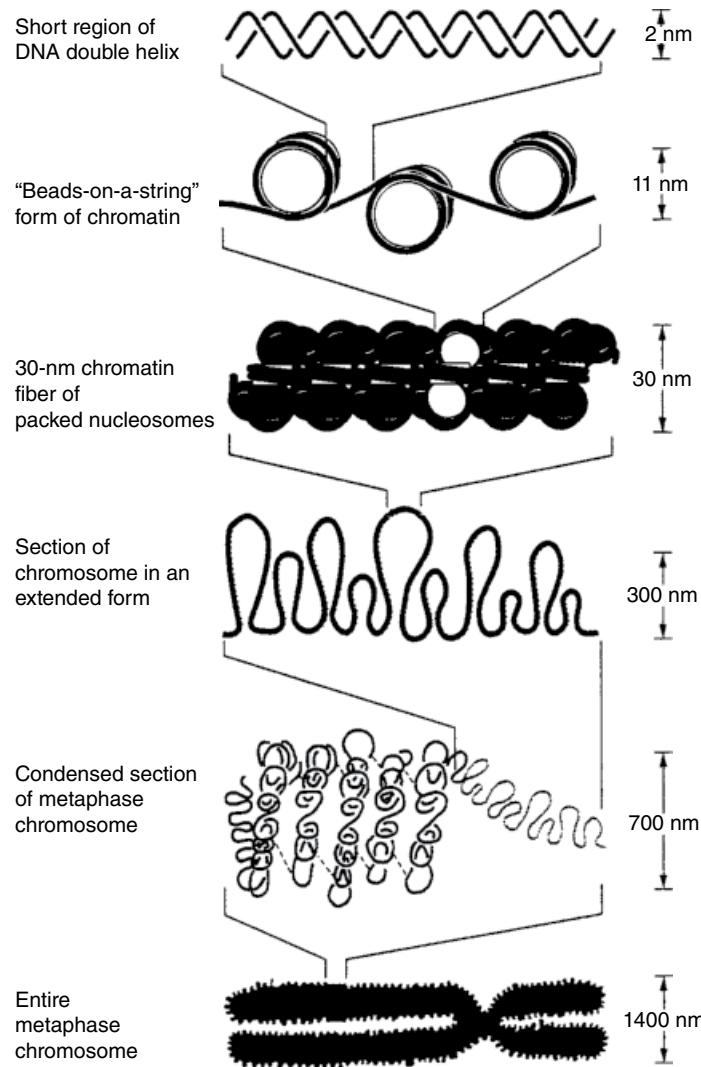


Figure 6.6 Various stages in the condensation of DNA and chromatin to form a metaphase chromosome. The dimensions indicate known sizes of intermediates, but the detailed structures are postulated. Alberts 2002 [72]. Reproduced with permission of Garland Science.

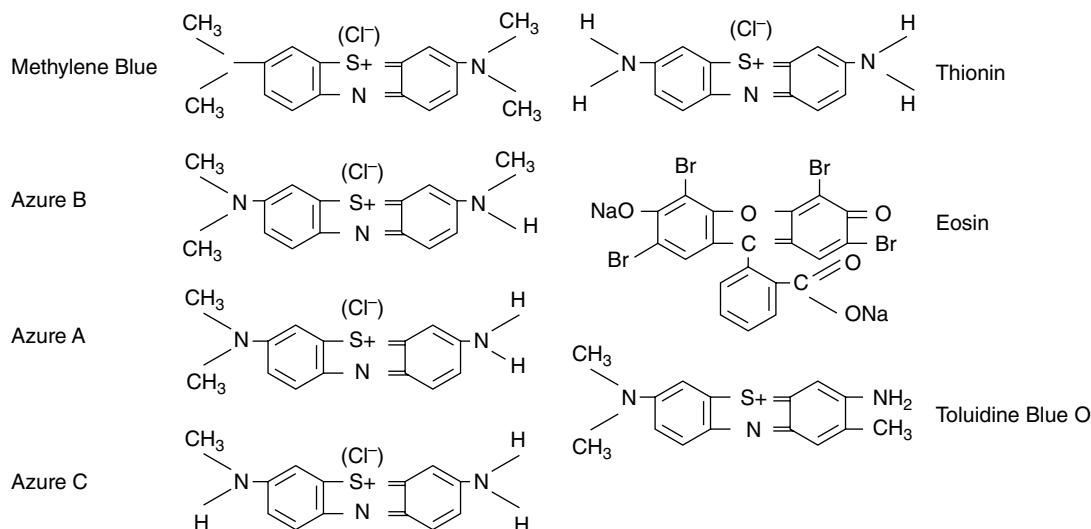


Figure 6.7 Structural formulas of the thiazin dyes, eosin Y, and toluidine blue. Giemsa stain components are mixtures of the basic thiazin dyes, which differ in the number of methyl groups attached to a core of two benzene rings bound together by a sulfur and a nitrogen molecule. Comings 1975 [95]. Reproduced with permission of Springer.

Sivak and Wolman [87] and Comings and Avelino [84] suggested that the nonhistone proteins are important in banding, because fixed chromosomes that had been treated in 0.2 N HCl for 4 hours (to remove all histones) still retained their ability to be subsequently banded. Burkholder and Duczek treated fixed CHO nuclei with 2× SSC, urea, NaCl-urea, and trypsin (all band-inducing agents) and studied their effects on protein extraction by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [88, 89]. The results indicated that G-banding techniques induce specific and reproducible changes in the proteins of isolated nuclei and suggested that they might induce similar changes in the proteins of mitotic chromosomes, thereby leading to banding patterns. It was noted that G-banding occurred in the presence or absence of histones. It seems, then, that nonhistone proteins have been more positively implicated in banding than histones, although exactly how they are involved is unknown [53, 84, 85, 88–94].

Dye structure and binding

Giemsa stain is a complex mixture of dyes that may vary in concentration, purity, and ratio. The main components are the basic aminophenothiazine dyes – azure A, azure B, azure C, thionin, and methylene blue-and the acidic dye, eosin. The thiazin dyes vary in the number of methyl groups attached to a core of two benzene rings bound together by nitrogen and a sulfur atom (Figure 6.8). Not only do manufacturer variation and lot-to-lot variation exist in the components, but also oxidation can occur, thereby changing the constitution even further.

Several studies have been done concerning the band-producing ability of the individual components of Giemsa stain. Methylene blue or any of the azures alone produces banding. Thionin, with no methyl groups, produces poor banding [95, 96]. Wyandt et al. [96] observed a general but imperfect correlation between banding quality and the number of methyl groups on the dye molecule, with methylene blue and azure B giving the best banding patterns. Eosin alone does not produce G-banding, and there is some debate over whether eosin is actually necessary for banding to occur. Comings suggested that it did not bind to DNA and was not necessary for banding to occur [95]. On the other hand, Sumner and Evans proposed that Giemsa staining is the result of a magenta-colored 2 : 1 thiazin–eosin compound that preferentially precipitates in hydrophobic regions of the DNA [90].

These two groups suggested somewhat different models for the binding of Giemsa to DNA. According to Sumner [90, 97], the initial stages involve the ionic bonding of methylene blue (thiazin) molecules alone in areas of the DNA where the phosphate groups are in the correct position to bind two thiazin molecules. Next, thiazin binds to eosin to form the magenta compound that precipitates in the hydrophobic regions, thereby freezing the active groups on the DNA to bind more thiazin and repeat the cycle. The resulting banding pattern is considered a consequence of an uneven distribution of hydrophobic groups along the chromosomes. The magenta complex has an absorption peak at 550 nm.



Figure 6.8 Example of an undertrypsinized metaphase. There are few bands and the contrast is very low between bands so small chromosome abnormalities would possibly be missed. Note that the trypsinization is uneven across the cell. This effect can be due to inconsistent slide drying during cell spreading. There are many causes for this phenomenon, including poor quality glass slides.

Comings [95] observed the magenta complex in gelatin films alone, and with azure A and thionin in the absence of eosin. He proposed that at low dye concentrations, the positively charged methylene blue molecules interact with the DNA phosphate groups by type I intercalative binding, thereby shifting the absorption peak from 660 to 675 nm. As the dye concentration is increased, it interacts with DNA by side-stacking (type II binding), with a concomitant metachromatic shift from 675 to 665 nm. In any case, DNA-phosphate anions are important: G-banding does not occur in the absence of DNA.

Another proposal for the action of Giemsa in chromosome staining is that of van Duijn and colleagues [98], who discussed the involvement of nucleosomes in Giemsa-staining of chromosomes. They concluded that neither the explanation of Sumner nor the theory of Comings was sufficient to explain the magenta color. Their study provided a detailed model concerning the effects of fixation, pretreatment, collapse and swelling, and staining of the chromosomes, along with supporting evidence. The model is discussed in relation to other types of banding and pretreatments. This model stands in sharp contrast to the widely accepted view that histones play no role in the banding phenomenon. Further studies will be necessary to prove or disprove the hypothesis.

Curtis and Horobin [99] initially studied the staining of chromosomes with the Romanowsky dyes, but eventually tested 39 basic dyes of varied structure on metaphase chromosomes [100]. They attempted to correlate the structural features of the dyes with the staining results and concluded that several factors are important for dye-chromosome affinity, including Coulombic forces and van der Waals attractions. They also suggested that the selectivity of G-banding is largely due to hydrophobic bonding, which is probably enhanced by the loss of hydrophilic histones.

Other dyes used to obtain the G-banding pattern are Wright and Leishman stains.

Structural and functional relationships of chromosome bands

Chromatin is divided into two main groups: heterochromatin and euchromatin. Constitutive heterochromatin is highly condensed, very repetitive, and transcriptionally inactive during interphase, in order for gene transcription to occur. One type of euchromatin, facultative heterochromatin, behaves like heterochromatin in a developmentally controlled manner, being transcriptionally inactive, late replicating, and condensed during interphase. The inactive X chromosome in the somatic cells of female mammals is an example of facultative heterochromatin.

Chromatin structure and function vary along the length of the chromosomes. Differential staining techniques reflect the packaging, base composition, and timing of DNA replication. Based on patterns obtained with AT- and GC-specific fluorochromes, G-bands are AT rich and R-bands are GC rich (see Chapter 1). The properties of chromosome bands were reviewed by Holmquist [101], who divided chromosome bands (300 bands/genome resolution) into five types or “flavors”: the AT-rich G-bands, and four types of R-bands with variations in both base composition and Alu content. R-bands consist of T- (telomeric) and non-T bands.

Although G-banding, R-banding, and other banding procedures are extremely valuable in clinical diagnosis, the precise histochemical basis of many of the techniques remains uncertain. In recent years, the emphasis has shifted from trying to understand the histochemical basis of dye/chromatin interactions to elucidating the structure–function relationship of chromosome bands. For a more in depth coverage of this topic, the reader is referred to reports by Burkholder [102], Craig and Bickmore [103], and Sumner [104], who reviewed these relationships with respect to basic chromosome structure, base composition, isochore content, gene density, location of CpG islands, location of interspersed repeats, DNase I sensitivity, condensation, replication timing, protein association and acetylation, and the frequency of induced and natural rearrangements.

Chromatin packaging and chromomeres

Differential chromatin packaging results in the characteristic pattern of meiotic chromomeres. Chromomeres are foci of chromatin condensation along the chromosomes, separated by more extended regions of chromatin. Giemsa staining at the pachytene stage without pretreatment results in densely staining, condensed chromomeres that correspond to mitotic G-bands [61]. The less condensed interchromomeric regions correspond to the R-bands.

Base composition and isochores

G-dark and G-light bands can be fractionated by buoyant density centrifugation into AT-rich and GC-rich fractions, respectively [105]. The cesium chloride buoyant density profile of human DNA shows several families of DNA molecules or isochores, each with its own modal density and base composition. Bernardi subdivided the genome into five isochore families (excluding satellite DNA and minor components such as ribosomal DNA) based on their GC content [106].

The GC-poorest isochores are L1 and L2; the GC-richest isochores are H1–H3, with H3 having the highest GC content. Sixty-two percent of the genome falls into the L1–L2 group, whereas 31% falls into the H1–H2 group, and 3% falls into the H3 group [107]. In situ hybridization using the various isochores as probes showed that G-bands are primarily composed of L1–L2; R-bands (R-bands exclusive of T-bands) are composed of roughly equal amounts of H1 and L1–L2, with a very small contribution of H2 and H3; T-bands are composed of H1, H2, and H3 [108, 109]. Approximately 75% of H3, the GC-richest isochore family, is located in the T-bands. Ikemura and Wada [110] demonstrated that a major portion of GC-rich genes are in the T-bands.

Gene density and CpG islands

The vast majority (80%) of more than 1,200 housekeeping and tissue-specific genes and oncogenes mapped to date reside in R-bands [106, 111, 112]. (Housekeeping genes are constitutively expressed in all cells in order to provide basic cellular maintenance; whereas, tissue-specific genes are expressed only in certain tissues, and expression may vary over time.) T-bands contain the highest concentration of genes in the human genome (approximately two thirds of the R-band genes) and show the highest level of transcriptional activity [101, 108].

CpG islands are unmethylated GC-rich clusters of DNA that are associated with the 5' ends of all housekeeping genes and about 40% of tissue-specific genes. As expected, >80% of CpG islands are located in R-bands [112–115].

Location of interspersed repeats

Interspersed repeat elements are families of related sequences that are dispersed throughout the genome. There are two main groups of interspersed repeats in the mammalian genome, called SINES and LINES (*Short and Long Interspersed ElementS*, respectively). The most frequently occurring members of each of these in the human genome are the Alu family of SINES and the L1 family of LINES. Alu sequences are 300 bp long, GC-rich (56% GC), and present in up to 10^6 copies/genome. L1 sequences are up to 6 kb long, AT-rich, and present in up to 10^4 – 10^5 copies/genome.

When Alu sequences are hybridized to metaphase chromosomes by in situ hybridization, the resulting pattern is an R-banded one. When L1 sequences are hybridized, the resulting pattern is a G-banded one [116, 117].

DNase I sensitivity

Active or potentially active genes are known to be hypersensitive to digestion with DNase I. When mammalian chromosomes are treated with DNase I, the pattern of sensitivity resembles R-banding [118–120].

Condensation

Chromosome condensation is a dynamic process that proceeds at different rates along the length of the chromosome. Chromosomes contain preferential regions of condensation. Drouin and colleagues [121–123] found that from prophase to late metaphase, the early-replicating R sub-bands fuse more readily than the late-replicating G sub-bands. In fact, R-positive bands usually replicate early and condense late, whereas adjacent G-positive bands replicate late but condense early. This may explain why G-positive bands seem to account for a larger proportion of the genome between prophase and metaphase condensation.

Replication timing

Replication banding patterns also show that R-bands replicate early in S phase, whereas G-bands replicate late [124–127]. The genome can be fractionated into early- and late-replicating regions by buoyant density centrifugation [128]. Early-replicating regions coincide with the G-light (GC-rich/R-band) regions, while late-replicating regions coincide with the G-dark (AT-rich/G-band) regions [129–131]. All housekeeping genes (those that are required for basic cellular metabolism and therefore are active in all cells) appear to replicate early. Tissue-specific genes (whether early or late) either replicate at the same stage of S phase regardless of the expression state, or replicate earlier when transcriptionally active than when inactive [128, 132]. The positive correlation between replication banding patterns and G/R bands suggests a direct relationship between transcriptional potential and replication.

Protein association

Acetylation and deacetylation of histones are dynamic processes whose exact relationship with transcription is unclear. Actively transcribing chromatin is maintained in a hypersensitive state. This can be demonstrated by immunofluorescence with an antibody to acetylated histones. Interphase nuclei show a pattern of variable (bright and dull) staining, whereas metaphase spreads show weak staining by comparison. Metaphases that are thought to be labeled soon after mitotic arrest (and therefore have not undergone significant deacetylation) are more brightly fluorescent. Acetylated histones, which are associated with active genes, are found principally in the R-bands [133]. HMG-I, which binds preferentially to AT-rich DNA, is found in G-bands and C-bands, but not in R-bands [134].

Recombination and breakage

R-bands appear to be the sites where initiation of meiotic pairing and recombination occur [135, 136]. T-bands in particular display a higher frequency of meiotic chiasmata, radiation-induced breakage and exchange, cancer-related rearrangements, and evolutionary rearrangements [101, 111, 137–140].

Technical aspects of G-bands

G-banding requires methanol/acetic acid-fixed cells spread onto slides; cytoplasmic background interferes with good G-bands, so proper cell dilution and spreading is essential. Slides are sufficiently dried (for 2–4 days at room temperature), or baked at low temperatures (e.g., 60 °C) for 2–18 hours, or high temperatures (e.g., 90–95 °C) for 20–60 minutes to dehydrate them (see Table 6.4). Slides that are insufficiently “aged” by natural means or by heating will not respond at all to the trypsin, no matter how long it is applied, and the chromosomes will appear unbanded. If slides are baked again, they will be more successful. Protease enzymes, such as trypsin or pancreatin, are then used on the slides to treat the cells; this rearranges the chromatin such that the dye will bind differentially and show G-bands. The time of exposure to the enzyme is critical to the quality of the banding (see Figures 6.8–6.10 for over- and undertrypsinized chromosomes). The enzyme is washed off, the stain is applied and subsequently rinsed off, the slide is dried, and then it is either mounted with a permanent resin under a coverslip to protect the cells from abrasion and dust, or the slide is left unmounted (see 6.1.2, Slide mounting). Unmounted slides can be immediately observed under the microscope as long as the immersion oil is not a stain solvent. Covered slides must allow time for the mounting medium to dry before it can be scanned.

One slide from each patient or each cell type (blood, amniotic fluid, etc.) should be banded first as a trial slide. This slide is judged with respect to trypsin time and concentration/activity, as well as stain effectiveness, all independent parameters that should be judged separately. Trypsin times are adjusted in 2–5 second increments, depending on how the chromosomes appear with an

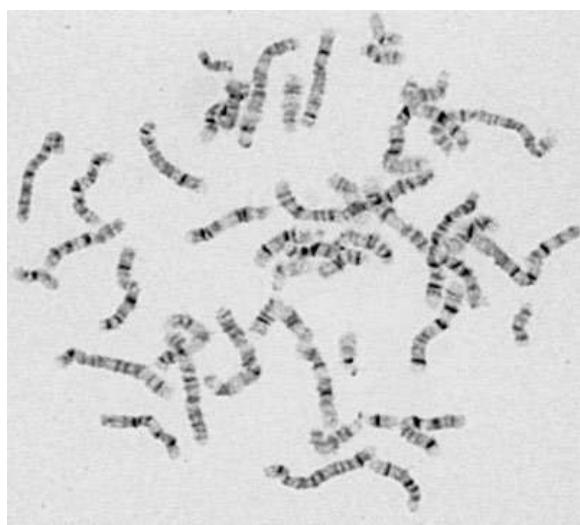


Figure 6.9 Example of an overtrypsinized G-banded metaphase. The chromosomes are still recognizable, but ghost-like with poor morphology. Telomere regions tend to be so light that they can appear deleted.



Figure 6.10 Example of a G-banded metaphase that are appropriately trypsinized. The chromosomes show good morphology, and the contrast is good but the telomere regions are still showing nicely. Image courtesy of Charles D. Bangs.

oil immersion high power lens (Figures 6.8–6.10). Staining times may also need to be adjusted in 5–30 second increments, depending upon how light or dark the chromosomes appear. Undertrypsinized chromosomes have indistinct bands and little contrast, similar to solid-stained chromosomes, but usually fuzzy (Figure 6.10). This appearance may also signify a need for further aging/heating of slides before banding can succeed. Overtrypsinized chromosomes have sharp bands and often appear frazzled at the ends. There is usually too much contrast between landmark bands and the telomeres fade out or disappear (Figure 6.11). Extremely overtrypsinized chromosomes are very pale after staining and may appear ghost-like and very swollen. Appropriately stained chromosomes are neither too dark nor too pale to analyze at the microscope (Figure 6.10), and should show a fair amount of contrast and a wide range of gray values. For example, certain chromosomes, including 1, 7, 11, 17, and 19, should be checked for differential contrast. Chromosomes 2, 4, and 6 are good for checking trypsinization and separation of medium dark bands. Judging slides takes practice and time, but is well worth the effort and leads to reproducible high-quality banding.

Trypsin exposure depends on its brand, concentration, and temperature, but may also be affected by preparation and working conditions. Recrystallized forms have a higher level of activity, thereby requiring less time or a lower concentration. A stock trypsin solution can be prepared in advance, and small aliquots can be frozen until needed. At lower temperatures, the working solution has decreased activity. Higher pH (approximately 8) and higher temperature increase the enzymatic action of the trypsin and may be useful with cells that appear to be resistant to standard concentrations. Exposing slides to trypsin in a vertical fashion (rather than horizontal) may enhance its activity slightly.

A fetal bovine serum rinse is helpful in stopping the action of the trypsin. Serum contains alpha-1-antitrypsin, which inhibits the trypsin by complexing it with the protein in the serum. Other rinses that are commonly used include pH 8.0 buffer, 0.85% (normal) saline, Hanks' balanced salt solution (HBSS), and 70% ethanol.

Slides must be thoroughly dry before mounting. Compressed air can be used for this purpose. Slides can also be dried on a 50 °C hotplate for 15–30 minutes or left out at room temperature to dry overnight before mounting. Blotting the slides dry with bibulous paper is not recommended, because it can scratch the chromosomes.

Wright stain is becoming increasingly popular for G-banding (GTW). It is extremely useful for high-resolution chromosome analysis, because it gives sharper resolution and reveals fine bands. It is also useful for *in situ* coverslips, which may have slightly more cytoplasmic background.

6.2.3 C-banding (CBG)

C-banding is used to visualize the constitutive heterochromatin at the centromeres and at the secondary constrictions of the chromosomes. It does not identify every chromosome as do the G- and R-band methods. It was originally seen in the autoradiography *in situ* hybridization method used by Pardue and Gall [45].

Constitutive heterochromatin was first described by Emil Heitz in 1928 [143]. This heterochromatin constitutes approximately 20% of the human genome [144]. It is located around the primary constriction of all of the human chromosomes and is most abundant at the secondary constrictions of chromosomes 1, 9, 16, and the long arm of the Y chromosome (Figure 6.11).

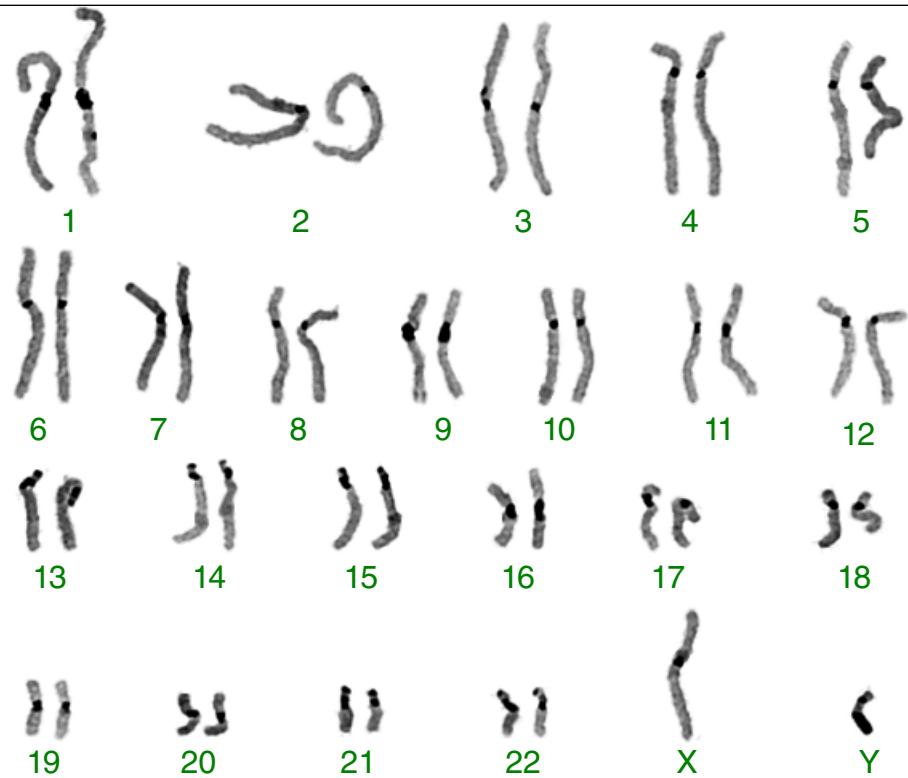


Figure 6.11 C-banding of a normal male cell. This cell was obtained following barium hydroxide incubation. Note large blocks of heterochromatin on chromosomes 1, 9, 16, and Y.

In other mammalian species, it is also located in telomeres and interstitial regions, as well as centromeric regions. Hsu reviewed the properties of constitutive heterochromatin: it is ubiquitous; is heteropyknotic; replicates late; contains highly repeated satellite and nonsatellite DNA sequences; is usually, but not always, centromeric in humans; contains few, if any, structural genes; is highly polymorphic; and is probably never transcribed [145].

The amount, stability, and evolutionary conservation of constitutive heterochromatin imply that it must have some functionally important role. Although review articles have postulated various effects of and functions for constitutive heterochromatin, supporting evidence for a functional role is minimal [146, 147]. Burkholder [148] suggests that transcriptionally inactive heterochromatin has several potential structural functions, for example, it may serve as a structural base on which the kinetochore is organized; it may participate in the interaction that holds sister chromatids together; or it may form a protective buffer zone between the segregation and spindle-related activities of the centromere and the genetically relevant euchromatin. Sumner suggests that if constitutive heterochromatin has a function, it probably occurs in oogenesis or spermatogenesis. Also, the key to its function might be in its position (commonly centromeric) and its composition (usually very large quantities of relatively short tandem repeats), rather than in actual DNA sequences or base composition [104].

Constitutive heterochromatin can be visualized with C-banding techniques, whereas facultative heterochromatin cannot.

Historical and theoretical perspectives

While establishing the localization of mouse satellite DNA by *in situ* hybridization, Pardue and Gall noted that the constitutive heterochromatin near the centromeres of mouse chromosomes, which had been treated with NaOH, stained more densely with Giemsa stain than the rest of the chromosomal regions [45]. With a modification of that technique, Arrighi and Hsu [144, 149] determined the location of constitutive heterochromatin in human and other mammalian chromosomes. They postulated that constitutive heterochromatin have repeated nucleotide sequences that reannealed more quickly than nonrepetitive sequences following alkaline denaturation.

Sumner modified these C-banding techniques, based on the observations that they were somewhat tedious and time-consuming, and that the NaOH was destructive, leading to severe distortion of some chromosomes and loss of others. He substituted barium

hydroxide for sodium hydroxide and called his method the BSG technique (*b*arium *h*ydroxide/*s*aline/*G*iems*a*) [150]. Salamanca and Armendares also recommended Ba(OH)₂ over NaOH, but they used a lower temperature (37 °C versus 50 °C) for pretreatment [151]. Several other modifications have been adopted in various laboratories, but all are basically minor variations that achieve the same end result [141, 142].

Two theories have been proposed for the production of C-bands. Very early studies suggested that alkaline pretreatment produced total denaturation of the chromosomes, and incubation in hot 2× SSC promoted rapid reannealing of the highly repetitive DNA located in C-band regions [144, 152, 153]. Later, autoradiographic studies showed that DNA is selectively extracted from the chromosome arms (euchromatin) and that a greater proportion of DNA is retained in the constitutive heterochromatin [153–155]. Holmquist showed that DNA is partially depurinated during the acid step and denatured during the alkaline step [156]. The apurinic sites are β-eliminated during the hot salt step, releasing small (200 bp) fragments of single-stranded DNA. Why this process selectively occurs in euchromatin is unknown.

Light microscopy and scanning electron microscopy support the second interpretation [157]. By light microscopy, the C-bands stain darkly with Giemsa, a DNA-specific dye, and the chromosome arms are pale. With scanning EM, the C-band regions have a smooth surface morphology, with a highly compact fine structure. Non-C-banded regions are loosely packed, and individual fibers are easily resolved, averaging 41 nm in diameter.

Biochemical evidence also supports the second interpretation. Using SDS-PAGE, Burkholder and Duczek found that the C-banding process caused extensive extraction of both histone and nonhistone proteins: HCl extracted large amounts of each of the five histones, as well as a few nonhistones. NaOH caused the loss of a large variety of nonhistones and some of each of the remaining histones [85, 94]. Ba(OH)₂ was more severe than NaOH and appeared to produce some protein degradation, especially with longer treatment. After the complete C-banding procedure, the residue consisted of a variety of nonhistones ($\geq 53,000$ Da) and histones, especially H3. There were small amounts of H2A and H4 and trace amounts of H1 and H2B. Comings and colleagues noted that SSC extracted soluble proteins that were extensively degraded and therefore not recovered by the trichloroacetic acid (TCA) precipitation used by Burkholder and Duczek [154]. They also found that approximately 60% of the DNA was extracted during the CBG procedure and suggested that certain proteins were associated with DNA and were closely involved in the condensation of constitutive heterochromatin, making it more resistant to dispersion and extraction. Burkholder and Weaver [93] and Burkholder and Duczek [85] also suggested that chromosomal protein-DNA interactions in the constitutive heterochromatic regions produced a configuration that was not susceptible to extraction.

The current thought is that the acidic, basic, and hot saline treatments extract the purine bases from the DNA and then selectively break the sugar-phosphate backbone of the euchromatic DNA fragments, allowing the DNA to be eluted out in the lightly stained regions. The heterochromatin in the C-band domains is darkly stained because it was not eluted.

Clinical significance

The C-banding (CBG) technique is primarily used to identify polymorphic variants in chromosomes, such as increases or decreases in the lengths of the heterochromatic regions (1qh, 9qh, 16qh, Yqh) and inversions or rearrangements of chromosome 9 or other chromosomes. The technique can also be used for the characterization of supernumerary or marker chromosomes (to determine to some degree whether it is composed of heterochromatin or euchromatin) and for the identification of specific chromosomes in somatic cell hybrids [158].

Numerous references address the measurement and classification of C-band variants [159–164]. From a practical standpoint, the method of Patil and Lubs seems to be fairly straightforward. The qh regions of chromosomes 1, 9, and 16 are compared with the average area of the short arms of chromosome 16 [162]. The reasons for choosing chromosome 16 are as follows: the length of the short arm of chromosome 16 is not appreciably changed by contraction; it is intermediate in size compared to the qh regions of 1, 9, and 16; the centromere of chromosome 16 is easily identified; and chromosome 16 itself is easily identified in a C-banded cell, thereby serving as an internal reference for each particular cell. Patil and Lubs [162] established five levels for C-band variants:

Level	Relative size
1	$\leq 0.5 \times 16p$
2	$>0.5\text{--}1.0 \times 16p$
3	$>1\text{--}1.5 \times 16p$
4	$>1.5\text{--}2 \times 16p$
5	$>2 \times 16p$

The examination of 5–10 C-banded cells of good quality is usually sufficient to define a variant chromosome.

Technical considerations

There are differing opinions on whether it is best to use freshly prepared slides or slides that have been aged a few days. The latter is preferable, because freshly prepared slides tend to show a pattern more like G-banding, and the chromosomes are somewhat fuzzy in appearance. In any case, the metaphases should be well spread with little or no cytoplasmic background.

Barium hydroxide treatment acts to denature the DNA of chromosomes, as does heat. Insufficient incubation in Ba(OH)₂ results in a more complete G-like banding pattern, whereas excessive incubation produces ghost-like swelling and distortion of the chromosomes. At 60 °C, the incubation times are somewhat too short to control easily, but at 37 °C, the times are inconveniently long. Using the barium hydroxide at 50 °C is a good compromise [150].

The 2× SSC incubation is a critical part of the procedure and is usually carried out at 60–65 °C for 1 hour. If the slides don't appear to be C-banded following Giemsa staining, they can be returned to the 2× SSC for another 15-minute increment. (The slides do not have to be destained in fixative first because 2× SSC itself will remove the stain.) Giemsa staining for C-banding should be performed at pH 6.8–7.2. At pH >8 or <6.4, differential staining between euchromatin and heterochromatin is obliterated [144].

Several references at the end of this chapter discuss sequential and simultaneous banding methods. Rubenstein et al. [165] described C-banding following Q-banding and sequential R-banding with acridine orange. De la Maza and Sanchez [166] described a method for simultaneous G- and C-banding of human chromosomes, using only HCl treatment, 2× SSC, alcohol dehydration, and Wright stain. They proposed the term "W bands" for this combined banding pattern. Denton et al. described a technique for simultaneously staining NORs and kinetochores, which causes both regions to appear black and the rest of the chromatin to appear yellow [167]. Tuck-Muller et al. [168] also described a method for combined C-banding and silver staining, where euchromatin is pale pink, heterochromatin is deep purple, and active NORs are black. De Braekeleer et al. [169] described a high-resolution banding technique using fluorodeoxyuridine synchronization and a G→C or R→C-banding technique.

6.2.4 G-11 staining

The G-11 technique, which specifically reveals the heterochromatic segment of chromosome 9, was described in 1972 [170, 171]. Juxtacentromeric staining may also be observed in other chromosomes, especially 1q, 4p, 5q, 7p, 10q, 17p, 20q, Yqh, and the centromeres and short arms of all of the D- and G-group chromosomes [170]. This technique involves staining metaphase preparations in buffered alkaline Giemsa stain. The heterochromatic 9qh region is stained reddish purple, but the rest of the chromosomes appear pale blue (Figure 6.12). The positive heterochromatic segments are also observed as reddish purple bodies in interphase cells. Understained chromosomes appear as uniformly pale blue; over-stained chromosomes are uniformly reddish purple.

Historical and theoretical perspectives

Gagne and Laberge reported that the proportion of nuclei and metaphases with G-11 positive staining was 25–30%; they attributed the low percentage to cellular damage incurred by the high pH [171]. The crucial points, in their estimation, were as follows: (a) the pH should be 11.3 to 11.9, with optimum results at 11.6. (b) New stain must be prepared for each batch of slides. (c) Three to four minutes in 45% glacial acetic acid (following 3 : 1 methanol–acetic acid fixation, but prior to slide preparation) seems to protect the cells from pH damage.

Wyandt et al. [172] attempted to elucidate the mechanism of G-11 banding by varying the conditions of staining. They determined that two components of Giemsa are necessary to obtain G-11 banding: azure (either A or B) and eosin Y. These components react under alkaline conditions to form an azure–eosinate complex. The conditions that are important for the interaction of these two dyes to produce G-11 banding are the absolute concentrations and ratio of the two dyes, the pH of the buffered staining solution, and the composition of the buffer used in the staining solution. The authors concluded that the differential denaturation of DNA does not appear to play a key role in the staining process, because at neutral pH, differentiation was not observed under conditions of high temperature or formamide incubation. Furthermore, neither moderately high temperatures nor formalin fixation affected G-11 banding at alkaline pH.

Wyandt and colleagues determined the optimum age of slides for G-11 stain to be 3 days to 1 month, the optimum staining time to be 10 minutes, and the optimum pH to be 11.6 (pH 11.3 was best for older slides, and pH 12 was best for slides <3 days old). Finally, they found that a combination of azure B and eosin Y gave results identical, if not superior, to those observed using Giemsa stain itself.

Magenis et al. described the use of the G-11 technique to study 1qh heteromorphism [173]. They found that the G-11 procedure produces more specific staining of the secondary constriction of chromosome 1 than does the C-banding technique. Although the G-11 band is smaller than the C-band region, it is always within the C-band region; therefore,

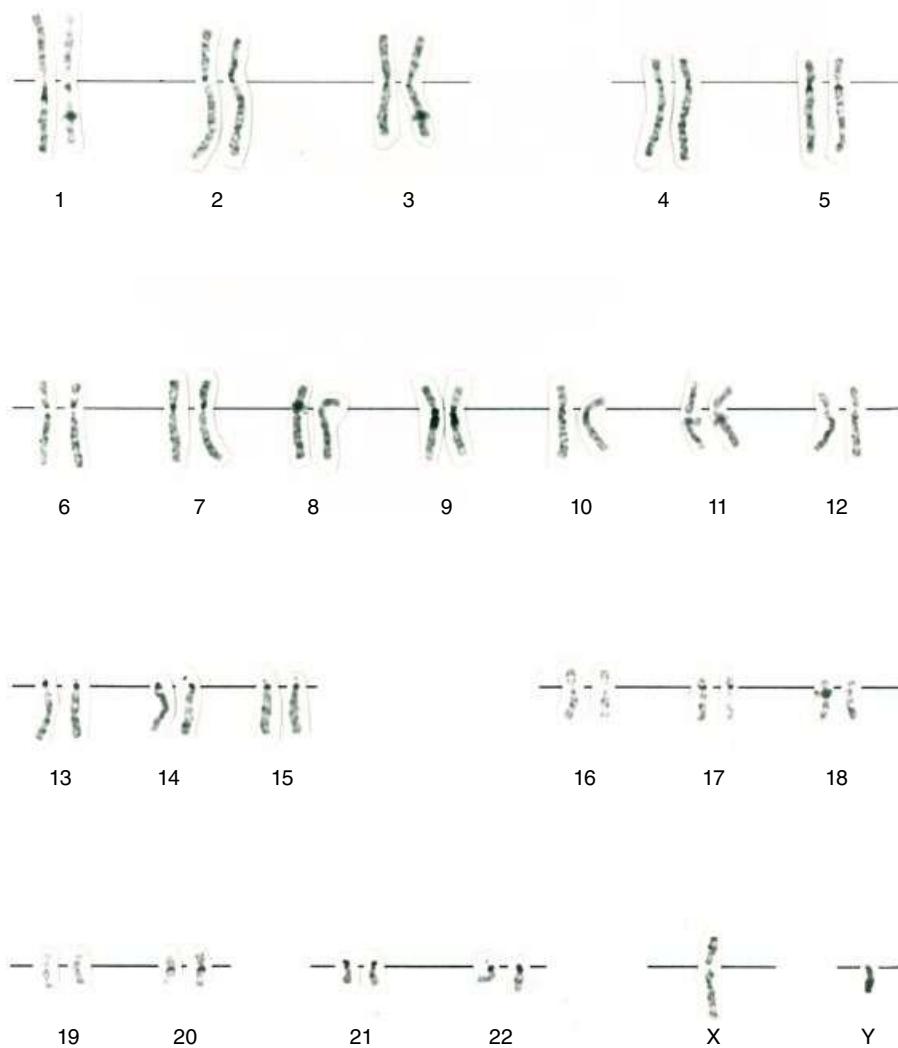


Figure 6.12 Karyogram of a G-11-stained metaphase. G-11 karyotypes show very distinct heterochromatin regions while retaining some G-bands for better identification of each homologue.

it appears that G-11 staining reveals a specific subset of heterochromatin. By studying chromosome 1 in several individuals, the authors found no direct correlation between the G-11 and C-band variants. These findings, in addition to *in situ* annealing studies, support the hypothesis that the heterochromatic region is composed of different satellite DNA sequences [174].

Clinical significance

Clinical applications include the use of G-11 staining to identify nonbanding centric fragments and occasional chromosome rearrangements, including pericentric inversions and translocations involving chromosome 9. Morse et al. [175] reported the clinical usefulness of this technique in hematologic studies. More accurate diagnoses were established in approximately 10% of patients (11 of 116) through the use of G-11 staining, using Alhadeff's modification (a combination of fluorescence plus Giemsa and G-11) with preliminary soaking in distilled water for 2 hours at 60 °C [176].

From a research standpoint, G-11 staining can be used to distinguish human (pink) from hamster (purple) chromosomes in somatic cell hybrids [176, 177]. G-11 staining might be useful in gene mapping and linkage studies and in the elucidation of the organization and structure of heterochromatic regions of chromosomes.

Technical considerations

Old slides (stained as well as unstained) can be destained and G-11-stained, and poor specimens can still be used to identify chromosome 9. Also, if necessary, the G-11 technique can be repeated on a previously treated slide.

6.2.5 Centromere/kinetochore staining

Although centromeres and kinetochores are biochemically and structurally distinct regions, they are functionally linked in the normal movement and segregation of mitotic chromosomes [178]. The centromere is the region of the primary constriction of mitotic chromosomes; it is also the locus that nucleates the formation of the kinetochore and directs the partitioning of chromosomes in mitosis [179]. The centromere is an integral part of the chromosome fiber, whereas the kinetochore is extrinsic to the DNA fiber and consists of several specialized proteins complexed with the centromeric DNA [180]. The kinetochore is the protein structure on the centromeres of the chromatids where the spindle fibers attach during cell division to pull sister chromatids apart [179].

Human centromeres consist of three structural domains: the kinetochore domain on the outer surface, the central domain on which the kinetochore rests, and the pairing domain, where the two sister chromatids are in contact. The mammalian kinetochore, as observed by electron microscopy, is a trilaminar disk-shaped structure, located on the lateral surface of each sister chromatid and resting on an underlying layer of densely packed heterochromatin. The kinetochore consists of three parallel plates on either of the primary constriction: an inner dense plate that is in contact with the centromeric heterochromatin, a middle electron-lucent zone, and an outer dense plate, into which the microtubules insert. An outermost fibrous corona is also observed in the absence of microtubules (Figure 6.13). Centromeres/kinetochores can be observed by Cd staining or by immunofluorescence using CREST and CENP antibodies. Both techniques will be discussed.

Cd staining

Historical and theoretical perspectives

The Cd staining procedure was described by Eiberg in 1974 [182]. It reveals two identical dots (very densely stained spheres, one of which is found on each chromatid) at the location of the centromere. These centromeric dots (hence the term Cd) may represent either organelles associated with the spindle fibers or a DNA protein complex that is heat- and alkali resistant. The dots are

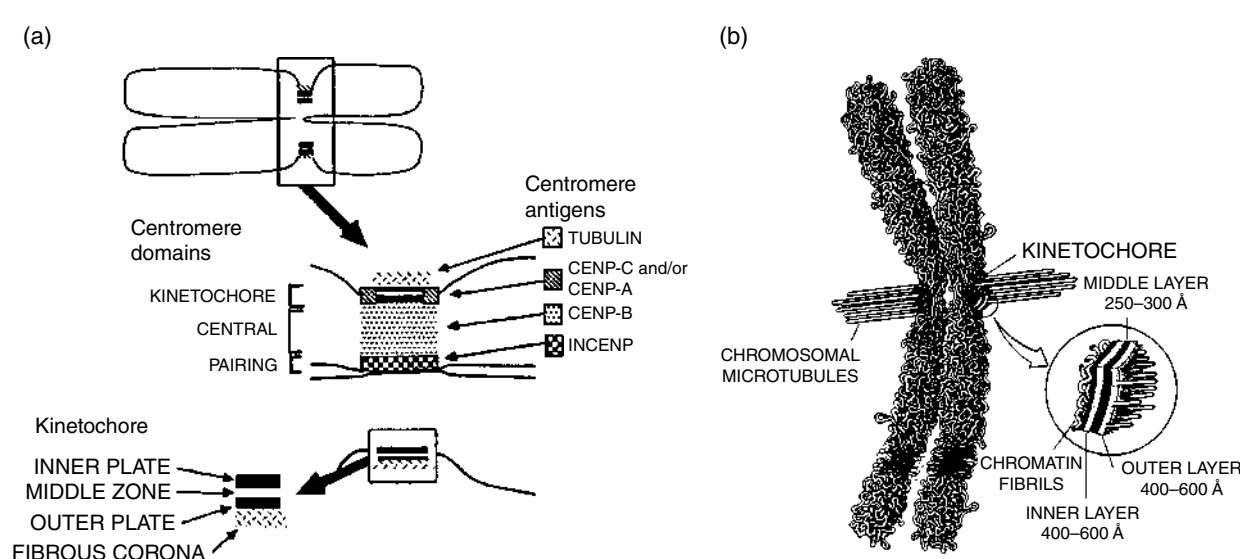


Figure 6.13 Diagrammatic representation of the mammalian centromere. (a) This figure shows the domains of the mammalian centromeres and their diagnostic antigens. The kinetochore plates have been shown as flat in this diagram for the purpose of simplicity. In reality, the kinetochore is slightly curved so that the borders of the outer plate contact the centromeric heterochromatin. Pluta 1990 [206]. Reproduced with permission of Elsevier. (b) Diagram of metaphase chromosome with kinetochore. Brinkley 1985 [178]. Reproduced with permission of Springer.

specific for centromeric regions, not secondary constrictions or other variable regions. Evans and Ross suggested that spotted centromeres might represent kinetochores or their associated proteins [183]. Thus, Cd-bands are not identical to C-bands.

Although C-banding techniques stain constitutive heterochromatin (which in the human is predominantly located in pericentromeric regions), the Cd-banding technique stains only active, or functional, centromeres. Inactive centromeres, also referred to as latent or suppressed centromeres, appear as nonconstricted regions that stain positively with C-banding but not with Cd-staining. The absence of a centromeric dot indicates that the kinetochore is either lost or altered, thereby preventing the attachment of spindle fibers. The alteration of kinetochore function is correlated with an alteration of the primary constriction.

Several types of abnormal chromosomes have been studied with both the Cd procedure and a C-banding procedure [184–186]. Studies of pseudodicentric chromosomes show that a Cd-band is present in only one of two C-banded sites, indicating one active and one inactive centromere. In all cases, chromatid separation is observed at the site that is not constricted, and is Cd-negative. True dicentric chromosomes with two active centromeres (both Cd-positive) can exist in the same cell as pseudodicentric chromosomes with only the active centromere being Cd-positive (e.g., a cell that contains both a dicentric iso Xp and an iso Xq or a tumor cell containing multiple dicentrics) [186].

Clinical significance

Cd-banding is recommended as an adjunct to C-banding for resolving active centromeres in Robertsonian translocations, dicentrics, pseudodicentrics, ring chromosomes, and other abnormal or supernumerary marker chromosomes.

Technical considerations

Eiberg found that culturing, hypotonic treatment, and fixation all influence the Cd banding pattern. His harvesting procedure is described in detail in reference 182.

Crest and CENP staining

Sera from patients with scleroderma contain several autoantibodies that recognize nuclear antigens, as characterized by different patterns of nuclear immunofluorescent staining. These patterns, observed in both interphase and mitotic cells, can be nucleolar, granular, speckled, or homogeneous. Metaphase cells exhibit two small fluorescent dots at the centromere.

Patients with the CREST variant of scleroderma (Calcinosis, Raynaud's phenomenon, Esophageal dys- motility, Sclerodactyly, and Telangiectasia) show a high frequency of antacentromere antibodies (ACAs) [187]. Several of these antibodies have been isolated and purified and have been instrumental in identifying domains within the tripartite kinetochore structure. Anticentromeric antigens that have been identified thus far include the *CEN*tromeric *P*roteins CENP-A (17 kDa), CENP-B (80 kDa), CENP-C (140 kDa), CENP-D (50 kDa), CENP-E (312 kDa), and CENP-F (367 kDa).

CENP-A is found in the centromere in nucleosome-like structures, in which it replaces one or both copies of histone H3 in the (H3–H4)₂ tetrameric core. It is structurally related to histone H3 and has similar biochemical characteristics. However, it also has sequences that are not related to histones or any other known mammalian CENPs. These nonhomologous regions are likely to be involved in localizing CENP-A to centromeric DNA, or in centromeric chromatin packaging and function [188, 189].

CENP-B is recognized by all ACA-positive sera [190] and CENP-B antibodies cross-react with CENP-A and CENP-C. CENP-B is distributed throughout the centromeric heterochromatin in the central domain and binds to a 17-bp sequence in alpha satellite DNA called the CENP-B box [191]. The amount of CENP-B varies from chromosome to chromosome, as does the amount of alphoid DNA [192]. CENP-B is absent from the kinetochore proper and therefore is unlikely to be involved in microtubule binding. Because it is a multidomain protein, it may have other functions (such as higher order folding and packaging) besides binding to alpha satellite DNA. CENP-B was shown to be present at both active and inactive centromeres in several different stable dicentric chromosomes [193, 194]; therefore, CENP-B is not sufficient in and of itself for kinetochore activity.

CENP-C (Figure 6.14) is present in approximately equal amounts at all human centromeres and as such is likely to be a component of an invariant chromosomal substructure, such as the kinetochore [193]. In fact, it appears to localize to the inner kinetochore domain [195]. CENP-C was not observed at the inactive centromere of a stable dicentric chromosome, suggesting that it might be essential for centromere/kinetochore activity [193]. Recent work shows that CENP-C may be required for the assembly of stable, functional kinetochores of normal size during interphase, and for a timely transition to anaphase [196].

From a temporal perspective, CENPs-A, -B, and -C are constitutively expressed and always associated with the centromere, whereas other chromosomal proteins, such as CENPs-D, -E, and -F, are only transiently associated with the centromere. CENP-D localizes to the kinetochore domain. Its sequence is highly homologous with the translated reading frame of RCC1, a gene that regulates the onset of mammalian chromosome condensation [197]. Therefore, CENP-D may also be a cell cycle control protein.

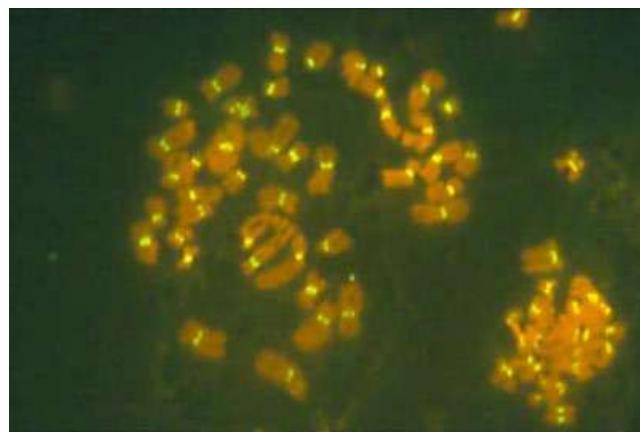


Figure 6.14 CENP-C antibody staining. Chromosomes are treated with antacentromere antibodies (ANA) from a patient's serum with CREST syndrome (calcinosis cutis, Raynaud's phenomenon, biliary cirrhosis). Then chromosomes are treated with fluorescence-conjugated goat antihuman IgG and counterstained with ethidium bromide. Scheres 1976 [326]. Reproduced with permission of Springer. See insert for color representation of this figure.

CENP-E is a kinesin-like motor protein in sequence and predicted structure [198–200]. It is absent during interphase, but the amount of protein in the cytoplasm rises sharply in late S and G2/M. During mitosis, it begins to appear at the centromere in prometaphase and increases as the cell progresses to metaphase. In early anaphase, CENP-E starts its migration to the midzone, and by telophase is found only in the midbody. After cytokinesis, it is probably discarded. As such, it is considered a chromosomal passenger protein [201]. CENP-E is required for metaphase-to-anaphase transition [199]; it appears to be one of the motors responsible for mammalian chromosome movement and/or spindle elongation [200]. CENP-E has two biochemically distinct microtubule binding domains, supporting its role as a microtubule crosslinker [202]. A recent report suggests that CENP-E and CENP-C are necessary for centromere activity in dicentric Robertsonian translocations [194].

CENP-F is the first kinetochore protein found to be part of the nuclear matrix [203, 204]. It, too, is cell cycle-regulated, and is transiently associated with the kinetochore. It appears earlier than any of the other known transient kinetochore proteins [205]. CENP-F is uniformly distributed throughout the nuclear matrix and gradually accumulates during the cell cycle until it reaches peak levels in G2/M. By prophase, it is present as discrete pairs of foci at all centromeres, with a concomitant reduction in nuclear staining. CENP-F is present on the surface of the outer plate of the fully differentiated kinetochore in prometaphase and metaphase chromosomes, and is still detectable after the onset of sister chromatid separation in early anaphase. By late anaphase, the protein is concentrated in the spindle midzone and intracellular bridge. CENP-F is also found throughout the cytoplasm of mitotic cells. The majority of CENP-F is degraded after mitosis. CENP-F may play a role in organizing the centromeric heterochromatin into the highly differentiated trilaminar kinetochore complex at the onset of G2, maturation and function of the kinetochore from G2 to anaphase, and/or intracellular bridge structure and function during anaphase/telophase.

In addition to the CENPs, several other proteins are associated with chromosomes, including the INCENPs (*Inner CENtromere Proteins*) and CLIPs (*Chromatid Linking Proteins*). These are discussed in several excellent reviews [179–181, 206].

Clinical significance

CREST and CENP staining by immunofluorescence are not performed routinely in most cytogenetics laboratories; they are used primarily as research tools to distinguish between active and inactive centromeres in dicentric, pseudodicentric, and Robertsonian translocations. These techniques can also be used to identify the proteins dated with cell division.

Technical considerations

Jeppeisen reviewed the application of immunofluorescence techniques to metaphase chromosomes [207]. A general outline of the methodology based on his review can be found in the submitted methods section.

With any kind of immunofluorescence staining, it is important to maintain *in vivo* cellular organization while retaining antigenicity and allowing access for antibodies. This can pose a problem when the objective is to study proteins, such as those found in and around the kinetochore. Standard techniques used in cytogenetic analysis – colchicine or Colcemid® treatment

to disrupt the mitotic spindle, hypotonic treatment to swell and disperse the chromosomes, and fixation with 3: 1 methanol-acetic acid – are not suitable for protein studies. To study the mechanisms of chromosome movement and segregation, disruption of the mitotic spindle may not be desirable. Furthermore, the use of an acidic fixative may significantly reduce or even abolish the antigenicity of the proteins, presumably by denaturation and extraction of basic proteins, such as histones. Alternative fixatives, such as organic solvents (e.g., methanol and acetone) or protein crosslinking agents (e.g., formaldehyde and glutaraldehyde) are preferred. However, because these fixatives do not provide adequate spreading of the chromosomes as does methanol–acetic acid fixative, it is necessary to spread the cells on the slide prior to fixation. This can be done by dropping the cells onto slides, making smears from concentrated cell suspensions, growing the cells directly on glass slides or coverslips, or sedimenting the cells onto the slides by cytocentrifugation. The last technique is the preferred method. Provided the correct cell density is used, the majority of the chromosomes and nuclei retain good morphology, though not comparable to that obtained with 3 : 1 fixed cells.

Following cytocentrifugation, it is important to permeabilize the cells prior to fixation in order to allow the antibodies access to their target antigens. The preferred method is to treat the cells briefly with potassium chromosome medium (KCM), an isotonic potassium chloride solution with 0.1% Triton X-100, a nonionic detergent. The detergent dissolves the lipid membrane, allowing access to the antibodies. KCM also provides a good medium in which to perform the antibody reactions for immunofluorescence.

6.2.6 Reverse banding (R-bands)

Giemsa R-banding

Reverse banding using heat and Giemsa (RHG) was first described by Dutrillaux and Lejeune [208]. This technique involves the incubation of slides in hot phosphate buffer with subsequent Giemsa staining. The resulting chromosome pattern shows darkly stained R-bands and pale G-bands.

Comings briefly reviewed the mechanism of R-banding and noted that the GC richness of the R-bands is a key factor [61]. The AT-rich regions are selectively, or more readily, denatured by heat, but the GC-rich regions remain intact. This is consistent with the fact that GC-specific fluorochromes (e.g., Chromomycin A3) also produce a reverse banding pattern.

Clinical significance and technical considerations

R-banding methods are useful for analyzing deletions or translocations that involve the terminal ends of chromosomes. Attempts at RHG banding have been successful and reproducible in some laboratories, but inconsistent in many others. Since its introduction, several modifications of this technique have been reported, as summarized by Verma and Lubs [209]. Bernheim and Berger reported a method in which the prepared slides were pretreated with a protein film comprised of fetal calf serum and 3 : 1 fixative. This film presumably stabilized the chromosome preparations under the harsh conditions of the R-banding treatment [210].

In many laboratories, RHG methods have been abandoned in favor of fluorescent R-banding techniques.

Fluorescent R-banding methods

Acridine orange R-banding (RFA)

Historical and theoretical perspectives

Acridine orange was originally used to stain untreated human [211] and mouse [212] chromosomes. Bobrow et al. [213] and Baserga and Castoldi [214] independently reported the use of acridine orange to obtain a reverse banding pattern of chromosomes.

Acridine orange (AO) is a base composition-independent fluorochrome that binds to DNA by intercalation, giving relatively uniform fluorescence along the length of the chromosome arms. The dye binds very little to non-nucleic acid cell components, but it fluoresces orange-red when bound to single-stranded nucleic acids and yellow-green when bound to double-stranded nucleic acids [215–217]. Following hot phosphate buffer treatment, R-bands are yellow-green, and G/Q-bands are orange-red. Bobrow and Madan [218] proposed that the structural organization of chromosomal DNA was differentially changed from double- to single-stranded DNA during the hot incubation period; however, Verma and Lubs [219] pointed out that after prolonged rinsing, the fluorescence became generally green, even though the state of naturation should not have been affected.

Although acridine orange is a nonspecific fluorochrome, it seems to exhibit base pair specificity when it is used following pretreatment with the antibiotics distamycin A (AT specific) or actinomycin D (GC specific). These base pair-specific

nonfluorescent antibiotics induce banding patterns by selectively altering AO binding sites in regions that are rich in the particular bases preferred by the ligands [220].

Several authors have reported the use of amethopterin, thymidine, or BrdU to produce synchronized cell cultures [221–226]. After harvesting and slide-making, the high-resolution chromosome preparations are stained with acridine orange, Hoechst, or Giemsa. The resulting patterns depend on the type of synchronization, timing, and sequence of chemical additives. A comparison of morphologic (structural) and dynamic (replication) banding techniques shows that all R-bands are not the same and all R-bands are not opposite in staining to all G-bands: R-bands by heating using Giemsa (RHG) and R-bands by BrdU using Giemsa (RBG) are 75–85% congruent, and RHG and GTG bands are 90% complementary [121]. The major factor that contributes to R-banding is the relative GC-richness of the R-bands themselves. As previously noted, Comings reviewed several observations which led to this conclusion [61].

With respect to proteins, Comings et al. [154] found that only about 3% of chromosomal proteins were extracted by R-banding (RHG). Burkholder and Duczek also reported that R-banding procedures, which required incubation in a hot saline or NaH_2PO_4 solution, had very little effect on the extraction of proteins from isolated nuclei, compared to a variety of C-banding techniques [94].

Electron microscopy suggests that there is a structural basis for R-banding [227]. Hot NaH_2PO_4 produced an adverse effect on chromatin structure with a reduction in overall electron density of the chromosomes themselves. Chromatin fibers had an increased electron density and were more aggregated in the R-band-positive regions than in the R-band-negative regions (the reverse of what is observed with G-banding treatments). The mechanism by which hot NaH_2PO_4 pretreatment produces differences in electron density is not clearly understood, although Burkholder suggested that alterations of DNA-protein interactions following differential denaturation may induce localized changes in the organization of the chromatin.

Technical considerations

Acridine orange preparations may range in color from uniformly green to uniformly red. The variation may result from improper incubation in buffer or improper staining. Insufficient incubation in buffer results in uniformly green fluorescence, which can be corrected by using a longer incubation or higher temperature. Excessive incubation in buffer results in uniformly red fluorescence, which can be corrected by using a shorter incubation or lower temperature. Insufficient staining (uniformly green fluorescence) can be corrected by longer staining or a higher dye concentration. Excessive staining (uniformly red fluorescence) can be corrected by additional rinsing, a shorter staining time, or a lower dye concentration [104].

Verma and Lubs [209, 219] outlined the following factors as critical for optimum R-banding with acridine orange:

1. Fixation
2. Length of time in buffer
 - a. Shorter incubation periods are necessary for older slides.
 - b. Fresh slides do not band clearly.
 - c. Slides older than 3 months stain uniformly red.
 - d. Slides 15–21 days old give the best results.
3. Conditions of buffer incubation
 - a. One slide should be incubated at a time.
 - b. The level of water in the water bath should be approximately the same as the level of buffer in staining jar.
 - c. The staining jar should be covered.
 - d. The slide should be agitated periodically to avoid patchy banding.
 - e. A pH of 6.5 is optimum.
4. Staining
 - a. Overstaining (uniformly orange-red) can be resolved by additional rinsing in buffer.
 - b. Understaining (uniformly green) can be resolved by restaining.
5. Mounting

For coverslip mounting, excess phosphate buffer should be pressed out.
6. Sequential staining
 - a. Q- followed by R- produces excellent results.
 - b. G- followed by R- gives variable results depending on the conditions.
 - c. R- followed by C- gives poor C-bands, if any.
 - d. C- followed by R- gives no R-bands.

For an example of acridine orange R-band staining methods, see submitted methods at the end of this chapter.

Chromomycin A3/methyl green R-banding (CA3/MG)

Historical and theoretical perspectives

Van de Sande et al. [228] originally recommended the use of the GC-specific antibiotic Chromomycin A3 to observe R-bands; however, better success was achieved with the structurally similar GC-specific antibiotic olivomycin. Schweizer [229] reported the use of methyl green as a pre-stain prior to Chromomycin A3 staining. His results with plant chromosomes were successful, but his attempts to similarly increase the contrast of Chromomycin A3-stained human chromosomes were inconclusive.

Sahar and Latt [230, 231] demonstrated clear fluorescent R-bands by the use of Chromomycin A3 and subsequent methyl green staining (Figure 6.15). The band differentiation was the result of electron energy transfer from the fluorescent Chromomycin A3 to the non-fluorescent methyl green. In this particular method, the donor, or primary stain, which is fluorescent by definition, is Chromomycin A3 [232]. This dye is a GC-specific antibiotic, which when complexed to calf thymus DNA has an excitation (absorption) maximum at 430 nm and an emission (fluorescence) maximum at 570 nm [233–235]. The use of Chromomycin A3 alone results in a faint R-band pattern [228].

Methyl green serves as the non-fluorescent acceptor counterstain in this technique. Methyl green is an AT-specific dye, which has its absorption maximum at 642 nm [236, 237]. This is close enough to the emission spectrum of Chromomycin A3 (570 nm) for energy to be transferred to methyl green instead of being emitted as fluorescence by Chromomycin A3. Specifically, A3 fluorescence is quenched by methyl green in areas where there are presumably fewer than 20 consecutive GC base pairs. These areas appear dull, whereas clusters of 20 or more GC base pairs are resistant to quenching and appear bright [230, 238].

Clinical significance

R-bands produced by the Chromomycin A3/methyl green technique generally provide the same information as bands produced by the Giemsa R-band or acridine orange R-band techniques. Similarly, the technique is useful for study of structural chromosome abnormalities and visualizing heteromorphisms. When Q- or C-band heteromorphisms are uninformative, R-band heteromorphisms may provide additional information. The Chromomycin A3/methyl green method has the advantage over other R-band methods because it will work the same no matter how old the slides are or whether they have been baked or not. Slides over a year old are suitable for banding with this technique. The R-banding method does not remove or destroy chromatin, and can be followed by other stains, such as NOR- and C-banding to obtain sequential stains on one cell.

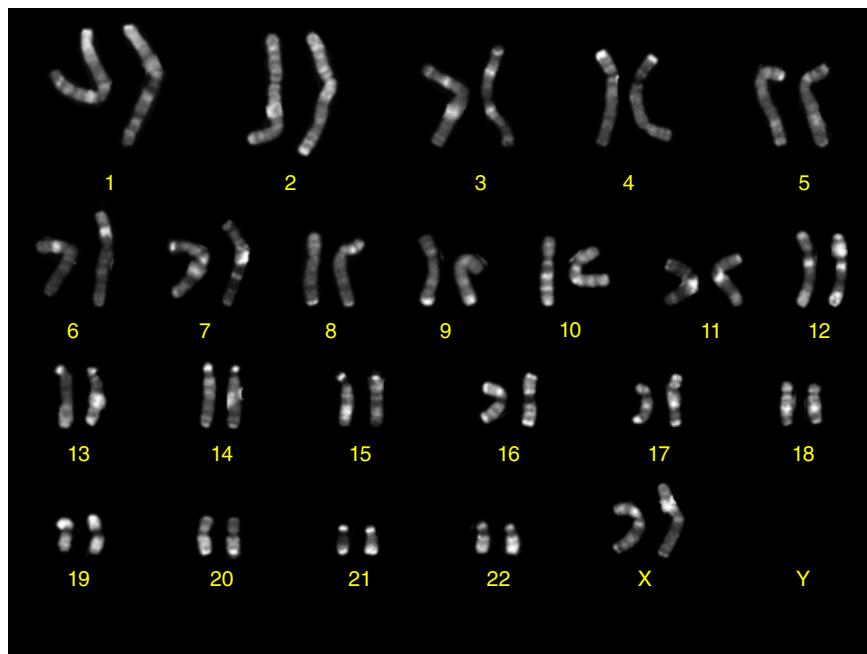


Figure 6.15 Reverse banding of a 46,XX cell by chromomycin A3 and methyl green. R-bands are the mirror image of Q- and G-bands, and are strong for showing the G-band light telomeric regions of the chromosomes that carry many genes but are difficult to stain well with GTG bands.

6.2.7 DAPI/distamycin A staining (DA-DAPI)

The DAPI/distamycin A fluorescent staining technique was first described by Schweizer et al. [239] as a method for labeling a specific subset of C-bands.

Historical and theoretical perspectives

DAPI, or 4',6'-diamidino-2'-phenylindole, is a fluorescent dye that has a fluorescence maximum of 450 nm when complexed with calf thymus DNA [240]. DAPI also has AT specificity, and it is considered to be the primary stain in this method. DAPI alone can yield good banding patterns in metaphase cells denatured in hot formamide using FISH methods.

Distamycin A (DA) is a nonfluorescent basic oligopeptide antibiotic that preferentially forms stable bonds to AT-rich double-stranded DNA. It does not intercalate, binding instead by hydrogen bonding and hydrophobic interaction. Distamycin A mainly binds to the chromosome arms, with patterns similar to Q-banding. Using calf thymus DNA, the UV absorption spectrum of the distamycin A-DNA complex peaks at 340 nm [241].

The combined staining of DAPI and distamycin A specifically highlights certain constitutive heterochromatin-containing regions, including 15p11, Yqh, and the C-band regions of chromosomes 1, 9, and 16 (Figure 6.16). Except for these DA-DAPI-positive regions, distamycin A obliterates all other fluorescent DAPI staining (although other chromosomes sometimes show a small bright band at the centromere). In terms of the C-band positive qh regions, the fluorescent intensity ranks as follows: 16qh > 1qh > 9qh. Interphase nuclei also show several distinct fluorescent chromocenters, which correspond to the bright DAPI areas.

The specific chemical properties of the DA-DAPI-positive heterochromatin related to this banding phenomenon are unknown. In general, all DA-DAPI-bright regions contain different classes of highly repetitive DNA, especially AT-rich satellite DNA [174, 242, 243]. All bright DA-DAPI regions are heterochromatic, but it is impossible to detect a simple relationship between the chromosomal distribution of the different satellite DNAs and the location of bright DA-DAPI hot spots. For example, 9qh and 15p11 are bright with DA-DAPI but not with DAPI alone. The centromere of chromosome 3 is DAPI-bright but not DA-DAPI-bright [239].

Donlon and Magenis [244] reported that the AT-specific dye methyl green (MG) could be used as a substitute for distamycin A. Methyl green gave essentially identical results, with the exception that the intercalary Q-bands (which appear following UV irradiation and fading of the DAPI fluorescence during microscopy) were more pronounced with methyl green than with distamycin A.

Schweizer reported the use of a triple stain – Chromomycin A3, distamycin A, and DAPI (GC/AT/AT) – to show the simultaneous fluorescent staining of R-bands and DA-DAPI bands in human chromosomes [245]. By selecting the appropriate excitation and emission filters, either R-bands or the DA-DAPI bands can be observed. In fact, in these triple-stained cells, the Chromomycin A fluorescence was found to be enhanced.

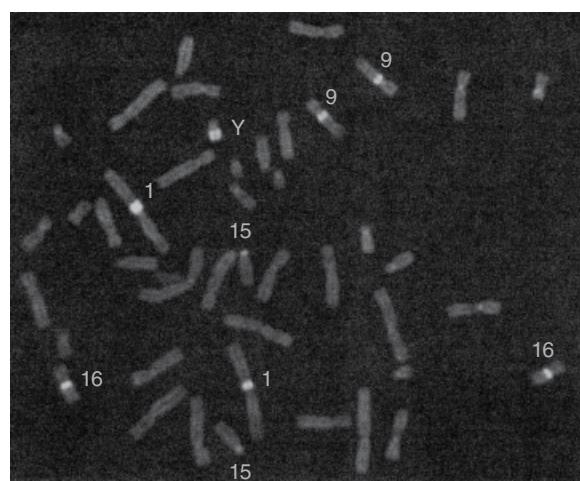


Figure 6.16 DA-DAPI staining. DA-DAPI (DAPI/distamycin A) stains show the heterochromatin of the 1, 9, 15p, 16, and Y very nicely.

Heng and Tsui reported a variation on the use of DAPI banding with fluorescence *in situ* hybridization (FISH) [246]. By controlling the degree of chromatin denaturation through formamide incubation, heat treatment, and/or high pH, they were able to produce three types of DAPI bands sequentially on the same set of BrdU-incorporated chromosomes. The pattern order was initially DAPI multibanding (equivalent to Q-banding), then partial C-banding including DA-DAPI banding, and finally C-banding. The authors also compared a one-step staining procedure using a mixture of DAPI and DA with the traditional two-step DA-DAPI staining method of Schweizer et al. [239]. They noted that much less DA was required to generate the DA-DAPI banding pattern when the stains were mixed; they suggested that for other types of fluorochrome banding with competitive stains, a mixed solution might also be more effective and more efficient in getting comparable or better results.

Currently, DAPI banding patterns (no DA is necessary) that include both a faint Q-band pattern and a bright C-banded pattern for certain heterochromatic regions are used to identify metaphase chromosomes in FISH studies when internal controls do not help. FISH performed using separate denaturation rather than codenaturation seems to yield the best bands for this purpose (Figure 6.17).

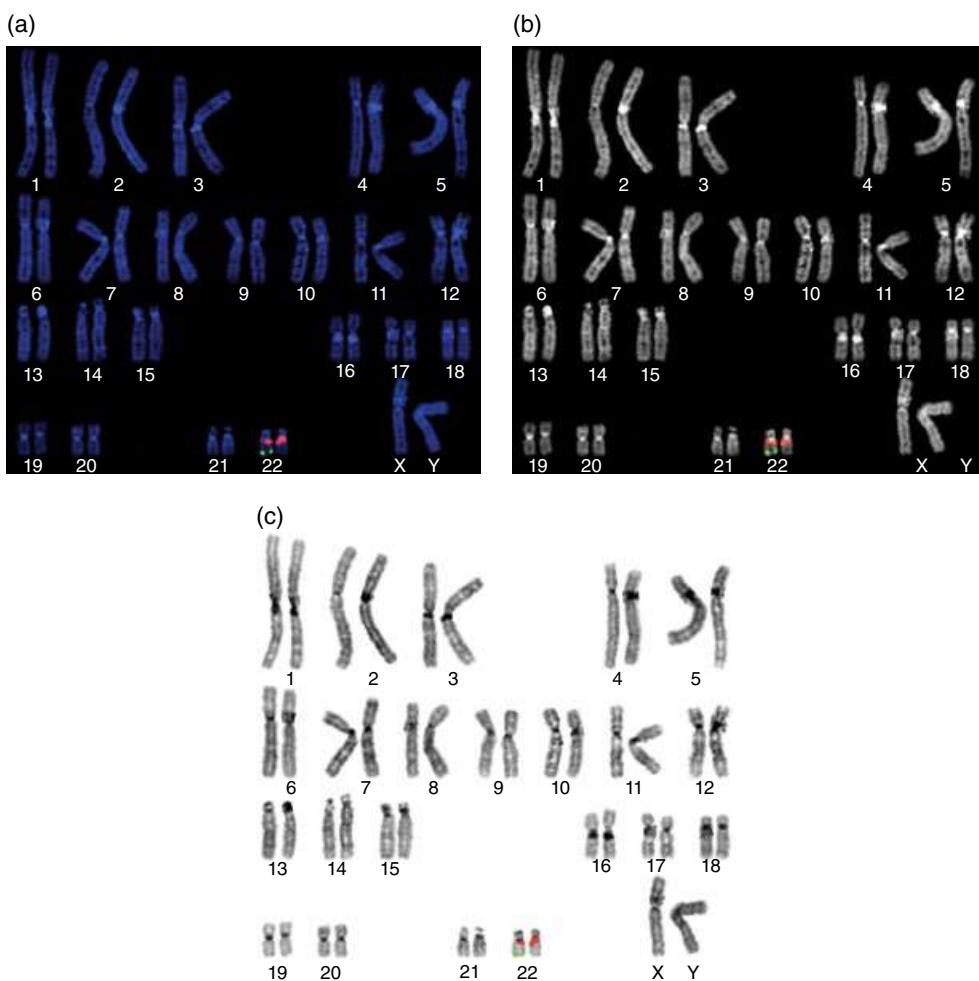


Figure 6.17 Cells from a FISH slide (TUPLE1/ARSA probes) are stained with DAPI to show a deletion of the TUPLE1 region. (a) In true colors. (b) Displayed in black and white to show bands and FISH. (c) Reversed DAPI to show as G-banding patterns. Courtesy of Applied Spectral Imaging. These Images were generated using Applied Spectral Imaging's GenASIs FISHView. See insert for color representation of this figure.

Clinical significance

From a clinical cytogenetic standpoint, the DA-DAPI technique is useful in identifying pericentromeric breakpoints in chromosomal rearrangements [247] and in identifying marker chromosomes that are too small for standard banding techniques to identify. For example, DA-DAPI can be used to help characterize bisatellited chromosome 15 markers (inv dup 15) [248] or other rearrangements of chromosome 15 [249, 250]. DA-DAPI is very helpful for detecting the fluorescent Yqh regions in Y autosome translocations. The QFQ-bright short arms of the acrocentric chromosomes (other than the 15q) do not usually fluoresce brightly with DAPI/DA. The method is therefore Y-discriminative [251–256].

6.2.8 Silver staining (AgNOR) for nucleolus organizing regions

The nucleolus is a highly basophilic body in the cell nucleus that is involved with the production and assembly of ribosomal subunits. The silver staining ability of the nucleolus in cytologic preparations was described in 1891 by Ruzicka [257], but the correlation of the nucleoli with chromosomal NORs did not occur until much later [258, 259].

The nucleolus organizing regions are distributed quite differently among various species [260–262]. In humans, NORs contain the genes for the production of 18S and 28S rRNA. They have also been found to contain ribosomal genes associated with proteins, such as upstream binding factor (UBF) and RNA polymerase I (RPI). These genes are clustered in 10 loci on the five pairs of acrocentric chromosomes (chromosomes 13, 14, 15, 21, and 22), demonstrated by *in situ* hybridization using tritiated rRNA [263, 264]. The amount of hybridization, and therefore the number of rDNA genes per NOR, is not constant. These variations are inherited in a Mendelian fashion [263, 265]. In the human, a theoretical maximum of 10 NORs per cell can be visualized, although six to eight is more likely (Figure 6.18) [266].

Miller and colleagues [267] showed that the silver staining method reflects the activity, and not merely the presence, of rRNA genes. Using mouse-human somatic cell hybrids in which human rRNA genes were suppressed, they determined that the absence of silver stain indicated that the human acrocentrics were not acting as nucleolar organizers in the preceding interphase in those particular cells. This was confirmed in mouse-human hybrids in which the suppressed mouse rRNA genes did not silver stain [268].

Verma et al. [269] noted that only acrocentric chromosomes with active, Ag-positive NORs were found in satellite associations and that unstained inactive NORs were not seen in associations.

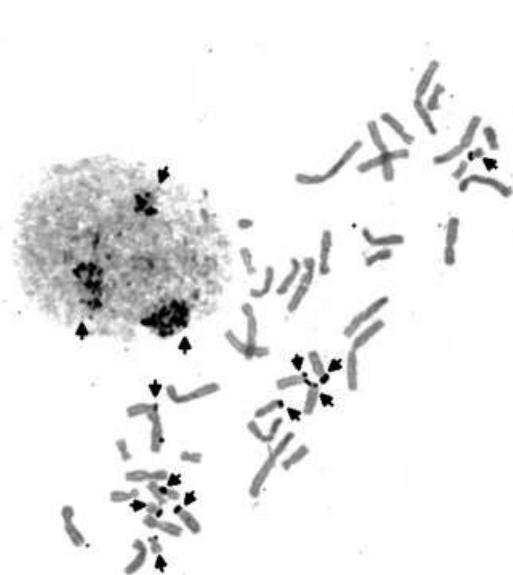


Figure 6.18 AgNOR staining. The nucleolus organizing regions are located on the short arms of the human acrocentric chromosomes. Arrowheads indicate NOR-positive D-group chromosomes and arrows indicate NOR-positive G-group.

Historical and theoretical perspectives, NOR staining

Prior to the silver-staining techniques that are currently used to study NORs, Matsui and Sasaki [270] described a method that they termed N-banding that involved the extraction of nucleic acids and histones either by strong acid treatments and heat or by DNase and RNase treatment. Their purplish-red N-bands correlated with the NORs and were also observed in the nucleoli of interphase nuclei in several species. They identified the N-band substances as acidic (nonhistone) proteins located at the satellites or stalks of the acrocentric chromosomes. Other variations on this method of N-banding soon followed for several eukaryotic species. Faust and Vogel argued that N-bands represented a specific heterochromatin adjacent to NORs [271] and Funaki et al. [260] defined the N-bands as structural nonhistone proteins specifically linked to NORs.

Howell et al. [272] were the first to report the use of silver nitrate to stain the NOR regions of acrocentric chromosomes. They called their technique the Ag-SAT (satellite) technique. It involved pretreating the slides in a 10% formalin solution, staining the chromosomes in ammoniacal silver stain, and developing them in 3% formalin.

Goodpasture and Bloom [273] reported another method for staining NORs, called the Ag-AS (ammoniacal silver) technique. They considered it an improvement over the Ag-SAT technique. It involved staining the slides in silver nitrate under a flood lamp and then developing them in a mixture of ammoniacal silver nitrate and 3% formalin.

Denton et al. [274] reported a two-step technique that was very similar to the Ag-AS procedure. Bloom and Goodpasture subsequently reported another improved technique, the Ag-I procedure, made more consistent by control of pH, temperature, concentration, and time [275]. In this method, they used 50% AgNO_3 for staining, but they omitted the use of the flood lamp and varied the time and temperature of incubation. The other solutions also remained the same, but the pHs and temperatures were more closely controlled. The authors found that the Ag-NORs could be visualized without the use of a developer. They offered several suggestions on ways to change the rate of development of NORs and to troubleshoot their technique. Finally, they showed that quinacrine staining prior to silver staining allowed positive identification of all the acrocentric or Ag-NOR positive chromosomes, and introduced the idea of counterstaining with Giemsa or orcein dyes.

A few more modifications of the silver staining technique were subsequently introduced. The single-step method of Howell and Black [276] utilized a colloidal developer to control the reduction of the silver. The first modification of this method used a 5% sodium thiosulfate fixation to eliminate silver precipitate from the slide [277]. Another modification used a 20 °C staining temperature to minimize background staining and various optical imaging processes (brightfield, darkfield, phase contrast, Nomarski differential interference contrast, and reflected light) to study NORs [278].

Until the mid-1970s, there was controversy over whether the NOR regions were located at the satellites (ISCN band p13) or stalks (p12) of human acrocentric chromosomes (see Chapter 7). Goodpasture et al. [266] demonstrated by combined brightfield and phase microscopy that NORs actually are located at the stalks and not at the satellites. This was confirmed, as was the equivalency of N-bands and Ag-positive NORs [279].

Variations in staining continued to appear in the literature, with emphasis placed on precise identification of positive-NOR-bearing chromosomes. Tantravahi et al. [280] demonstrated that silver staining could be done after Q-, C-, G-, or R-banding procedures. Zankl and Bernhardt described a sequential silver staining and Giemsa banding technique [281]. These techniques, like that of Bloom and Goodpasture [275], involved sequential staining and sequential photography. Lau and colleagues reported a technique for simultaneous silver staining and fluorescence (quinacrine or Hoechst) [282], and Howell and Black [283] described a technique for simultaneous silver staining and Giemsa banding. Dipierri and Fraisse described a technique for silver staining and R-banding (RHG) [284], and Rufas et al. described a method for simultaneous silver staining and C-banding [285]. All of these had the advantage of eliminating duplicate observation and photography.

The obvious question arises as to exactly what cellular component is stained by the silver. The action of the staining appears to be the reduction of the silver nitrate solution by a nucleolar protein into native silver, which is dense and shows as a small, dark region wherever it stains. After the formalin developer is applied, additional silver is apparently deposited at the site, and the black color at the site of the NOR is darkened and enlarged [266, 286]. Schwarzacher et al. [287] stated that the silver staining materials are protein components of the ribonucleic protein and are therefore not part of the chromosomes themselves. Electron micrographs demonstrated silver grains to the side or around the stalks, but not on the chromosomal material itself.

Fuaniki et al. showed that extraction of nucleic acids by TCA-HCl or hot phosphate buffer did not affect the stainability of the NORs [260]. Various studies showed that silver staining is not destroyed by DNase, RNase, TCA, HCl, or H_2SO_4 but that it is destroyed by trypsin and pronase [272, 273, 287]. Consequently, the silver-stainable substance is a protein rather than a nucleic acid, and it is an acidic (nonhistone) protein, as previously thought by Faust and Vogel [271] and Matsui [288]. This protein appears with the synthesis of rRNA; the silver deposits are larger in interphase and prophase than in metaphase, suggesting that the protein is produced during interphase [287, 289, 290]. Hubbell et al. [291] identified a single protein responsible for cytologic silver staining in Novikov hepatoma cells.

Buyss and Osinga [292] identified an abundance of protein-bound sulphydryl and disulfide groups at active NORs by first treating chromosomes with fluorescent sulphydryl- and disulfide-specific reagents and then staining them with silver. They concluded that these reducing groups are responsible for reducing Ag^+ to visible metallic silver (Ag^0).

Ochs and Busch [293] found that a monoclonal antibody to protein C23 blocked NOR-staining in chromosomes and silver staining in interphase nuclei. They suggested that C23 is probably the major, and perhaps the only, silver-staining protein of the nucleolus and that it is directly or indirectly associated with rDNA.

AgNORs are structural and functional parts of the nucleolus, and they contain all of the elements necessary for ribosomal RNA synthesis. Two proteins involved in rRNA transcription and processing, nucleolin and nucleophosmin, are associated with interphase AgNORs and are responsible for their stainability with silver methods. Since the number of AgNORs is associated with RNA transcriptional activity, quantitating them can yield some information about how rapidly cells are proliferating [294].

The AgNOR protein is associated with the actively transcribing rRNA genes, indicating that it is involved in the processing or packaging of rRNA but not in rRNA transcription [289]. Raman and Sperling favor this protein as a more probable rDNA-binding protein that may keep the DNA sequences accessible to the transcriptional machinery [295]. This idea was alluded to but challenged by Haaf et al. [296], who noted positive silver staining even under conditions of rDNA inhibition. Positive silver staining was correlated with decondensed as well as transcriptionally active NORs, and it was suggested that the Ag-positive protein may be a substrate for decondensation. Finally, Spector et al. demonstrated by NOR-staining and immunofluorescence that protein C23 and the AgNOR protein both seem to be associated with rDNA-containing structures [297].

Silver stained acrocentric chromosomes can have two sites of silver appearing as two dots, they can show only one black dot on one chromatid, or they may show very heavy staining appearing as a rod-shaped structure on the short arm [298]. A chromosome that appears to have no silver stain may just need more time in the stain to show a small NOR region. Because silver stain is dependent upon the expression of the genes, inactive NORs will not stain with silver; however, it is possible to make a FISH probe made from the 18S and 28S ribosomal genes that is designed to work on any NOR whether it is active or not.

Because NOR size and presence is inherited, the amount of silver stain present on each chromosome and the number of staining NORs differ among individuals. Usually, out of 10 possible sites (a pair each of the 13, 14, 15, 21, and 22 chromosomes), only six to eight will be silver stain positive. Silver-positive regions are occasionally found in unusual locations (4q, Yq) and the presence of more than one silver-staining region on one chromosome can help delineate the presence of double stalks.

Silver staining is useful for distinguishing between a fragile site and an inserted NOR, which can both appear as a simple gap in a chromosome arm in some patients [299]. If the gap contains silver staining material, it is not a fragile site but rather an NOR. Such insertions have been seen in many human chromosomes. Double stalks usually show NOR staining material at both stalk regions.

Technical considerations of NOR staining

There have been many modifications of the silver staining technique since it was introduced to cytogenetics in 1975. The technique of Howell and Black [276] is simple and reproducible, and is the most commonly used in cytogenetics laboratories today. It involves the use of a 50% AgNO_3 solution and a formic acid–gelatin developer that are sandwiched between a coverslip and the slide with the chromosome preparation. This slide is heated to about 70 °C for a few minutes in an oven or on a hot plate until the mixture becomes golden-brown. The slide is rinsed and counterstained with a light Giemsa or Wright stain.

Technical problems include over- and underexposure to the silver stain, precipitated silver staining in the background, and difficulty in determining which chromosomes are staining due to solid stained material. Fresh slides usually work the best for silver staining, and trypsinized (G-banded) slides are unreliable due to loss of the protein on the NOR region. Some troubleshooting tips include:

- If the material appears underexposed, the slide can be flooded again and stained for an additional period of time.
- Overstaining requires beginning with a fresh slide.
- Background staining is usually related to overstaining (too long on the hotplate). Certain batches of silver stain seem to yield more background than others, and the developer can affect background, as well.
- Gelatin source is considered critical by some.

In order to determine which chromosomes are being stained with the silver stain, sequential banding to silver staining is useful. Q-bands or R-bands work very well for this purpose; however, they will not work after the silver staining and must be done previous to it [283]. Coverslipping the slide is thought to cause the silver stain to destain [280, 300]. If C-banding is performed prior to AgNOR staining, some of the C-bands will be removed by the AgNOR stain method [285].

When acrocentric chromosomes form into satellite associations, it becomes difficult to score the NOR regions, so it is best to look for cells with nonassociated acrocentrics. The larger the silver staining region on a chromosome, the more likely it is to exhibit associations.

6.3 5-bromo-2'-deoxyuridine methodologies

Initiation of DNA synthesis is multifocal and follows a particular pattern for each chromosome [301]. DNA synthesis includes incorporation of new bases into the new DNA strand, including thymidine. Areas that have replicated may be visualized by the incorporation of a thymidine analog, 5-bromo-2'-deoxyuridine (BrdU) (Figure 6.19), into cells during synthesis and then examining the chromosomes in a subsequent metaphase using fluorescent dyes that exhibit differential fluorescence in the presence of a substituted strand. These methods may be used to determine inactivation in structurally abnormal X chromosomes. In the female, X chromosome inactivation is random; it is accompanied by a delay in both the initiation and completion of DNA synthesis [302–305]. Late replication banding permits the identification of the early- and late-replicating X chromosomes, as well as identifying the early- and late-replicating regions of the other chromosomes. The mechanism of action is through the process of 5-bromo-2'-deoxyuridine incorporation into the chromatids and subsequent staining with one of several dyes (Hoechst 33258, acridine orange, propidium iodide) that are quenched in the presence of BrdU. The BrdU may be pulsed early in synthesis (S) or late in S with corresponding dye quenching of the parts of the chromosomes that are replicating at the time of the pulse.

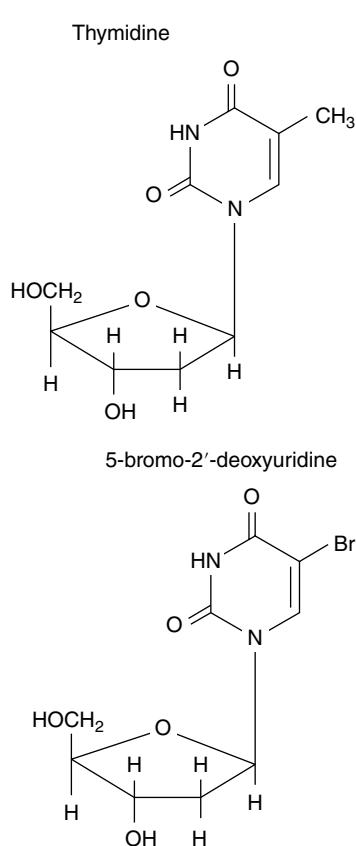


Figure 6.19 Chemical structures of thymidine and 5-bromo-2'-deoxyuridine. Note the only difference between these two structures is that a methyl group (CH₃) has been substituted with a bromine molecule (Br) in the 5-bromo-2'-deoxyuridine (from ref. 320), making BrdU ideal for substituting into synthesizing chromatin. BrdU has the capability of quenching the fluorescence of the Hoechst 33258 stain, and makes it possible to visualize where synthesis has taken place. McCaw 1977 [317]. Reproduced with permission of Springer.

In addition, BrdU may be used to visualize sister chromatid exchanges by growing the cells for two complete cycles (Figures 6.20–6.24). Peripheral blood lends itself to these methods, because lymphocytes are somewhat synchronous after the addition of phytohemagglutinin to stimulate growth (Chapter 3). However, the BrdU substitution methods also can be used with long-term cultures successfully.

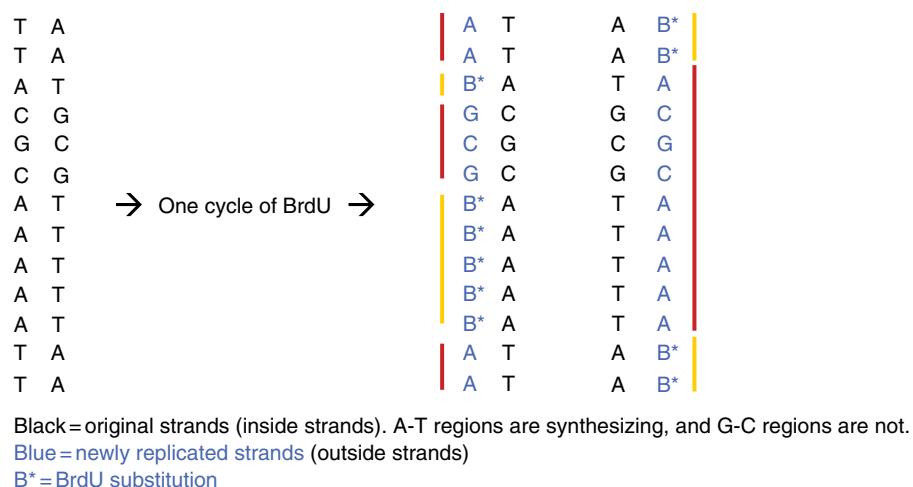


Figure 6.20 Incorporation of BrdU (B) into DNA during synthesis. The BrdU will substitute in the part of the strand that was synthesizing at the time it was added (orange section) but not in the section that was not synthesizing (gold). The orange sections will be quenched and represent the late replicating (labeling) chromatin if the BrdU was added late in DNA synthesis and the early replicating chromatin if the BrdU was added at the beginning of DNA synthesis and then rinsed out of the culture (Figure 6.21). See insert for color representation of this figure.

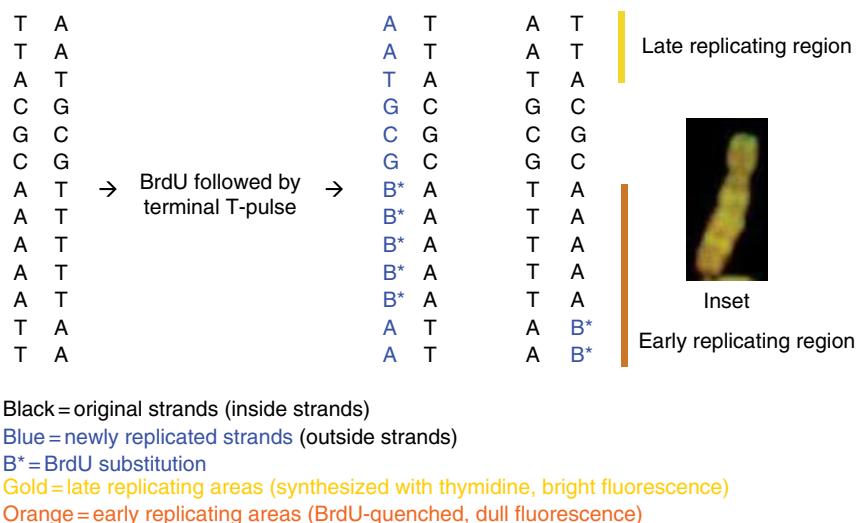


Figure 6.21 Incorporation of BrdU (B*) into early replicating DNA, followed by a terminal thymidine (T) pulse. BrdU substitutes for thymidine only in the regions that were replicating early in the S period so the early replicating sections will be quenched. The BrdU is removed from the culture, and the rest of the DNA synthesizes with thymidine during the last part of S. All BrdU substituted DNA will be quenched (dimly stained) when stained with Hoechst 33258, acridine orange, or propidium iodide, giving rise to the replication band patterns (inset). See insert for color representation of this figure.

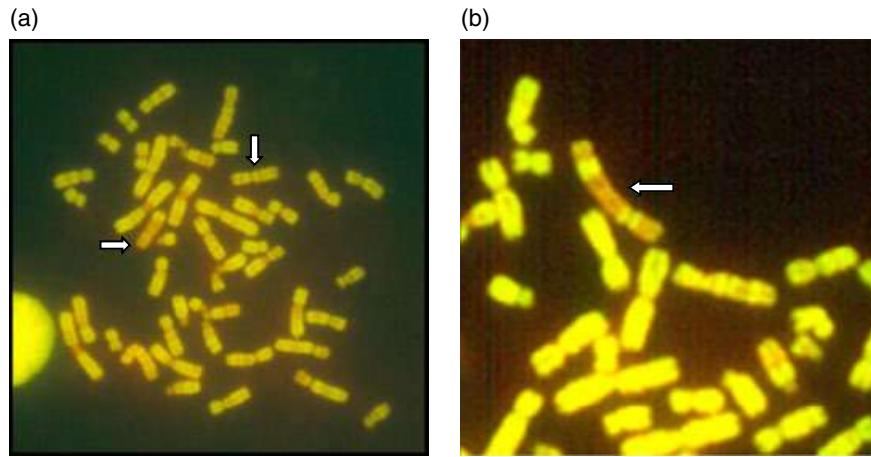


Figure 6.22 Replication banding. The images represent replication banding using a 6-hour terminal pulse with BrdU (final concentration 10 µg/mL) and stained with acridine orange (0.01%). (a). Normal cell. Note: early (bright, upper right) and late (dull, lower left) replicating X (arrows). (b). Late replication banding pattern of a pseudo-isodicentric X chromosome with break and fusion points at Xq28; psu idic(X)(q28). Chamla 1976 [327]. Reproduced with permission of Springer. See insert for color representation of this figure.

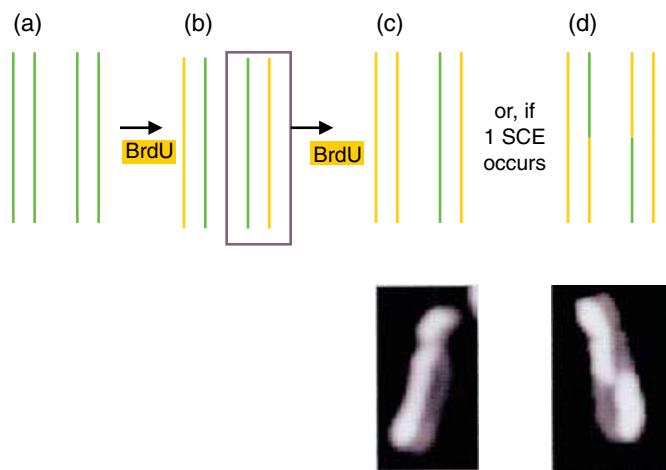


Figure 6.23 Sister chromatid exchange (SCE). Diagrammatic representation of BrdU substitution for two cell cycles make sister chromatid exchanges apparent. The two original chromatids (shown in green) of a chromosome, each with two DNA strands (a), replicate in the presence of BrdU to make two new chromatids (b), each of which has a DNA strand with BrdU substitution (shown in gold) at the thymidine bases. Each of these unifilarly substituted chromatids will partially quench Hoechst stain equally to its sister chromatid, so there will not yet be any difference in the way the two chromatids appear on a fluorescence microscope. Then each of these chromatids goes through a second division in the presence of BrdU (c), so for each of these green/gold chromatids (e.g., the one in the box) there will be two daughter chromatids, one daughter chromatid with one native, green strand plus a BrdU substituted gold strand, and another daughter chromatid with two substituted (gold) strands. The double-substituted chromatid will quench the Hoechst dye much more than the single-substituted chromatid, so the eye will perceive one dark (quenched) and one bright chromatid on each chromosome in the cell. If there is an exchange between the chromatids of a chromosome, it will be visible as a chromosome with quenched and non-quenched sections that alternate between chromosome arms (d). The singly-substituted chromatid sections (orange-green) will be bright, and the doubly-substituted sections (orange-orange) will be dull. See insert for color representation of this figure.

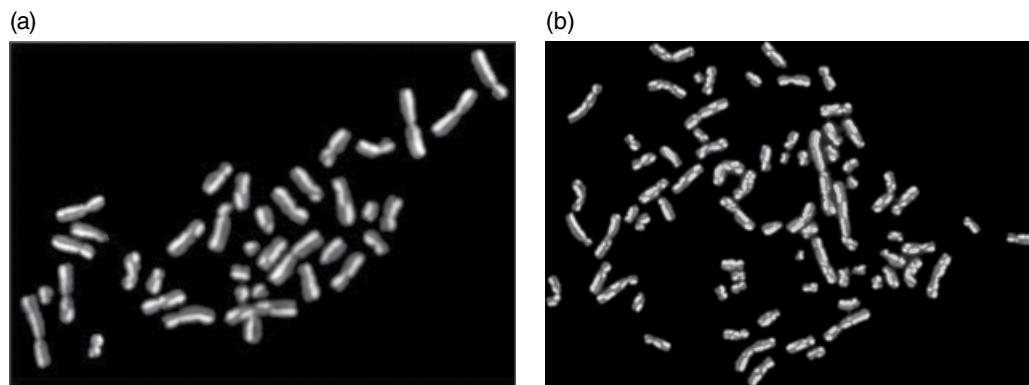


Figure 6.24 Sister chromatid exchange. (a) Sister chromatid exchanges in a metaphase cell from a normal individual. (b) Sister chromatid exchanges in a metaphase cell from a patient with Bloom syndrome. Image (a) courtesy of Amy Hanlon-Newell. Image (b) courtesy of Nichole Owen.

A method known as lateral asymmetry [315] is also possible using BrdU substitution methods. It results in differential brightness between chromatids in which there is a high concentration of thymidine, such as the 1, 16 and Y long arms. It has not become a valuable tool in the cytogenetics laboratory and will not be discussed in this chapter.

6.3.1 Historical and theoretical perspectives

By incorporating tritiated thymidine into growing cell populations and assaying for replicated DNA by autoradiography, Taylor et al. [306] demonstrated that all chromosome segments do not replicate synchronously. The technique had limitations, however, especially for resolving isolabeling of adjuvant segments of chromosomes.

Several years later, Latt [124] reported a fluorometric technique for analyzing DNA replication. By incorporating the thymidine analog BrdU (Figure 6.19) into chromosomal DNA, he was able to demonstrate partial quenching of the fluorescence of the bisbenzimidazole dye 33258 Hoescht that was bound to the BrdU-substituted regions. Pulsing the cells with thymidine late in the S period allowed late-replicating chromosome segments to be identified.

Most of the routine replication banding techniques employ the same basic culture technique of initial BrdU or thymidine incorporation with a terminal pulse of one or the other compounds. Variations include the use of acridine orange [221, 222, 224–226, 307] or Giemsa stain following photodegradation of the BrdU–DNA–Hoechst complex [308–311].

The dye 33258 Hoechst – p-(5-(5-(4-Methyl-1-piperazinyl)-1H-2-benzimidazolyl)-1H-2-benzimidazolyl)phenol trihydrochloride – was originally used on mouse chromosomes by Hilwig and Groppe to demonstrate that the centromeres (i.e., heterochromatin) of mouse chromosomes fluoresced more brightly than surrounding chromatin [312]. Weisblum and Haenssler [313] reported that the fluorescence of Hoechst-DNA complexes increased proportionally with AT content. AT-rich DNA sequences enhanced dye binding, and, except at very high DNA/dye ratios, the fluorescence efficiency of bound dye was greater with AT-rich DNA than with GC-rich DNA [314]. The AT binding specificity therefore resembles that of distamycin A and netropsin (both nonfluorescent dyes of similar structure). Like these two dyes, Hoechst does not appear to bind by intercalation. Chromosomal proteins apparently exclude the dye from approximately half of the DNA sites available.

The absorption maximum of the Hoechst-DNA complex is 356 nm and the fluorescence maximum is 465 nm [314]. The fluorescence properties of the dye depend strongly on pH, as will be noted later.

Hoechst dye alone (without BrdU incorporation) gives a bright Q-band-like pattern with poor contrast between bands. Latt demonstrated that the substitution of the base analog 5-bromo-2'-deoxyuridine for thymidine causes a decrease in the fluorescence of the Hoechst dye-DNA complex [124]. With one cycle of BrdU incorporation, the fluorescence intensity is decreased by approximately two thirds and with two cycles, by another one third of the previous cycle [315]. Similarly, in solution, Hoechst/poly(dA-BrdU) complexes have a quantum yield one fourth that of Hoechst/poly(dA-dT) complexes [314]. The actual mechanism by which quenching occurs is not known. Quenching caused by BrdU is negligible at pH 4 (little differentiation of bands) but increases with pH to about pH 7.5. At approximately pH 8, quenching is present, but the extent

of it depends on the buffer ion in the mounting medium. The use of higher ionic strength (0.4 M versus 0.01 M NaCl) buffer for staining also enhances apparent fluorescence differentiation [314].

With regard to metaphase chromosome preparations, Latt demonstrated that one cycle of BrdU incorporation results in unifilar (single strand) substitution, two cycles result in bifilar substitution (both strands) with sister chromatid differentiation, and three or more cycles result in various patterns of substitution. These results are consistent with semiconservative DNA replication [124, 315] (Chapter 1).

Late replication banding patterns are apparent after one cycle of BrdU substitution with a terminal pulse of thymidine prior to harvest. Sister chromatid differentiation for sister chromatid exchange (SCE) analysis is apparent after two rounds of BrdU substitution. The staining techniques for replication banding, SCEs, and lateral asymmetry are identical.

6.3.2 Replication banding

BrdU is incorporated into chromosomes during the S phase (DNA synthesis) of the cell cycle. The existing thymidine-containing DNA serves as a template for the newly synthesized DNA (Figure 6.20). All BrdU-substituted chromatin will exhibit dull fluorescence with 33258 Hoechst and some other dyes.

If BrdU is present during only the beginning of the cell cycle and is replaced with thymidine several hours before harvest (called a T or thymidine pulse), those regions that replicate early will incorporate BrdU. Those that replicate late will contain thymidine (Figure 6.21). In this situation, the late-replicating thymidine-containing regions will fluoresce brightly with 33258 Hoechst, whereas the early replicating BrdU-containing regions will be quenched.

Alternatively, exposure of cells to thymidine at the beginning of the cell cycle and pulsing with BrdU during the last few hours of culture (called a B or BrdU pulse) will produce dull late-replicating regions and bright early-replicating regions when stained with Hoechst.

Latt found that following a T pulse, the resulting Hoechst-bright bands are similar to Q-bands, with a few exceptions. The heterochromatic regions of chromosomes 1, 9, and 16, which are Q-negative, replicate late and are thus very bright with Hoechst. The Hoechst-bright bands are sharper than Q-bands and delineate regional replication order. Latt also noted that cellular and homologue asynchrony exist; these differences are interpreted as fluctuations in sequences in which different chromosome regions complete replication from cell to cell and homologue to homologue.

With specific reference to the two X chromosomes in a human female, the early replicating X terminates replication in bands Xp21, Xq21, and Xq27, as does the male X chromosome. The late-replicating X terminates replication in bands Xp23, Xq21, and Xp21 (in order of decreasing intensity with Hoechst) [316]. An alternative late X pattern observed in 5 to 10 percent of lymphocytes shows Xq25 and Xq27 replicating last. Latt also observed asynchrony between multiple late replicating X chromosomes, as well as prominent patterns of late replication in bands Xq21 and Xq23 or Xq25 and Xq27 in isochromosomes of the X. Kaiser-McCaw and Latt studied parthenogenetic benign ovarian teratomas and identified the presence of an inactive late replicating X, demonstrating that fertilization was not required for inactivation [317]. Figure 6.22 shows replication-banded chromosomes treated with a terminal pulse of BrdU, 10 µg/mL final concentration, stained with acridine orange (0.01%). The late-labeled normal X is shown in one picture, and a late-labeled pseudo-isodicentric X is shown in the other (psu idic(X)(q28)).

Grzeschik and colleagues [311] generally confirmed Latt's earlier findings of X chromosome replication and homologue asynchrony. Both Grzeschik et al. [311] and Latt et al. [310] used a method similar to Wolff and Perry's fluorescence plus Giemsa (FPG) technique, which involves exposing Hoechst-stained chromosomes to light, washing in 2x SSC at 65 °C, and staining in Giemsa [308, 309]. Exposure to short-wavelength light promotes selective degradation of the BrdU-substituted DNA. The subsequent hot salt treatment elutes the broken fragments of DNA, thereby leading to reduced staining with Giemsa. Hoechst-dull bands stain weakly with Giemsa, and Hoechst-bright bands stain dark with Hoechst stain alone, for example, fluorescence microscopy is unnecessary, thus eliminating the problem of rapid fading. Slides are permanently stained with Giemsa for brightfield microscopy.

Clinical significance of replication banding

The most important clinical use of late replication banding is in the study of sex chromosome abnormalities (supernumerary X chromosomes, X chromosome deletions, balanced/unbalanced X;X or X;autosome translocations) and in quick scanning of 45,X individuals for rare 46,XX cells [318]. Also, because late-replicating T-pulse bands generally correspond to Q-bands, with the advantage of being much sharper, breakpoints may be more precisely determined using the banding pattern. A translocated piece may have Q-banding intensity similar to that of several different chromosome regions, making it difficult to determine the origin. Differences in replication timing (and therefore band

intensity) with this method may help differentiate among several possible origins. From a research standpoint, cellular replication kinetics can be studied by BrdU incorporation.

6.3.3 Sister chromatid exchanges

Sister chromatid exchanges (SCEs) represent the interchange of DNA between replication products at apparently homologous loci, and at low levels constitute normal occurrences. Although the molecular basis for SCE formation is not completely understood, it presumably involves DNA damage and repair [319, 320]. SCEs appear as sharp demarcations in the intensity of staining along a chromatid, with the opposite staining pattern on the adjacent chromatid (Figure 6.23).

SCE induction requires the cells to pass through S phase. Lesions that occur during the G2 phase of the cell cycle do not give rise to SCEs until after the cell has divided and proceeded through the next S phase.

Using BrdU concentrations between 10^{-5} M and 10^{-4} M, the number of baseline SCEs per cell ranges from 5 to 15 in normal individuals. Patients with Bloom syndrome have greatly elevated SCEs/cell. In normal cells, the distribution of SCEs among the chromosomes is a function of chromosome length. Sequential staining techniques indicate that SCEs are located in quinacrine-pale, Giemsa-negative bands or at band junctions.

Clinical significance

From a clinical standpoint, SCE analysis has been used to aid in the diagnosis of several inherited diseases characterized by chromosome fragility and predisposition to the development of neoplasia. These include Bloom syndrome, Fanconi anemia, ataxia-telangiectasia, and Xeroderma pigmentosum. These chromosome fragility diseases show differential response to SCE-inducing agents, indicating that there are probably several types of DNA repair defects.

From a research standpoint, SCE analysis is one method of assaying DNA damage. Many SCE-inducing agents are mutagens, carcinogens, or both. Small quantities of certain compounds, such as alkylating agents, induce SCEs well below the concentration needed to cause gross chromosomal damage. However, some agents that are known to cause chromosome breaks and rearrangements (e.g., radiation) are not very effective at inducing SCEs. Therefore, the combined analysis of chromosome aberrations and SCEs can be helpful to screen potentially mutagenic or carcinogenic substances.

6.3.4 Technical considerations for replication banding and SCEs

Because individual cell types have different replication kinetics, it is important to study cell cycle time in order to schedule the addition of BrdU and/or thymidine accurately. For replication banding, human adult lymphocytes generally require 40–50 hours in BrdU-containing medium, followed by a 5- to 7-hour T pulse, for late-replicating bands. Human newborn cells, which divide more rapidly, are adjusted accordingly (44 hours in BrdU-containing medium, 6-hour T pulse). The schedule employed for early replicating bands requires 65–69 hours of growth in thymidine-containing medium followed by a 5- to 7-hour B pulse. The culture medium employed during the time that BrdU is present should not contain thymidine, because it can interfere with the incorporation of BrdU.

With regard to the possible effects of BrdU on cell replication, Tice et al. showed no inhibition of cellular kinetics at concentrations of BrdU below 35 mm [321]. Below 7.5 mm, they were unable to classify all cells, so they selected 25 mm BrdU to analyze cellular kinetics. Willard and Latt concluded that BrdU did not have a significant effect on the temporal order of band replication, because the banding patterns of the T- and B-pulse procedures are complementary and independent of BrdU concentration, and because the autoradiographic grain distributions over X chromosomes from both protocols were similar [322].

Additives that may improve culture conditions include fluorodeoxyuridine, uridine, and deoxycytidine. By inhibiting thymidine kinase and the methylation of dUMP to dTMP, fluorodeoxyuridine inhibits endogenous deoxythymidine production by the cells. Uridine overcomes some of the potentially toxic effects of the fluorodeoxyuridine. Deoxycytidine improves cell growth and increases the number of second division cells after three days. The cells must survive to second division or the response will be biased due to nonsurvival of severely damaged cells.

Cells containing BrdU-substituted chromosomes are light sensitive. Light exposure below 313 nm may cause chromosome damage, an increase in the number of SCEs, or cell death. Cultures should be protected from light.

Hoechst-stained slides should also be protected from light because the fluorescence fades very rapidly. Photograph or capture cells quickly and analyze cells from images rather than under the microscope. Making slides permanent with the FPG method obviates these problems that are encountered with fluorescence.

Note: Because BrdU can replace thymidine during DNA replication, it can cause mutations; its use, therefore, is potentially a health hazard.

6.4 T-banding/CT-banding

6.4.1 Historical and theoretical perspectives

Telomeric (or terminal) banding was first reported by Dutrillaux, who used two types of controlled thermal denaturation followed by staining with either Giemsa or acridine orange [221]. The T-bands apparently represent a subset of the R-bands and are more strictly telomeric.

Scheres [323] observed the production of both C- and T-bands (CT-bands) in human chromosomes after heat treatment at a high pH and staining with the cationic dye “Stains-All” (4,5,4'5'-dibenzo-3,3'-diethyl-9-methyl-thiacarbocyanidebromide) [324]. This dye stains RNA purple, DNA blue, and proteins red. The kinds of banding that appear depend on both the pH and the type of salt used. Depending on conditions, G-bands, R-bands, CT-bands, or heterochromatic staining of some human acrocentric short arms will result [325]. Ba(OH)₂ alone, with subsequent staining, produces R-bands, with positive staining of the 9qh. Incubation in 2× SSC weakens the R-bands, making the centromeric regions more prominent. Incubation at 85 °C results in staining the short arms of some of the variant acrocentric human chromosomes.

Further work by Scheres concerned the role of cations in the alkaline pretreatment [323]. A comparison of monovalent hydroxides – NaOH, KOH, LiOH – and divalent hydroxides – Ba(OH)₂, Sr(OH)₂, Ca(OH)₂, Mg(OH)₂, NH₂OH, tetramethylammonium hydroxide, or ethylammonium hydroxide – showed that divalent cations produced similar R-banding patterns and were less damaging to the chromosomes than monovalent cations. The R-banding ability corresponded with the strength of the base, so in order of strength, Ba(OH)₂, Sr(OH)₂, Ca(OH)₂, and Mg(OH)₂, will all produce R- or T-bands. Both ammonium bases and hydroxides of monovalent alkali metals had a strong disintegrating effect on the chromosomes, and short or dilute treatment produced G-banding. Although the mechanisms for such R- and T- banding are unknown, Scheres [323] suggested that divalent cations have a strong stabilizing effect on the chromosomes, perhaps in conjunction with alkaline denaturation.

Chalma and Ruffie [326] described a method of CT-banding that was slightly different from the Scheres method. The T-banding methods of Dutrillaux and the CT-banding methods of Scheres and Chalma and Ruffie are described in the literature [325, 327, 328].

6.4.2 Technical considerations

Standard culture methods are appropriate. Slides should be aged for a few days prior to staining. With Giemsa the chromosomes are pale, unbanded, and difficult to see. With acridine orange staining for various lengths of time, the chromosomes appear as follows: >15 minutes: green at telomeres, otherwise orange; 15–20 minutes: green at telomeres, orange areas are less intense; 30 minutes or more: orange color is gone, intercalating R-bands appear.

6.5 Antibody banding and restriction endonuclease banding

Although antibody banding and restriction endonuclease banding are not routinely used in cytogenetics, they can help to learn more about the base composition and DNA structure that comprise metaphase chromosomes and interphase chromatin. Interested readers can refer to the second edition of this book for details [152].

6.6 Destaining slides

All oil and/or mounting medium should be thoroughly removed by soaking the slide in two to three changes of fresh xylene (a known carcinogen) or a xylene substitute, such as Propar, limonene, or Histoclear, and thoroughly dried before destaining. There are several methods for destaining slides prior to proceeding with another staining or banding procedure. The easiest and probably most commonly used technique involves treating the slides with 3 : 1 methanol–acetic acid fixative. Old fixatives can be saved for this purpose following harvesting procedures. Other methods include using a series of graded alcohols or simply rinsing with methyl or ethyl alcohol.

Destaining methods can be used to destain Giemsa, quinacrine, Hoechst, DAPI, Distamycin A, Chromomycin A3, and methyl green; however, destaining C-banded or silver-stained slides is difficult, and the chromatin does not lend itself to further staining procedures after being submitted to these harsh methods.

6.7 FISH DAPI bands

The use of DAPI counterstain is helpful in FISH methods, especially after separate denaturation, for identifying the chromosomes that are associated with the banding patterns produced. The chromosomes appear to have a mild Q-band pattern except that the DA-DAPI regions remain bright on the 1qh, 15p, and 16qh (Figure 6.17). See DAPI/Distamycin A staining (DA-DAPI).

6.8 Sequential staining

It is occasionally desirable to perform more than one staining procedure on the same preparation. If clinical case material is limited, it is advisable to attempt the sequential stain on control slides to be sure the method works before attempting the sequential stains.

The best rule of thumb for the order in which to carry out sequential staining is that the most destructive procedures (C-bands, AgNOR) should be carried out last, and the least destructive should be performed first (Q-bands, solid stain). Fluorescence Q-, R-, and DAPI stains are not very destructive unless they are overexposed to the UV light during observation, which can photo-degrade the chromatin. G-banding with trypsin removes some protein, and usually follows fluorescent banding. If G-bands are performed before Q-bands, some of the Q-bands will resemble G-bands (e.g., the light heterochromatin with Q-bands – 1q and 16q – will appear bright instead). Between each stain method, it is important to thoroughly remove all oil from the slide, remove all stain from the cells, and dry the slide completely.

If cells are to be banded in conjunction with FISH studies, it is best to do the banding before the FISH, as the hot formamide in the probe will irreversibly change the chromatin. The exception is that sometimes the slide can be stained in Giemsa after the FISH step to produce C-bands.

Fluorescence methods, for example, Q-bands and R-bands using distamycin/methyl green or acridine orange, are the easiest to use prior to FISH methods for sequential identification of particular chromosomes followed by FISH. In our laboratory, we have successfully performed two different FISH hybridizations sequentially to an R-band method with DI/methyl green.

Acknowledgments

Thanks to Charles Dana Bangs, Jack Spurbeck, and Michael Brown for providing illustrations for this chapter. Thanks to Marilyn Arsham and Karen Gustashaw, who wrote the chapters in the first three editions of this book and whose work constitutes the bulk of the chapter in this edition as well. Thanks to Turid Knutsen for formatting the methods.

References

1. Paris Conference (1971): Standardization in human cytogenetics. Birth Defects: Original Article Series. VIII (7). The National Foundation, New York 1972; also in *Cytogenetics* 1972;11:3l3–362.
2. ISCN (2016): *An International System for Human Cytogenetic Nomenclature*. McGowan-Jordan J, Simons A, Schmid M (eds). Basel: S Karger, 2016.
3. Paris Conference (1971), Supplement (1975): *Standardization in Human Cytogenetics: Birth Defects: Original Article Series. XI (9)*. The National Foundation, New York: 1975; also in *Cytogenet Cell Genet* 1975; l4:201–238.
4. Dallapiccola B, Ricci N. Observations on specific Giemsa staining of the Y and on selective oil destaining of the chromosomes. *Humangenetik* 1975;26:251–255.
5. Lisse IM, Kudahi B. Destaining effects of immersion oils on Giemsa-stained chromosomes (R-bands). *Ann Genet* (Paris) 1977;20:136–140.
6. Giloh H, Sedat JW. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. *Science* 1982;217:1252–1255.
7. Johnson GD, Nogueira Araujo GMdeC. A simple method of reducing the fading of immunofluorescence during microscopy. *J Immunol Methods* 1981;43:349–350.
8. Johnson GD, Davidson RS, McNamee KC, Russell G, Goodwin D, Holborow EJ. Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. *J Immunol Methods* 1982;55:231–242.
9. Caspersson T, Farber S, Foley GE, Kudynowski J, Modest EJ, Simonsson E, Wagh U, Zech L. Chemical differentiation along metaphase chromosomes. *Exp Cell Res* 1968;49:219–222.
10. Caspersson T, Zech L, Modest EJ, Foley GE, Wagh U, Simonsson E. DNA-binding fluorochromes for the study of the organization of the metaphase nucleus. *Exp Cell Res* 1969;58:128–140.
11. Caspersson T, Zech L, Modest EJ, Foley GE, Wagh U, Simonsson E. DNA-binding fluorochromes for the study of the organization of the metaphase nucleus. *Exp Cell Res* 1969;58:141–152.
12. Caspersson T, Zech L, Johansson C. Analysis of human metaphase chromosome set by aid of DNA-binding fluorescent agents. *Exp Cell Res* 1970;62:490–492.

13. Caspersson T, Zech L, Johansson C. Differential binding of alkylating fluorochromes in human chromosomes. *Exp Cell Res* 1970;60:315–319.
14. Caspersson T, Zech L, Johansson C, Modest EJ. Identification of human chromosomes by DNA-binding fluorescent agents. *Chromosoma* 1970; 30:215–227.
15. Caspersson T, Zech L, Modest EJ. Fluorescent labeling of chromosomal DNA: superiority of quinacrine mustard to quinacrine. *Science* 1970;170:762.
16. Caspersson T, Lomakka G, Zech L. The 24 fluorescence patterns of the human metaphase chromosomes—distinguishing characters and variability. *Hereditas* 1971;67:89–102.
17. Lin CC, van de Sande JH. Differential fluorescent staining of human chromosomes with daunomycin and adriamycin—the D-bands. *Science* 1975;190:61–63.
18. Brookes P. Reaction of alkylating agents with nucleic acids. In: Plattner PA, ed. *Cancer Chemotherapy*. Amsterdam: Elsevier 1964;32–43.
19. Loveless A. On the inactivation of bacteriophages, micro-organisms, and transforming principles by alkylating agents. In: *Genetic and Allied Effects of Alkylating Agents*. London: Butterworths, 1966;125–150.
20. Kurnick NB, Radcliffe IE. Reaction between DNA and quinacrine and other antibacterials. *J Lab Clin Med* 1962;60:669–688.
21. O'Brien RL, Olenick JG, Hahn FE. Reactions of quinine, chloroquine, and quinacrine with DNA and their effects on the DNA and RNA polymerase reactions. *Proc Natl Acad Sci USA* 1966;55:1511–1517.
22. Vosa CG. Heterochromatin recognition with fluorochromes. *Chromosoma* 1970;30:366–372.
23. Weisblum B, de Haseth PL. Quinacrine, a chromosome stain specific for deoxyadenylate-deoxythymidylate-rich regions in DNA. *Proc Natl Acad Sci USA* 1972;63:629–632.
24. Ellison JR, Barr HJ. Quinacrine fluorescence of specific chromosome regions. Late replication and high A:T content in Samoia leonensis. *Chromosoma* 1972;36:375–390.
25. Comings DE, Kovacs BW, Avelino E, Harris DC. Mechanisms of chromosome banding. V. Quinacrine banding. *Chromosoma* 1975;50:111–145.
26. Gottesfeld JM, Bonner J, Radda GK, Walker IO. Biophysical studies on the mechanism of quinacrine staining of chromosomes. *Biochemistry* 1974;13:2937–2945.
27. Michelson AM, Monny C, Kovoar A. Action of quinacrine mustard on polynucleotides. *Biochemie* 1972;54: 1129–1136.
28. Pachmann U, Rigler R. Quantum yield of acridines interacting with DNA of defined base sequence. *Exp Cell Res* 1972;72:602–608.
29. Selander RK, de la Chapelle A. The fluorescence of quinacrine mustard with nucleic acids. *Nature New Biol* 1973;245:240–243.
30. Weisblum B, de Haseth PL. Nucleotide specificity of the quinacrine staining reaction for chromosomes. *Chromosomes Today* 1973;4:35–51.
31. Dev VG, Warburton D, Miller OJ, Miller DA, Erlanger BF, Beiser SM. Consistent pattern of binding of anti-adenosine antibodies to human metaphase chromosomes. *Exp Cell Res* 1972;74:288–293.
32. Schreck RR, Warburton D, Miller OJ, Beiser M, Erlanger BF. Chromosome structure as revealed by a combined chemical and immunochemical procedure. *Proc Natl Acad Sci USA* 1973;70:804–807.
33. Dev VG, Grewal MS, Miller DA, Kouri RE, Hutton JJ, Miller OJ. The quinacrine fluorescence karyotype of *Mus musculus* and demonstration of strain differences in secondary constrictions. *Cytogenetics* 1971; 10:436–451.
34. Rowley JD, Bodmer WF. Relationship of centromeric heterochromatin to fluorescent banding patterns of metaphase chromosomes in the mouse. *Nature* 1971;231:503–506.
35. Latt SA, Gerald PS. Staining of human metaphase chromosomes with fluorescent conjugates of polylysine. *Exp Cell Res* 1973;81:401–406.
36. Sumner AT. Suppression of quinacrine banding in human chromosomes by mounting in organic media. *Chromosoma* 1977;64:337–342.
37. Sumner AT. The distribution of quinacrine on chromosomes as determined by X-ray microanalysis. I. Q-bands on CHO chromosomes. *Chromosoma* 1981;82:717–734.

38. Sumner AT. Mechanisms of quinacrine binding and fluorescence in nuclei and chromosomes. *Histochemistry* 1986;84:566–574.
39. Lorber BJ, Grantham M, Peters J, Willard HF, Hassold TJ. Nondisjunction of chromosome 21: Comparisons of cytogenetic and molecular studies of the meiotic stage and parent of origin. *Am J Hum Genet* 1992;51: 1265–1276.
40. Latt SA, Brodie S, Munroe SH. Optical studies of complexes of quinacrine with DNA and chromatin: implications for the fluorescence of cytological chromosome preparations. *Chromosoma* 1974;49: 17–40.
41. Bosman FT, van der Ploeg M, Geraedts JP. Influence of Q- and G-banding on the Feulgen-stainability of human metaphase chromosomes. *Histochem J* 1977;9:31–42.
42. Nakagome Y, Oka S, Higurashi M. Quinacrine and acridine R-banding without a fluorescence microscope. *Hum Genet* 1978;40:171–176.
43. Lin CC, van de Sande JH, Smink WK, Newton DR. Quinacrine fluorescence and Q-banding patterns of human chromosomes. I. Effects of varying factors. *Can J Genet Cytol* 1975;17:81–92.
44. McIlvaine TC. A buffer solution for colorimetric comparison. *J Biol Chem* 1921;49:183–186.
45. Pardue ML, Gall JG. Chromosomal localization of mouse satellite DNA. *Science* 1970;168:1356–1358.
46. Sumner AT, Evans HJ, Buckland RA. New technique for distinguishing between human chromosomes. *Nature New Biol* 1971;232:31–32.
47. Evans HJ, Buckton KE, Sumner AT. Cytological mapping of human chromosomes: results obtained with quinacrine fluorescence and the acetic-saline-Giemsa technique. *Chromosoma* 1971;35:310–325.
48. Patil SR, Merrick S, Lubs HA. Identification of each human chromosome with a modified Giemsa stain. *Science* 1971;173:821–822.
49. Drets ME, Shaw MW. Specific banding patterns of human chromosomes. *Proc Natl Acad Sci USA* 1971;68:2073–2077.
50. Schnell W. Analysis of the human karyotype using a reassociation technique. *Chromosoma* 1971;34:448–454.
51. Seabright M. A rapid banding technique for human chromosomes. *Lancet* 1971;2:971–972.
52. Wang HC, Federoff S. Banding in human chromosomes treated with trypsin. *Nature New Biol* 1972;235:52–53.
53. Kato H, Moriwaki K. Factors involved in the production of banded structures in mammalian chromosomes. *Chromosoma* 1972;38:105–120.
54. Burkholder GD. The ultrastructure of G- and C-banded chromosomes. *Exp Cell Res* 1975;90:269–278.
55. Bahr GF, Larsen PM. Structural “bands” in human chromosomes. *Adv Cell Mol Biol* 1974;3:191–212.
56. Golomb HM, Bahr GF. Human chromatin from interphase to metaphase: a scanning electron microscopic study. *Exp Cell Res* 1974;84:79–87.
57. McDay RDG. The mechanism of G and C banding in mammalian metaphase chromosomes. *Chromosoma* 1973;44:1–14.
58. Musio A, Mariani T, Frediani C, Sbrana I, Ascoli C. Longitudinal patterns similar to G-banding in untreated human chromosomes: evidence from atomic force microscopy. *Chromosoma* 1994;103:225–229.
59. Ambros PF, Sumner AT. Correlation of pachytene chromomeres and metaphase bands of human chromosomes, and distinctive properties of telomeric regions. *Cytogenet Cell Genet* 1987;44:223–228.
60. Okada TA, Comings DE. Mechanisms of chromosome banding. III. Similarity between G-bands of mitotic chromosomes and chromomeres of meiotic chromosomes. *Chromosoma* 1974;48:65–71.
61. Comings DE. Mechanisms of chromosome banding and implication for chromosome structure. *Annu Rev Genetic* 1978;12:25–46.
62. Harrison CJ, Britch M, Allen TD, Harris R. Scanning electron microscopy of the G-banded human karyotype. *Exp Cell Res* 1981;134:141–153.
63. Ross A, Gormley IP. Examination of surface topography of Giemsa-banded human chromosomes by light and electron microscopic techniques. *Exp Cell Res* 1973;81:79–86.
64. Utsumi KR. Scanning electron microscopy of Giemsa-stained chromosomes and surface-spread chromosomes. *Chromosoma* 1982;86:683–702.
65. Hsu TC. Longitudinal differentiation of chromosomes. *Annu Rev Genet* 1973;7:153–176.

66. Kato H, Yosida TH. Banding patterns of Chinese hamster chromosomes revealed by new techniques. *Chromosoma* 1972;36:272–280.
67. Lee CLY, Welch JP, Lee SHS. Banding of human chromosomes by protein denaturation. *Nature New Biol* 1973;241:142–143.
68. Utakoji T. Differential staining patterns of human chromosomes treated with potassium permanganate. *Nature* 1972;239:168–169.
69. Utakoji T. Differential staining patterns of human chromosomes treated with potassium permanganate and its blocking by organic mercurials. In: Wahrman J, Lewis KR, eds. *Chromosomes Today*, vol 4. Jerusalem: Keter, 1973;53–59.
70. Sumner AT. Involvement of protein disulphides and sulphhydryls in chromosome banding. *Exp Cell Res* 1973;83:438–442.
71. Sumner AT. Distribution of protein sulphhydryls and disulphides in fixed mammalian chromosomes, and their relationship to banding. *J Cell Sci* 1984;70:177–188.
72. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. *Molecular Biology of the Cell*. New York: Garland, 2002.
73. Lewin B. *Genes IX*. Boston: Jones and Bartlett, 2007.
74. Freifelder D. *Molecular Biology*. Boston: Jones and Bartlett, 1987.
75. Adolph KW, Cheng SM, Laemmli UK. Role of nonhistone proteins in metaphase chromosome structure. *Cell* 1977; 12:805–806.
76. Adolph KW, Cheng SM, Paulson JR, Laemmli UK. Isolation of a protein scaffold from mitotic HeLa cell chromosomes. *Proc Natl Acad Sci USA* 1977;74:4937–4941.
77. Hadlaczky G, Sumner AT, Ross A. Protein-depleted chromosomes. II. Experiments concerning the reality of chromosome scaffolds. *Chromosoma* 1981;81:557–567.
78. Paulson JR, Laemmli UK. The structure of histone-depleted metaphase chromosomes. *Cell* 1977;12:817–828.
79. Lewis CD, Laemmli UK. Higher order metaphase chromosome structure: evidence for metalloprotein interactions. *Cell* 1982;29:171–181.
80. Earnshaw WC, Heck MMS. Localization of topoisomerase II in mitotic chromosomes. *J Cell Biol* 1985; 100:716–725.
81. Gasser SM, Laemmli UK. A glimpse at chromosomal order. *Trends Genet* 1987;3:16–22.
82. Saitoh Y, Laemmli UK. Metaphase chromosome structure: Bands arise from a differential folding of the highly AT-rich scaffold. *Cell* 1994;76:609–622.
83. Sumner AT, Evans HJ, Buckland RA. Mechanisms involved in the banding of chromosomes with quinacrine and Giemsa. I. The effects of fixation in methanol-acetic acid. *Exp Cell Res* 1973;81:214–222.
84. Comings DE, Avelino E. Mechanisms of chromosome banding. II. Evidence that histones are not involved. *Exp Cell Res* 1974;86:202–206.
85. Burkholder GD, Duczek LL. The effect of chromosome banding techniques on the proteins of isolated chromosomes. *Chromosoma* 1982;87:425–435.
86. Hancock JM, Sumner AT. The role of proteins in the production of different types of chromosome bands. *Cytobios* 1982;35:37–46.
87. Sivak A, Wolman SR. Chromosomal proteins in fixed metaphase cells. *Histochemistry* 1974;42:345–349.
88. Burkholder GD, Duczek LL. Proteins in chromosome banding. I. Effect of G-banding treatments on the proteins of isolated nuclei. *Chromosoma* 1980;79:29–41.
89. Burkholder GD, Duczek LL. The effect of chromosome banding techniques on the histone and nonhistone proteins of isolated chromatin. *Can J Biochem* 1982;60:328–337.
90. Sumner AT, Evans HJ. Mechanisms involved in the banding of chromosomes with quinacrine and Giemsa. II. The interaction of the dyes the chromosomal components. *Exp Cell Res* 1973;81:223–236.
91. Vogel W, Faust J, Schmid M, Siebers JW. On the relevance of nonhistone proteins to the production of Giemsa banding patterns on chromosomes. *Humangenetik* 1974;21:227–236.
92. Matsukuma S, Utakoji T. Uneven extraction of protein in Chinese hamster chromosomes during G-staining procedures. *Exp Cell Res* 1976;97:297–303.
93. Burkholder GD, Weaver MG. DNA-protein interactions and chromosome banding. *Exp Cell Res* 1977;110:251–262.

94. Burkholder GD, Duczek LL. Proteins in chromosome banding. II. Effect of R- and C-banding treatments on the proteins of isolated nuclei. *Chromosoma* 1980;79:43–51.
95. Comings DE. Mechanisms of chromosome banding. IV Optical properties of the Giemsa dyes. *Chromosoma* 1975;50:89–110.
96. Wyandt HE, Anderson RS, Patil SR, Hecht F. Mechanisms of Giemsa banding. II. Giemsa components and other variables in G-Banding. *Hum Genet* 1980;53:211–215.
97. Sumner AT. Dye binding mechanisms in G-banding of chromosomes. *J Microsc* 1980;119:397–406.
98. van Duijn P, van Prooijen-Knegt AC, van der Ploeg M. The involvement of nucleosomes in Giemsa staining of chromosomes. A new hypothesis on the banding mechanism. *Histochemistry* 1985;82:363–376.
99. Curtis DJ, Horobin RW. Staining banded human chromosomes with Romanowsky dyes: some practical consequences of the nature of the stain. *Humangenetik* 1975;26:99–104.
100. Curtis D, Horobin RW. Chromosome banding: Specification of structural features of dyes giving rise to G-banding. *Histochem J* 1982;14:911–928.
101. Holmquist GP. Review article: Chromosome bands, their chromatin flavors, and their functional features. *Am J Hum Genet* 1992;51:17–37.
102. Burkholder GD. The basis of chromosome banding. *Appl Cytogenet* 1993;19:181–186.
103. Craig JM, Bickmore WA. Chromosome bands—flavours to savour. *Bioessays* 1993;15:349–354.
104. Sumner AT. Functional aspects of the longitudinal differentiation of chromosomes. *Eur J Histochem* 1994;38:91–109.
105. Bernardi G, Olofsson B, Filipski J, Zerial M, Salinas J, Cuny G, Meunier-Rotival M, Rodier F. The mosaic genome of warm-blooded vertebrates. *Science* 1985;228:953–958.
106. Bernardi G. The isochores organization of the human genome. *Annu Rev Genet* 1989;23:637–661.
107. Mouchiroud D, D'Onofrio G, Aissani B, Macaya G, Gautier C. The distribution of genes in the human genome. *Gene* 1991;100:181–187.
108. Saccone S, De Sario A, Valle GD, Bernardi G. The highest gene concentrations in the human genome are in telomeric bands of metaphase chromosomes. *Proc Natl Acad Sci USA* 1992;89:4913–4917.
109. Saccone S, De Sario A, Wiegant J, Raap AK, Valle GD, Bernardi G. Correlations between isochores and chromosomal bands in the human genome. *Proc Natl Acad Sci USA* 1993;90:11929–11933.
110. Ikemura T, Wada K-N. Evident diversity of codon usage patterns of human genes with respect to chromosome banding patterns and chromosomes: relation between nucleotide sequence data and cytogenetic data. *Nucl Acids Res* 1991;19:4333–4339.
111. Trent JM, Kaneko Y, Mitelman F. Report of the committee on structural changes in neoplasia. *Cytogenet Cell Genet* 1989;51:533–562.
112. Bickmore WA, Sumner AT. Mammalian chromosome banding—an expression of genome organization. *Trends Genet* 1989;5:144–148.
113. Holmquist GP. Evolution of chromosome bands: molecular ecology of noncoding DNA. *J Mol Evol* 1989;28:469–486.
114. Larsen F, Gundersen G, Lopez R, Prydz H. CpG islands as gene markers in the human genome. *Genomics* 1992;13:1095–1107.
115. Craig JM, Bickmore WA. The distribution of CpG islands in mammalian chromosomes. *Nature Genetics* 1994;7:376–382.
116. Manuelidis L, Ward DC. Chromosomal and nuclear distribution of the Hind III 1.9-kb human DNA repeat segment. *Chromosoma* 1984;91:28–38.
117. Korenberg JR, Rykowski MC. Human genome organization: Alu, lines, and the molecular structure of metaphase chromosome bands. *Cell* 1988;53:391–400.
118. Kerem BS, Goitein R, Diamond G, Cedar H, Marcus M. Mapping of DNase I sensitive regions on mitotic chromosomes. *Cell* 1984;38:493–499.
119. de la Torre J, Sumner AT, Gosálvez J, Stuppia L. The distribution of genes on human chromosomes as studied by in situ nick translation. *Genome* 1992;35:890–894.
120. Sumner AT, de la Torre J, Stuppia L. The distribution of genes on chromosomes: A cytological approach. *J Mol Evol* 1993;37:117–122.

121. Drouin R, Lemieux N, Richer C-L. High-resolution R-banding at the 1250-band level. III. Comparative analysis of morphologic and dynamic R-band patterns (RHG and RBG). *Hereditas* 1991;114:65–77.
122. Drouin R, Lemieux N, Richer C-L. Chromosome condensation from prophase to late metaphase: Relationship to chromosome bands and their replication time. *Cytogenet Cell Genet* 1991;57:91–99.
123. Kuroiwa T. Asynchronous condensation of chromosomes from early prophase to late prophase as revealed by electron microscopic autoradiography. *Exp Cell Res* 1971;69:97–105.
124. Latt SA. Microfluorometric detection of deoxyribonucleic acid replication in human metaphase chromosomes. *Proc Natl Acad Sci USA* 1973;70:3395–3399.
125. Ganner E, Evans HJ. The relationship between patterns of DNA replication and of quinacrine fluorescence in the human chromosome complement. *Chromosoma* 1971;35:326–341.
126. Dutrillaux B, Couturier J, Richer C-L, Viegas-Pequillnot E. Sequence of DNA replication in 277 R- and Q-bands of human chromosomes using a BrdU treatment. *Chromosoma* 1976;58:51–61.
127. Vogel W, Autenrieth M, Speit G. Detection of bromodeoxyuridine-incorporation in mammalian chromosomes by a bromodeoxyuridine-antibody. I. Demonstration of replication patterns. *Human Genet* 1986;72:129–132.
128. Goldman MA, Holmquist GP, Gray MC, Caston LA, Nag A. Replication timing of genes and middle repetitive sequences. *Science* 1984;224:686–692.
129. Holmquist GP, Caston LA. Replication time of interspersed repetitive sequences. *Biochem Biophys Acta* 1986;868:164–177.
130. Holmquist GP. The magic of cytogenetic technology. In: Obe G, Basler A, eds. *Cytogenetics*. Heidelberg: Springer-Verlag, 1987.
131. Holmquist GP. DNA sequences in G-bands and R-bands. In: Adolph KW, ed. *Chromosomes and Chromatin*. Boca Raton: CRC Press, 1988; 76–121.
132. Hatton K, Dhar V, Brown E, Iqbal A, Stuart S, Didamo V, Schildkraut C. The replication program of active and inactive genes in mammalian cells. *Mol Cell Biol* 1988;8:2149–2158.
133. Turner BM. Histone acetylation and control of gene expression. *J Cell Sci* 1991;99:13–20.
134. Disney JE, Johnson KR, Magnuson NS, Sylvester SR, Reeves R. High-mobility group protein HMG-I localizes to G/Q- and C-bands of human and mouse chromosomes. *J Cell Biol* 1989;109:1975–1982.
135. Ashley T. Prediction of mammalian meiotic synaptic and recombination behavior of inversion heterozygotes based on mitotic breakpoint data and the possible evolutionary consequences. *Genetics* 1990;83:1–7.
136. Chandley AC. A model for effective pairing and recombination at meiosis based on early replicating sites (R-bands) along chromosomes. *Human Genet* 1986;72:50–57.
137. Nakagome Y, Chiyo H. Non-random distribution of exchange points in patients with structural rearrangements. *Am J Hum Genet* 1976;28:31–41.
138. Buckton KE. Identification with G- and R-banding of the position of breakage points induced in human chromosomes by in vitro X irradiation. *Int J Radiat Biol* 1976;29:475–488.
139. San Roman C, Bobrow M. The sites of radiation induced-breakage in human lymphocyte chromosomes, determined by quinacrine. *Experimental Mutat Res* 1973;18:325–331.
140. Morad M, Jonasson J, Lindsten J. Distribution of mitomycin C induced breaks on human chromosomes. *Hereditas* 1973;74:273–282.
141. Hack MS, Lawce HJ, eds. *The Association of Cytogenetic Technologists Cytogenetics Laboratory Manual*. San Francisco: The Association of Genetic Technologists, 1980.
142. Barch MJ, ed. *The ACT Cytogenetics Laboratory Manual*, 2nd ed. New York: Raven Press, 1991.
143. Heitz E. Das heterochromatin der moose. I. Pringsheims Jb. Wiss. Botany 1928;69:762–818.
144. Arrighi FE, Hsu TC. Localization of heterochromatin in human chromosomes. *Cytogenetics* 1971;10:81–86.
145. Hsu TC. Symposium no. 14: Chromosome structure. A possible function of constitutive heterochromatin: the bodyguard hypothesis. *Genetics* 1975;79:137–150.
146. John B. The biology of heterochromatin. In: Verma RS, ed. *Heterochromatin: Molecular and Structural Aspects*. Cambridge: Cambridge University Press, 1988; 1–147.

147. Pardue ML, Hennig W. Heterochromatin: Junk or collectors item? *Chromosoma* 1990;100:3–7.
148. Burkholder GD. The analysis of chromosome organization by experimental manipulation. In: Gustafson JP, Appels R. *Chromosome Structure and Function. Impact of New Concepts*. New York: Plenum Press, 1988;1–52.
149. Hsu TC, Arrighi FE. Distribution of constitutive heterochromatin in mammalian chromosomes. *Chromosoma* 1971;34:243–253.
150. Sumner AT. A simple technique for demonstrating centromeric heterochromatin. *Exp Cell Res* 1972;75:304–306.
151. Salamanca F, Armendares S. C bands in human metaphase chromosomes treated by barium hydroxide. *Ann Genet (Paris)* 1974;17:135–136.
152. Gustashaw KM. Chromosome Stains. In: Barch MJ, ed. *The ACT Cytogenetics Laboratory Manual*, 2nd ed. New York: Raven Press, 1991;205–269.
153. Hsu TC, Arrighi FE. Service of in situ nucleic acid hybridization to biology. In: Caspersson T, Zech L, ed. *Chromosome Identification: Techniques and Applications in Biology and Medicine. Nobel Symposia*. 23. New York: Academic Press, 1973.
154. Comings DE, Avelino E, Okada TA, Wyandt HE. The mechanism of C- and G-banding of chromosomes, *Exp Cell Res* 1973;77:469–493.
155. Pathak S, Arrighi FE. Loss of DNA following C-banding procedures. *Cytogenet Cell Genet* 1973;12:414–422.
156. Holmquist G. C-banding, depurination and beta-elimination. *J Cell Biol* 1977;75:140a.
157. Jack EM, Harrison CJ, Allen DT, Harris R. The structural basis for C-banding. *Chromosoma* 1985;91:363–368.
158. Chen TR, Ruddle FH. Karyotype analysis utilizing differentially stained constitutive heterochromatin of human and murine chromosomes. *Chromosoma* 1971;34:51–72.
159. Azumi J, Nakagome Y, Matsunaga E. A new approach in the evaluation of C-positive variants in man. *Jpn J Hum Genet* 1979;24:99–104.
160. Brown T, Robertson FW, Dawson BM, Hanlin SJ, Page BM. Individual variation of centric heterochromatin in man. *Hum Genet* 1980;55:367–373.
161. Muller H, Klinger HP, Glasser M. Chromosome polymorphism in a human newborn population. II. Potentials of polymorphic chromosome variants for characterizing the idiogram of an individual. *Cytogenet Cell Genet* 1975;15:239–255.
162. Patil SR, Lubs HA. Classification of qh regions in human chromosomes 1, 9, and 16 by C-banding. *Hum Genet* 1977;38:35–38.
163. Phillips RB. New C band markers of human chromosomes: C band position variants. *J Med Genet* 1980;17:380–385.
164. Zanenga R, Mattevi MS, Erdtmann B. Smaller autosomal C band sizes in blacks than in Caucasoids. *Hum Genet* 1984;66:286.
165. Rubenstein CT, Verma RS, Dosik H. Centromeric banding I of sequentially Q- and R-banded human chromosomes. *Hum Genet* 1978;40:279–283.
166. de la Maza L, Sanchez O. Simultaneous G and C banding of human chromosomes. *J Med Genet* 1976;13:235–236.
167. Denton TE, Brooke WR, Howell WM. A technique for the simultaneous staining of both nucleolar organizer regions and kinetochores of human chromosomes with silver. *Stain Technol* 1977;52:311–313.
168. Tuck-Muller CM, Bordson BL, Kane mm, Hamilton AE. A method for combined C-banding and silver staining. *Stain Technol* 1984;59:265–268.
169. DeBrackeleer M, Keushnig M, Lin CC. A high-resolution C-banding Technique. *Can J Genet CytoI* 1986;28:317–322.
170. Bobrow M, Madan K, Pearson PL. Staining of some specific regions of human chromosomes, particularly the secondary constriction of No. 9. *Nature New Biol* 1972;238:122–124.
171. Gagne R, Laberge C. Specific cytological recognition of the heterochromatic segment of number 9 chromosome in man. *Exp Cell Res* 1972;73:239–242.
172. Wyandt HE, Wysham DG, Minden SK, Anderson RS, Hecht F. Mechanisms of Giemsa banding of chromosomes. I. Giemsa-11 banding with azure and eosin. *Exp Cell Res* 1976;102:85–94.
173. Magenis RE, Donlon TA, Wyandt HE. Giemsa-11 staining of chromosome 1: a newly described heteromorphism. *Science* 1978;202:64–65.

174. Gosden JR, Mitchell AR, Buckland RA, Clayton RP, Evans HJ. The location of four human satellite DNAs on human chromosomes. *Exp Cell Res* 1975;92:148–158.
175. Morse HG, Hays T, Patterson D, Robinson A. Giemsa-11 technique. Applications in the chromosomal characterization of hematologic specimens. *Hum Genet* 1982;61:141–144.
176. Alhadeff B, Velivasakis M, Siniscalco M. Simultaneous identification of chromatid replication and of human chromosomes in metaphases of mouse somatic cell hybrids. *Cytogenet Cell Genet* 1977;19:236–239.
177. Bobrow M, Cross J. Differential staining of human and mouse chromosomes in interspecific cell hybrids. *Nature* 1974;251:77–79.
178. Brinkley B, Tousson A, Valdivia MM. The kinetochore of mammalian chromosomes: Structure and function in normal mitosis and aneuploidy. In: Dellarco V, Voytek P, Hollander A, eds. *Aneuploidy: Etiology and Mechanisms*. New York: Plenum Press, 1985;243–267.
179. Earnshaw WC, Tomkiel JE. Centromere and kinetochore structure. *Curr Opin Cell Biol* 1992;4:86–93.
180. Willard HE. Centromeres of mammalian chromosomes. *Trends Genet* 1990;6:410–416.
181. Wolfe SL. *Biology of the Cell*. Belmont, CA: Wadsworth, 1972.
182. Eiberg H. New selective Giemsa technique for human chromosomes, Cd staining. *Nature* 1974;248:55.
183. Evans HJ, Ross A. Spotted centromeres in human chromosomes. *Nature* 1974;249:861–862.
184. Daniel A. Single Cd band in dicentric translocations with one suppressed centromere. *Hum Genet* 1979;48:85–92.
185. Lambiase S, Maraschio P, Zuffardi O. The Cd technique identifies a specific structure related to centromeric function. *Hum Genet* 1984;67:214–215.
186. Maraschio P, Zuffardi O, Lo Curto F. Cd bands and centromeric function in dicentric chromosomes. *Hum Genet* 1980;54:265–267.
187. Moroi Y, Peebles C, Fritzler MJ, Steigerwald J, Tan EM. Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc Natl Acad Sci USA* 1980;77:1627–1631.
188. Palmer DK, O'Day K, Trong HL, Charbonneau H, Margolis RL. Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proc Natl Acad Sci USA* 1991;88:3734–3738.
189. Sullivan KF, Hechenberger M, Masri K. Human CENP-A contains a histone H3 related histone fold domain that is required for targeting to the centromere. *J Cell Biol* 1994;127:581–592.
190. Earnshaw W, Bordwell B, Marino C, Rothfield N. Three human chromosomal autoantigens are recognized by sera from patients with anti-centromere antibodies. *J Clin Invest* 1986;77:426–430.
191. Matsumoto H, Matsukata H, Muro Y, Nozaki N, Okazaki T. A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J Cell Biol* 1989;109:1963–1973.
192. Cooke CA, Bernat RL, Earnshaw WC. CENP-B: A major human centromere protein located beneath the kinetochore. *J Cell Biol* 1990;110:1475–1488.
193. Earnshaw WC, Ratrie H III, Stetten G. Visualization of centromere proteins CENP-B and CENP-C on a stable dicentric chromosome in cytological spreads. *Chromosoma* 1989;98:1–12.
194. Sullivan BA, Schwartz S. Identification of centromeric antigens in dicentric Robertsonian translocations: CENP-C and CENP-E are necessary components of functional centromeres. *Human Molecular Genetics* 1995;4:1289–1298.
195. Saitoh H, Tomkiel J, Cooke CA, Ratrie H, Maurer M, Rothfield NF, Earnshaw WC. CENP-C, an autoantigen in scleroderma, is a component of the human inner kinetochore plate. *Cell* 1992;70:115–125.
196. Tomkiel J, Cooke CA, Saitoh H, Bernat RL, Earnshaw WC. CENP-C is required for maintaining proper kinetochore size and for a timely transition to anaphase. *J Cell Biol* 1994; 125:531–545.
197. Bischoff FR, Maier G, Tilz G, Ponstingl H. A 47-kDa human nuclear protein recognized by antikinetochore autoimmune sera is homologous with the protein encoded by RCC1, a gene implicated in onset of chromosome condensation. *Proc Natl Acad Sci USA* 1990;87:8617–8621.
198. Compton DA, Yen TJ, Cleveland DW. Identification of novel centromere/kinetochore-associated proteins using monoclonal antibodies generated against human mitotic chromosome scaffolds. *J Cell Biol* 1991;112:1083–1097.
199. Yen TJ, Compton DA, Wise D, Zinkowski RP, Brinkley BR, Earnshaw WC, Cleveland DW. CENP-E, a novel human centromere-associated protein required for progression from metaphase to anaphase. *EMBO J* 1991;10:1245–1254.

200. Yen TJ, Li G, Schaar B, Szilak I, Cleveland DW. CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature* 1992;359:536–539.
201. Mackay AM, Earnshaw WC. The INCEP's: Structural and functional analysis of a family of chromosome passenger proteins. *Cold Spring Harbor Symp Quant Biol* 1993;58:697–706.
202. Liao H, Li G, Yen TJ. Mitotic regulation of microtubule cross-linking activity of CENP-E kinetochore protein. *Science* 1994;265:394–398.
203. Rattner JB, Rao A, Fritzler MJ, Valencia DW, Yen TJ. CENP-F is a ca 400 Kda kinetochore protein that exhibits a cell cycle-dependent localization. *Cell Motil Cytoskel* 1993;26:214–226.
204. Liao H, Winkfein RJ, Mack G, Rattner JB, Yen TJ. CENP-F is a protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis. *J Cell Biol* 1995;130:507–518.
205. Brinkley BR, Ouspenski I, Zinkowski RP. Structure and molecular organization of the centromere-kinetochore complex. *Trends Cell Biol* 1992;2:15–21.
206. Pluta AF, Cooke CA, Earnshaw WC. Structure of the human centromere at metaphase. *Trends Biochem Sci* 1990;15:181–185.
207. Jeppesen P. Immunofluorescence techniques applied to mitotic chromosome preparations. In: Gosden J, ed. *Methods in Molecular Biology*. Totowa, NJ: Humana Press. 1994;253–285.
208. Dutrillaux B, Lejeune J. Technique d'analyse du caryotype humain. *CR Acad Sci* 1971;272:2638–2640.
209. Verma RS, Lubs HA. A simple R-banding technique. *Am J Hum Genet* 1975;27:110–117.
210. Bernheim A, Berger R. A simple method for improving the reproducibility of the R-banding technique. *Hum Genet* 1981;57:432–433.
211. de la Chapelle A, Schroder J, Selander RK. Repetitious DNA in mammalian chromosomes. *Hereditas* 1971;69:149–153.
212. Stockert JC, Lisanti JA. Acridine-orange differential fluorescence of fast- and slow-reassociating chromosomal DNA after *in situ* DNA experimental and reassociation. *Chromosoma* 1972;37:117–130.
213. Bobrow M, Collacott HEAC, Madan K. Chromosome banding with acridine orange. *Lancet* 1972;2:1311.
214. Baserga A, Castoldi GL. Applicazione allo studio delle zone eterocromatiche dei cromosomi umani della fluorescenza differenziale mediante arancio di experime. Fondazione Carlo Erba. Simposio Internazionale sul DNA Satellite 1972.
215. Rigler R. Microfluorometric characterization of intracellular nucleic acids and nucleoproteins by acridine orange. *Acta Physiol Scand* 1966;67(Suppl):267.
216. Rigler R. Acridine orange in nucleic acid analysis. *Ann NY Acad Sci* 1969;157:211–224.
217. Rigler R, Killander D, Bolund L, Ringertz NR. Cytochemical characterization of deoxyribonucleoprotein in individual cell nuclei. Techniques for obtaining heat experimental curves with the aid of acridine orange microfluorimetry and ultraviolet microspectrophotometry. *Exp Cell Res* 1969;55:215–224.
218. Bobrow M, Madan K. The effects of various banding procedures on human chromosomes studied with acridine orange. *Cytogenet Cell Genet* 1973;12:145–156.
219. Verma RS, Lubs HA. Additional observations on the preparation of R-banded human chromosomes with acridine orange. *Can J Genet Cytol* 1976;18:45–50.
220. Lin CC, Jorgenson KF, van de Sande JH. Specific fluorescent bands on chromosomes produced by acridine orange after prestaining with base specific non-fluorescent DNA ligands. *Chromosoma* 1980;79:271–286.
221. Dutrillaux B. Obtention simultanee de plusieurs marquages chromosomiques sur les memes preparations, après traitement par le BrdU. *Humangenetik* 1975;30:297–306.
222. Dutrillaux B. Traitements experimental par le BrdU et coloration par acridine orange: obtention de marquages R. *Chromosoma* 1975;52:261–273.
223. Dutrillaux B. The relationship between DNA replication and chromosome structure. *Hum Genet* 1977;35:247–253.
224. Dutrillaux B, Viegas-Pequignot EV. High resolution R- and G-banding on the same preparation. *Hum Genet* 1981;57:93–95.
225. Pai GS, Thomas GH. A new R-banding technique in clinical cytogenetics. *Hum Genet* 1980;54:41–45.
226. Schollmayer E, Schafer D, Frisch B, Schleiermacher E. High resolution analysis and differential condensation in RBA-banded human chromosomes. *Hum Genet* 1981;59:187–193.

227. Burkholder GD. The ultrastructure of R-banded chromosomes. *Chromosoma* 1981;83:473–480.
228. van de Sande JH, Lin CC, Jorgensen KF. Reverse banding on chromosomes produced by a guanine-cytosine specific DNA binding antibiotic: olivomycin. *Science* 1977;195:400–402.
229. Schweizer D. R-banding produced by DNase I digestion of chromomycin-stained chromosomes. *Chromosoma* 1977;64:117–124.
230. Sahar E, Latt SA. Enhancement of banding patterns in human metaphase chromosomes by energy transfer. *Proc Natl Acad Sci USA* 1978;75:5650–5654.
231. Sahar E, Latt SA. Energy transfer and binding competition between dyes used to enhance staining differentiation in metaphase chromosomes. *Chromosoma* 1980;79:1–28.
232. Schweizer D. Counterstain-enhanced chromosome banding. *Hum Genet* 1981;57:1–14.
233. Behr W, Honikel K, Hartmann G. Interaction of the RNA polymerase inhibitor Chromomycin with DNA. *Eur J Biochem* 1969;9:82–92.
234. Jensen RH, Langlois RG, Mayall BH. Strategies for choosing a deoxyribonucleic acid stain for flow cytometry of metaphase chromosomes. *J Histochem Cytochem* 1977;25:954–964.
235. Ward D, Reich E, Goldberg IH. Base specificity in the interaction of polynucleotides with antibiotic drugs. *Science* 1965;149:1259–1263.
236. Muller W, Gautier F. Interactions of heteroaromatic compounds with nucleic acids. A-T specific, non-intercalating DNA ligands, *Eur J Biochem* 1975;54:385–394.
237. Kurnick NB, Foster M. Methyl green, III. Reaction with deoxyribonucleic acid, stoichiometry, and behavior of the reaction product. *J Gen Physiol* 1950;34:147–159.
238. Latt SA, Juergens LA, Matthews DJ, Gustashaw KM, Sahar E. Energy transfer-enhanced chromosome banding. An overview. *Cancer Genet Cytogenet* 1980;1:187–196.
239. Schweizer D, Ambros P, Andrle M. Modification of DAPI banding on human chromosomes by prestaining with a DNA-binding oligopeptide antibiotic, Distamycin A. *Exp Cell Res* 1978;111:327–332.
240. Lin MS, Comings DE, Alfi OS. Optical studies of the interaction of 4,6-diamidino-2-phenylindole with DNA and metaphase chromosomes. *Chromosoma* 1977;60:15–25.
241. Zimmer CH, Puschendorf B, Grunieke H, Chandra P, Venner H. Influence of netropsin and Distamycin A on the secondary structure and template activity of DNA. *Eur J Biochem* 1971;21:269–278.
242. Buhler EM, Tsuchimoto T, Jurik LP. Satellite DNA III and alkaline Giemsa staining. *Humangenetik* 1975;26:329–333.
243. Miller OJ, Schnedl W, Allen J, Erlanger BF. 5-Methylcytosine localized in mammalian constitutive heterochromatin. *Nature* 1974;251:636–637.
244. Donlon TA, Magenis RE. Methyl green is a substitute for distamycin A in the formation of distamycin A/DAPI bands. *Hum Genet* 1983;65:144–146.
245. Schweizer D. Simultaneous fluorescent staining of R-bands and specific heterochromatic regions (DA-DAPI bands) in human chromosomes. *Cytogenet Cell Genet* 1980;27:190–193.
246. Heng HHQ, Tsui L-C. Modes of DAPI banding and simultaneous in situ hybridization. *Chromosoma* 1993;102:325–332.
247. Magenis E, Brown MG, Chamberlin J, Donlon T, Hepburn D, Lamvik N, Lovrien E, Yoshitomi M. Resolution of breakpoints in a complex rearrangement by use of multiple staining techniques: Confirmation of suspected 12p12.3 intraband deletion by dosage effect of LDHB. *Am J Med Genet* 1981;9:95–103.
248. Wisniewski L, Hassold T, Heffelfinger J, Higgins JV. Cytogenetic and clinical studies in five cases of inv dup (15). *Hum Genet* 1979;50:259–270.
249. Chamberlin J, Magenis RE. Parental origin of de novo chromosome rearrangements. *Hum Genet* 1980;53:343–347.
250. Ledbetter DH, Riccardi VM, Au WW, Wilson DP, Holmquist GP. Ring chromosome 15: Phenotype, Ag-NOR analysis, secondary aneuploidy, and associated chromosome instability. *Cytogenet Cell Genet* 1980;27:111–122.
251. Buys CHCM, Anders GJPA, Borkent-Ypma IMM, Blenkers-Blatter IAM, van der Hoek-van der Veen AY. Familial transmission of a translocation Y/14. *Hum Genet* 1979;53:125–127.
252. Cooke HJ, Noel B. Confirmation of Y/autosome translocations using recombinant DNA. *Hum Genet* 1979;50:39–44.

253. Mitchell AR, Spowart G. The detection of Y chromosome material in cases of suspected Y-autosome translocation. *Cytogenet Cell Genet* 1979;25:79.
254. Noel L. Translocation de l'heterochromatine distale du chromosome Y sur un chromosome. 4 observations familiales. D. Med. Thesis, University of Grenoble; 1979.
255. Spowart G. Reassessment of presumed Y/22 and Y/15 translocations in man using a new technique. *Cytogenet Cell Genet* 1979;23:90–94.
256. Schnedl W, Abraham R, Dann O, Geber G, Schweizer D. Preferential fluorescent staining of heterochromatic regions in human chromosomes 9, 15, and the Y by D287/170. *Hum Genet* 1981;59:10–13.
257. Ruzicka V. Zur geschichte und kenntnis der feineren structur der nucleolen centraler nervzellen. *Anat Anz* 1891;16:557–563.
258. Ferguson-Smith MA, Handmaker SD. Observations on the satellites human chromosomes. *Lancet* 1961;1:638–640.
259. Ohno S, Trujillo JM, Kaplan WD, Kinoshita R. Nucleolus organizers in the causation of chromosome anomalies in man. *Lancet* 1961;2:123–125.
260. Funaki K, Matsui S, Sasaki M. Location of nucleolar organizers in animal and plant chromosomes by means of an improved N-banding technique. *Chromosoma* 1975;49:357–370.
261. Hsu TC, Spirito SE, Pardue ML. Distribution of 18–28S ribosomal genes in mammalian genomes. *Chromosoma* 1975;53:25–36.
262. Tantravahi R, Miller DA, Dev VG, Miller OJ. Detection of nucleolus organizer regions in chromosomes of human, chimpanzee, gorilla, orangutan and gibbon. *Chromosoma* 1976;56:15–27.
263. Evans HJ, Buckland RA, Pardue ML. Distribution of 18–28S ribosomal genes in the human genome. *Chromosoma* 1974;48:405–426.
264. Henderson AS, Warburton D, Atwood KC. Location of ribosomal DNA in the human chromosome complement. *Proc Natl Acad Sci USA* 1972;69:3394–3398.
265. Taylor EF, Martin-DeLeon PA. Familial silver staining patterns of human nucleolus organizer regions (NORs). *Am J Hum Genet* 1981;33:67–76.
266. Goodpasture C, Bloom SE, Hsu TC, Arrighi FE. Human nucleolus organizers: The satellites or the stalks? *Am J Hum Genet* 1976;28:559–566.
267. Miller DA, Dev VG, Tantravahi R, Miller OJ. Suppression of human nucleolus organizer activity in mouse-human somatic hybrid cells. *Exp Cell Res* 1976;101:235–243.
268. Miller OJ, Miller DA, Dev VG, Tantravahi R, Croce CM. Expression of human and suppression of mouse nucleolus organizer activity in mouse-human somatic cell hybrids. *Proc Natl Acad Sci USA* 1976;73:4531–4535.
269. Verma RS, Rodriguez J, Shah JV, Dosik H. Preferential association of nucleolar organizing human chromosomes as revealed by silver staining technique at mitosis. *Mol Gen Genet* 1983;190:352–354.
270. Matsui SI, Sasaki M. Differential staining of nucleolus experiment in mammalian chromosomes. *Nature* 1973;246:148–150.
271. Faust J, Vogel W. Are N-bands selective staining of specific heterochromatin? *Nature* 1974;249:352–353.
272. Howell WM, Denton TE, Diamond JR. Differential staining of the satellite regions of human acrocentric chromosomes. *Experientia* 1975;31:260–262.
273. Goodpasture C, Bloom SE. Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. *Chromosoma* 1975;53:37–50.
274. Denton TE, Howell WM, Barrett JY. Human nucleolar organizer chromosomes: satellite associations. *Chromosoma* 1976;55:81–84.
275. Bloom SE, Goodpasture C. An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. *Hum Genet* 1976;34:199–206.
276. Howell WM, Black DA. Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: A 1-step method. *Experientia* 1980;36:1014–1015.
277. Gold JR, Ellison JR. Silver staining for nucleolar organizing regions of vertebrate chromosomes. *Stain Technol* 1983;58:51–55.

278. Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ. Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. *Histochem J* 1986;18:5–14.
279. Ferraro M, Archidiacono N, Pelliccia F, Rocchi M, Rocchi A, de Capoa A. Secondary constrictions and nucleolus organizer regions in man. *Exp Cell Res* 1977;104:428–430.
280. Tantravahj R, Miller DA, Miller OJ. Ag-staining of nucleolus organizer regions of chromosomes after Q-, C-, G-, or R- banding procedures. *Cytogenet Cell Genet* 1977;18:364–369.
281. Zankl H, Bernhardt S. Combined silver staining of the nucleolus organizing regions and Giemsa banding in human chromosomes. *Hum Genet* 1977;37:79–80.
282. Lau YF, Pfeiffer RA, Arrighi FE, Hsu TC. Combination of silver and fluorescent staining for metaphase chromosomes. *Am J Hum Genet* 1978;30:76–79.
283. Howell WM, Black DA. A rapid technique for producing silver-stained nucleolus organizer regions and trypsin-Giemsa bands on human chromosomes. *Hum Genet* 1978;43:53–56.
284. Dipierri JE, Fraisse J. A simple combined Ag-I/RHG technique for metaphase chromosomes. *Hum Genet* 1983;64:286–287.
285. Rufas JS, Gosalvez J, Lopez-Fernandez C, Cardoso H. Complete dependence between AG NORs and C-positive heterochromatin revealed by simultaneous AG-NOR C-banding method. *Cell Biol Int Rep* 1983;7:275–281.
286. Das NK. Demonstration of a non-RNA nucleolar fraction by silver staining. *Exp Cell Res* 1961;26:428–431.
287. Schwarzacher HG, Mikelsaar AV, Schnedl W. The nature of the Ag-staining of nucleolus organizer regions. Electron- and light-microscopic studies on human cells in interphase, mitosis, and meiosis. *Cytogenet Cell Genet* 1978;20:24–39.
288. Matsui S. Structural proteins associated with ribosomal cistrons in *Xenopus laevis* chromosomes. *Exp Cell Res* 1974;88:88–94.
289. Hubbell HR, Lau YF, Brown RL, Hsu TC. Cell cycle analysis and drug inhibition studies of silver staining in synchronous HeLa cells. *Exp Cell Res* 1980;129:139–147.
290. Lau YF, Arrighi FE. Studies of the squirrel monkey, *Saimiri sciureus*, genome. I. Cytological characterization of chromosomal heterozygosity. *Cytogenet Cell Genet* 1976;17:51–60.
291. Hubbell HR, Rothblum LI, Hsu TC. Identification of a silver binding protein associated with the cytological silver staining of actively transcribing nucleolar regions. *Cell Biol Int Rep* 1979;3:615–622.
292. Buys CHCM, Osinga J. Abundance of protein-bound sulphydryl and disulfide groups at chromosomal nucleolus organizing regions. *Chromosoma* 1980;77:1–11.
293. Ochs RL, Busch H. Further evidence that phosphoprotein C23 (110 kD/pI 5.1) is the nucleolar silver staining protein. *Exp Cell Res* 1984;152:260–265.
294. Chiou-Nan S, Azadeh A, Wright A. Nucleolar organization, growth control and cancer. *Epigenetics* 2010;5(3):200–205.
295. Raman R, Sperling K. Patterns of silver staining on NORs of prematurely condensed muntjac chromosomes following RNA inhibition. *Exp Cell Res* 1981;135:373–378.
296. Haaf T, Weis H, Schindler D, Schmid M. Specific silver staining of experimentally undercondensed chromosome regions. *Chromosoma* 1984;90:149–155.
297. Spector DL, Ochs RL, Busch H. Silver staining, immunofluorescence, and immunoelectron microscopic localization of nucleolar phosphoteins B23 and C23. *Chromosoma* 1984;90:139–148.
298. Héliot L, Mongelard F, Klein C, O'Donohue MF, Chassery JM, Robert-Nicoud M, Usson Y. Nonrandom distribution of metaphase AgNOR staining patterns on human acrocentric chromosomes. *J Histochem Cytochem* 2000;48(1):13–20.
299. Guttenbach M, Haaf T, Steinlein C, Caesar J, Schinzel A, Schmid M. Ectopic NORs on human chromosomes 4qter and 8q11: rare chromosomal variants detected in two families. *J Med Genet* 1999;36:339–342.
300. Hsu TC. Polymorphism in human acrocentric chromosomes and the silver staining method for nucleolus organizer regions. *Karyogram* 1981;7:45–47.
301. Latt SA. Fluorescence analysis of late DNA replication in human metaphase chromosomes. *Somat Cell Genet* 1975;1:293–321.
302. German JL III. DNA synthesis in human chromosomes. *Trans NY Acad Sci* 1962;24:395–407.
303. Lyon MF. X-chromosome inactivation and developmental patterns in mammals. *Biol Rev* 1972;47:1–35.

304. Petersen AJ. DNA synthesis and chromosomal asynchrony. *J Cell Biol* 1964;23:651–654.
305. Priest JH, Heady JE, Priest RE. Delayed onset of replication of human X chromosomes. *J Cell Biol* 1967;35:483–486.
306. Taylor JH, Woods RS, Hughes WL. The organization and duplication of chromosomes as revealed by autoradiographic studies using tritium-labelled thymidine. *Proc Natl Acad Sci USA* 1957;43:122–127.
307. Korenberg JR, Freedlander EF. Giemsa technique for the detection of sister chromatid exchanges. *Chromosoma* 1974;48:355–360.
308. Perry P, Wolff S. New Giesma method for the differential staining of sister chromatids. *Nature* 1974;251:156–158.
309. Wolff S, Perry P. Differential Giemsa staining of sister chromatids and the study of sister chromatid exchanges without autoradiography. *Chromosoma* (Berl.) 1974;48:341–353.
310. Latt SA, Willard HF, Gerald PS. BrdU-33258 Hoechst analysis of DNA replication in human lymphocytes with supernumerary or structurally abnormal X chromosomes. *Chromosoma* 1976;57:135–153.
311. Grzeschik KH, Kim MA, Johannsmann R. Late replicating bands of human chromosomes demonstrated by fluorochrome and Giemsa staining. *Humangenetik* 1975;29:41–59.
312. Hilwig I, Gropp A. Decondensation of constitutive heterochromatin in L cell chromosomes by a benzimidazole compound (“33258 Hoechst”). *Exp Cell Res* 1973;81:474–477.
313. Weisblum B, Haensler E. Fluorometric properties of the bisbenzimidazol derivative Hoechst 33258, a fluorescent probe specific for AT-concentration in chromosomal DNA. *Chromosoma* 1974;46:255–260.
314. Latt SA, Wohlleb JC. Optical studies of the interaction of 33258 Hoechst with DNA, chromatin, and metaphase chromosomes. *Chromosoma* 1975;52:297–316.
315. Latt SA. Microfluorometric analysis of deoxyribonucleic acid replication kinetics and sister chromatid exchanges in human chromosomes. *J Histochem Cytochem* 1974;22:478–491.
316. Latt SA. Microfluorometric analysis of DNA replication in human X chromosomes. *Exp Cell Res* 1974;86:412–415.
317. Kaiser-McCaw B, Latt SA. X-chromosome replication in parthenogenetic benign ovarian teratomas. *Hum Genet* 1977;253–264.
318. Beauchesne MT. Late replication banding. *Karyogram* 1981;7:24–26.
319. Latt SA, Schreck R, Loveday K, Shuler C. In vitro and in vivo analysis of sister chromatid exchange. *Pharmacol Rev* 1978;30:501–535.
320. Gustashaw KM. Sister chromatid exchanges. *Karyogram* 1981;7:18–23.
321. Tice R, Schneider EL, Rary JM. The utilization of bromodeoxyuridine incorporation into DNA for the analysis of cellular kinetics. *Exp Cell Res* 1976;102:232–236.
322. Willard HF, Latt SA. Analysis of deoxyribonucleic acid replication in human X chromosomes by fluorescence microscopy. *Am J Hum Genet* 1976;28:213–227.
323. Scheres JMJC. Production of C and T bands in human chromosomes after heat treatment at high pH and staining with “Stains-All.” *Humangenetik* 1974;23:311–314.
324. Dahlberg AE, Dingman CW, Peacock AC. Electrophoretic characterization of bacterial polyribosomes in agarose-acrylamide composite gels. *J Mol Biol* 1969;41:139–147.
325. Scheres JMJC. CT banding of human chromosomes. Description of the banding technique and some of its modifications. *Hum Genet* 1976;31:293–307.
326. Scheres JMJC. CT banding of human chromosomes. The role of cations in the alkaline pretreatment. *Hum Genet* 1976;33:167–174.
327. Chamla Y, Ruffie M. Production of C and T bands in human mitotic chromosomes after heat treatment. *Hum Genet* 1976;34:213–216.
328. Dutrillaux B. Nouveau système de marquage chromosomique: les bandes T. *Chromosoma* 1973;41:395–402.
329. Powell C, Winkelmann RK, Veneczel-Lemarchard F, Spurbeck JL, Schroeter AL. The anticentromere antibody: disease specificity and clinical significance. *Mayo Clin Proc* 1984;59:700–706.
330. Spurbeck JL, Adams SA, Stupca PJ, Dewald GW. Primer on medical genomics, part XI: visualizing human chromosomes. *Mayo Clin Proc* 2004;79(1):58–75.

Contributed protocols section

IMPORTANT: No protocol included in this manual should be used clinically unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements of all chemicals used in the laboratory.

Protocol 6.1 Conventional Giemsa staining (unbanded)

From the Third Edition of the AGT Cytogenetics Laboratory Manual, contributed anonymously

I. Materials

1. Buffer: Gurr's buffer tablets, pH 6.8 (Gibco® #10582-013):
Dissolve 1 tablet in 1 liter of water. If no Gurr's buffer tablets are available, an equivalent buffer can be made by adding 0.469 g NaH₂PO₄ plus 0.937 g Na₂HPO₄, q.s. to a liter with water
2. Giemsa stain:
Stock Giemsa: Gibco® #10092-013
Working Giemsa: 3 mL stock Giemsa in 48.5 mL Gurr's buffer
3. 5 N HCl
4. Water

II. Method

1. Place slides in a Coplin jar or staining dish.
2. Prepare the working stain and add it into the jar with the slides.
3. Stain for 7–10 minutes.
4. Decant the stain and rinse slides in two changes of distilled water.
5. Air-dry or blow-dry slides.
6. Optional: coverslip with mounting medium for permanent storage.

Protocol 6.2 Leishman's stain

From the Third Edition of the AGT Cytogenetics Laboratory Manual, contributed anonymously

I. Materials

1. Leishman's Stain: Sigma Aldrich #L6254.
Make stock solution at 0.15% in 100% methanol.
2. Buffer: Gurr's Buffer Tablets, pH 6.8 (Gibco® #10582-013)
3. Working stain: Leishman stain stock, diluted 1 : 4 with buffer

II. Method

1. Stain slides 3–5 minutes in working stain.
2. Rinse well with buffer. If stain is too intense, wash longer in buffer. If it is too weak, re-stain the slides.
3. Blot dry with bibulous paper or air dry with an air jet.
4. Mount slides if desired in mounting medium.

Protocol 6.3 Quinacrine mustard chromosome staining (Q-bands)

Contributed by Oregon Health & Science Center, Knight Diagnostic Cytogenetics Laboratory, Portland, Oregon

Safety warnings

Quinacrine mustard dihydrochloride is a possible carcinogen, mutagen, and teratogen. It is highly toxic, either by contact with skin or inhaled or swallowed. It is an irritant and a neurological hazard. Use gloves when working with solutions or powder,

and when weighing powder, use gloves, mask, and laboratory coat. Do not breathe dust or vapor. In case of contact with eyes or skin, rinse immediately with plenty of water for at least 15 minutes, remove contaminated clothing and shoes, and see a physician as soon as possible.

I. Principle

The Q-banding method identifies each chromosome by its pattern, and like G-bands, is good for recognizing certain structural and numerical abnormalities. In addition, Q-staining differentially stains the variable heterochromatic regions of certain chromosomes such as the Yq and the acrocentric short arms. These variations in the heterochromatic regions are very individual, specific and inherited, and can be used to distinguish individuals and determine chromosome origin. This banding method does not preclude other stains sequential to it, including FISH, and slides may be easily destained in fixative and re-stained. Quinacrine mustard dihydrochloride is a member of the acridine family of dyes having a core structure of three flat aromatic rings. The quinacrine molecule contains a terminal mustard configuration in the molecular side chain. There appears to be a correlation between strong QM binding and distribution of heterochromatin determined by thymidine labeling. Also, brighter fluorescence reflects DNA with a high (A+T)/(G+C) ratio. Base composition, however, does not appear to be the only explanation for banding. There is evidence that quinacrine binds by binding predominately due to nonhistone proteins; hence, the exact molecular mechanism of fluorescent Q-banding is still not completely understood. The method involves making slides according to usual methods, re-hydrating, exposing the slides to the quinacrine stain in the dark to prevent quenching of the stain, and rinsing in buffer and water to reduce the background fluorescence. Slides are mounted with a coverslip in buffered, saturated sucrose solution, which acts as a semi-permanent mounting medium. Slides are viewed using a fluorescent microscope with an excitation filter of about 455 nm and an emission filter of 436–495 nm. The excitation wavelength causes the quinacrine molecule to fluoresce by energizing the electrons to jump to the next shell, and when they return, the excess energy is emitted as light.

II. Materials

Supplies

1. Coverslips, 24 × 60 mm
2. Pasteur pipettes, 5¾ inch
3. Coplin jars

Reagents

1. Quinacrine mustard dihydrochloride, Fluka, obtained from Sigma, #22535, 100 mg (or, old Sigma #Q-2000, or old Gibco® #130765). Shelf life is at least 10 years.
Dissolve 3 mg of QM in several drops of methanol, and then add up to 10 mL of distilled water. Dilute to 50 mL with McIlvaine's buffer, pH 7.0. Final concentration of stain is 6 µg/mL. Store stain in a foil-covered Coplin jar in the refrigerator, inside a styrofoam container for light-tightness. Stain is good for 2–3 months.
2. McIlvaine's Buffer: there are two components to the buffer, which yield different pHs with different proportions of the components. A pH of 7.0 results from a mixture of 164.67 mL of solution A and 35.3 mL of solution B for each 200 mL desired as follows:
 - a. Solution A: 28.4 g disodium phosphate (Na_2HPO_4)/liter of distilled water
 - b. Solution B: 21 g citric acid ($\text{C}_6\text{H}_8\text{O}$)/liter of distilled water.
3. Sucrose, 60%, in McIlvaine's buffer, pH 7.0.
Weigh out 6 g sucrose. Add to a 15 mL centrifuge tube with about 7–8 mL of McIlvaine's buffer, pH 7.0, and place in a 37–50 °C water bath overnight to dissolve. Vortex to complete the solution, and bring the final volume to 10 mL with McIlvaine's buffer.
4. Methanol
5. Ethanol, 100%, 95%, and 50% in water

III. Method

1. Make slides as usual, and bake at 90–95 °C for 20 minutes. Cool slides.
2. Run slides through the following steps:
 - a. 100% ethanol, 2 minutes
 - b. 95% ethanol, 2 minutes

- c. 50% ethanol, 2 minutes
 - d. Deionized water, 2 minutes
 - e. McIlvaine's buffer, 5 minutes
 - f. QM stain solution, 2-4 minutes
 - g. McIlvaine's buffer, 10 minutes
 - h. Deionized water, 10 minutes
 - i. Deionized water, 10 minutes
3. Mount slides with sucrose/buffer solution, using 1 small drop on each end of the slide, adding the coverslip with as few bubbles as possible, and then squeeze out excess sucrose gently between layers of paper towels (excess thickness will result in "flare"). Store slides in the dark until use. Slides may be sequentially stained with other stains by soaking off coverslip in distilled water, and placing slide in 3 : 1 methanol-acetic acid fixative for 5 minutes. Then allow slide to dry, and continue with the next procedure as usual.

IV. References and suggested readings

1. Caspersson T, Zech L, Johansson C. Analysis of human metaphase chromosomes by aid of DNA-binding fluorescent agents. *Exp Cell Res.* 1970; 62:490–492.
2. Caspersson T, Lomakka G, Zech L. The 24 fluorescence patterns of the human metaphase chromosomes—distinguishing characters and variability. *Hereditas* 1971; 67:89–102.
3. Gustashaw K. Chromosome Stains. In: Barch M, Knutsen T, Spurbeck J, eds., *The AGT Cytogenetics Laboratory Manual*, 3rd ed. New York, Lippincott-Raven, 1997, pp. 266–268.

C-Banding protocols

Protocol 6.4 C-banding

Contributed by the Department of Pathology, Monmouth Medical Center, Long Branch, New Jersey

I. Principle

C-banding is a special staining technique in which the primary heterochromatic regions and centromeres of all the chromosomes are darkly stained. Secondary constrictions of chromosomes 1, 9, and 16 and the long arm of Y are intensely stained. The chromosome arms will appear pale or lightly stained. This technique is useful for studying chromosomal variants or polymorphisms.

II. Materials

Specimen

C-banding can be performed on metaphases from all types of specimens (slides or coverslips). This technique can be done on previously G-banded spreads after they are de-stained (see Note 1, Steps to destain previously G-banded chromosomes). Slide preparations should be baked overnight in a 60 °C oven, or in a 95 °C oven for 50 minutes.

Supplies

1. Coplin jars
 2. Deionized water
 3. 0.7 N Ba(OH)₂ (Sigma #B2507) (5%: 5 g/100 mL distilled water). Make fresh and mix well before using.
 4. 2× SSC
- Dissolve 17.535 grams sodium chloride (Sigma #S9888) and 8.82 grams sodium citrate (Sigma #S464) in 1000 mL distilled water. Store at room temperature. Expiration 6 months.

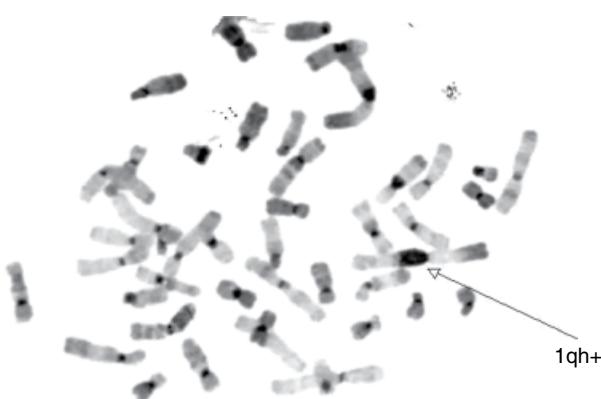


Figure 6.25 C-Banding. Male C-banded metaphase showing a large heterochromatic region on the long arm of chromosome 1 (1qh+). Courtesy of the Department of Pathology, Monmouth Medical Center, Long Branch, New Jersey.

5. 60 °C oven and 90 °C oven.
6. Giemsa stain. Dissolve 5 mL Giemsa (Harleco #620G-75) in 45 mL PO₄ buffer (Gurr buffer tablets, Gibco® Invitrogen #10582-013). Make fresh daily.

III. Method

1. Place slide in saturated Ba(OH)₂ at 37 °C for 15 minutes.
2. Rinse three times in deionized distilled water
3. Place slide in 2× SSC solution that has been heated and kept at 60 °C for 2 hours.
4. Rinse three times in deionized distilled water.
5. Stain in 10% Giemsa solution for 5 minutes (3 minutes for coverslips).

Analysis

The heterochromatin region should be darkly stained in all chromosomes, with a faint outline of the euchromatic material. The amount of C-banding of each chromosome may vary from individual to individual. Polymorphisms are pronounced in chromosomes 1, 9, 16 and the Y chromosome (see Figure 6.25).

IV. Notes

(Editors' Note: Xylene is a known carcinogen. If possible, a Xylene substitute is recommended.)

1. Steps to destain previously G-banded chromosomes
 - a. Soak slide in Xylene until oil is removed (5–10 minutes).
 - b. Place slide in Xylene-100% ethanol mixture for 2 minutes.
 - c. Place slide in 100% ethanol for 2 minutes.
 - d. Place slide in 95% ethanol for 2 minutes.
 - e. Place slide in 70% ethanol/1% HCl for 2–3 minutes until slide is decolorized.
 - f. Rinse slide in 70% ethanol and air dry.

V. References and suggested readings

1. Warburton D. Current techniques in chromosome analysis. *Pediatr Clin North Am* 1980; 27(4):753–769.
2. Houghton J. Techniques for identification of human chromosomes. *Sci Prog* 1974; 61:461–472.

3. Craig-Holmes A. Polymorphism of human C-band heterochromatin. I. Frequency of variants. *Am J Hum Genet* 1973; 25:181–192.
4. Arighi FE, Hsu TC. Staining constitutive heterochromatin and Giemsa crossbands of mammalian chromosomes. In Yunis JJ, ed, *Human Chromosome Methodology*, 1974. New York: Academic Press; 59–71.

Protocol 6.5 C-banding

Anonymous Contributor

I. Principle

The purpose of this protocol is to observe heterochromatic DNA. The C-banding technique stains constitutive heterochromatin around the centromeres of chromosomes. Such regions are heteromorphic (vary in size) on some chromosomes. In particular, regions on chromosomes 1, 9, and 16, and the short arms of the acrocentric chromosomes (numbers 13–15 and 21–22) vary in size from one individual to another. Such variations are heritable. The C-banding technique is useful for detecting dicentric chromosomes, for determining parental origin of certain chromosomes, and for deciding, in certain instances, whether extra chromosomal material is genetically important or genetically inert (i.e., if C-band positive).

II. Materials

Specimens

Amniotic fluids, products of conception, peripheral blood, and bone marrow.

Reagents

Note: Make sure that all glassware used is scrupulously cleaned and rinsed with sterile bottled water (Baxter).

1. Concentrated HCl (12 N). Store in acid cabinet. Expiration: See label on bottle.
2. Barium hydroxide (Fisher #B47-250). Store in chemical cabinet.
3. Sodium chloride (Fisher #S671-500). Store in chemical cabinet. Expiration: See label on bottle.
4. Trisodium citrate (Fisher #S279-500). Store in chemical cabinet. Expiration: See label on bottle.
5. Gurr Buffer Tablets, pH 6.8 (Gallard-Schlesinger #331932). Store at room temperature. Expiration: See label on bottle.
6. Gurr Giemsa Stain (Gallard-Schlesinger #350862M). Store at room temperature. Expiration: See label on bottle.
7. Wright Stain (Sigma #W-3000). Store at room temperature, away from light. Add 500 mL methanol to a light-tight bottle and begin stirring. Slowly add 1.5 g Wright stain to the methanol. Continue stirring overnight. Using two layers of filter paper, gravity-filter the stain into a clean, dry, light-tight bottle. Allow stain to age for a minimum of 10 days before testing, but it can take 3–6 months before reaching its ideal quality. Run test slides of each tissue type being banded prior to using at a staining session. Filter every month, or as needed. Store at room temperature, away from light. Once dissolved with methanol, reagent must be kept in a flammable storage cabinet and used under the fume hood. Waste must be collected, safely stored, and discarded via flammable waste procedures. Because this stain generally improves with age, expiration is indefinite; therefore, careful monitoring is required to determine when the quality is starting to deteriorate, generally 12–18 months from date of preparation, unless the manufacturer's expiration of the stock reagent supersedes this date.
8. 1 N HCl stock solution. Dilute 4.15 mL concentrated hydrochloric acid (HCl 12 N) to 50 mL with sterile bottled water. This gives a 1 N solution. This solution needs to be made fresh each time that it is used. CAUTION: Initially add acid to water slowly with mixing - this step should be performed in chemical fume hood with proper equipment.
9. HCl working solution. Dilute 10 mL of stock HCl solution with 40 mL sterile bottled water. Discard after use with running tap water in sink.
10. Barium hydroxide stock solution. Dissolve 2.57 g of barium hydroxide $[\text{Ba}(\text{OH})_2]$ in 50 mL of sterile bottled water. This solution must be made fresh each time it is used.
11. Barium hydroxide working solution. Make this solution after HCl incubation. Make sure stock barium hydroxide solution is well mixed before making up working solution. Dilute 15 mL of the stock solution $[\text{Ba}(\text{OH})_2]$ to 35 mL sterile bottled water.
12. 2× SSC (pH 7.0). Dissolve 9.0 g sodium chloride (NaCl) and 5.0 g trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) in 300 mL sterile bottled water in a 500 mL volumetric flask. Add enough sterile water to make 500 mL.

13. Gurr's buffer solution, pH 6.8. Add one Gurr tablet (pH 6.8) to a labeled 100 mL bottle and add 100 mL sterile bottled water. Discard unused portion daily.
14. Gurr's Giemsa stain. Dilute 2.0 mL Gurr's Giemsa with 48 mL Gurr's buffer solution.
15. Wright Stain. Dilute 3.0 mL of prepared Wright Stain in 30 mL of Gurr's buffer solution.

Quality control

If chromosomes are stained too lightly, time in Ba(OH)₂ should be reduced. Use thirty-second intervals. Too little contrast requires a longer time in Ba(OH)₂.

Aging slides (optional treatment for slides less than 2 weeks of age):

1. Prewarm 40 mL 2× SSC pH 7.0 (1 : 10 dilution of 20× SSC adjusted to pH 7.0 with concentrated HCl) in a Coplin jar to 37 °C in a water bath. Place prepared slides in the Coplin jar and incubate for 30 minutes.
2. Rinse slides with sterile bottled water over sink
Treatment of slides in 2× SSC artificially ages the chromosomes, making them less sensitive to over-denaturation. This treatment is not necessary if the slides are more than 2 weeks old.

III. Method

1. Place slides in Coplin jar of working HCl for 1 hour.
2. Rinse in sterile bottled water over sink.
3. Make up barium hydroxide [Ba(OH)₂] solution. Place slides in Coplin jar of Ba(OH)₂ working solution for 45 seconds to 1.5 minutes. New slides require less time. Older slides (more than 2 days old) may require longer time. Slides that have been destained or are new should be exposed for only 50 seconds.
4. Rinse in sterile bottled water over sink.
5. Incubate in 60 °C water bath in 2× SSC (pH 7.0) for 1 hour. If cells seem to wash off in this treatment, see Note 1, Troubleshooting.
6. Rinse in sterile bottled water over sink.
7. Place slides in Giemsa or Wright stain in Coplin jar for 5 minutes.
8. Rinse in sterile bottled water over sink.
9. Air dry slides at room temperature.

IV. Notes

1. Troubleshooting
If a loss of metaphases are observed after treatment at 60 °C, artificially age a new slide in 2× SSC (pH 7.0) for 30 minutes at 37 °C before C-banding.

V. References

1. Sumner AT. A simple technique for demonstrating centromeric heterochromatin. *Exp Cell Res* 1972; 75:304–306.
2. Oncor. *Chromosome In-Situ Hybridization System*. Oncor, Inc., Maryland. 1994.

Protocol 6.6 C-banding

Contributed by Oregon Health & Science University, Knight Diagnostic Laboratories, Portland, Oregon

Safety warning

Barium hydroxide is corrosive and may cause burns. Flush for 15 minutes in case of contact. Wear gloves, lab coat and goggles when in use.

I. Principle

The variable chromosomal heterochromatic regions (constitutive heterochromatin) containing noncoding DNA can be detected by employing C-banding procedures. These techniques involve the differential solubilization of fragmented DNA from euchromatin

by depurination DNA denaturation and chain breakage at the depurinated sites produced by HCl, NaOH, or Ba(OH)₂ with heat. Since heterochromatin is more condensed than euchromatin (more nonhistone protein and DNA), the DNA is less accessible to the destructive forces applied and more resilient to solubilization, allowing binding of more Giemsa dye.

II. Materials

Specimen

Well-labeled, well-spread metaphase chromosome slides

Supplies

1. Coplin jars with lids
2. Unstained chromosome slides

Reagents

1. Saturated solution of Ba(OH)₂
Add Ba(OH)₂ to deionized water until it will no longer go into solution. Filter into Coplin jar just prior to use.
2. 2× SSC: 17.56 g NaCl, 8.82 g Na Citrate/1 L H₂O
3. 4% Giemsa: 48 mL Gurr's Buffer (pH 6.8) and 2 mL Giemsa blood stain
4. 70% and 95% ethanol

III. Method

1. Heat 2× SSC in a Coplin jar to 60 °C in a water bath.
2. Wash slides in 95% ethanol
3. Dip slides five to eight times in a solution of 0.9% NaCl.
4. Put slides in filtered BaOH for 30 seconds to 1 minute.
5. Rinse slides in 70% ethanol to remove BaOH.
6. Rinse slides in 0.9% NaCl.
7. Place slides in 2× SSC and incubate at 60 °C for 1.5 hours.
8. Remove slides and allow them to cool briefly.
9. Rinse slides in distilled water.
10. Stain in 4% Giemsa for 40 minutes.
11. Rinse in water and blow dry with air jet.

IV. References

1. Arrighi FE, Hsu TC. Localization of heterochromatin in human chromosomes. *Cytogenetics* 1971; 10:81–86.
2. Comings DE, Avelino E, Okada T, Wyandt HE. The mechanism of C- and G-banding of chromosomes. *Exp Cell Res* 1973; 77:469–493.
3. Holmquist G. The mechanism of C-banding: depurination and β-elimination. *Chromosoma* 1979; 72:203–224.

Protocol 6.7 C-banding of blood slides

Contributed Department of Pathology and Laboratory Medicine, Cytogenetics Laboratory, Vancouver General Hospital, Vancouver, British Columbia, Canada

Safety warning

Ba(OH)₂ powder is hazardous! Use gown, gloves, and mask and work in fume hood. Dispose of barium hydroxide solution in sealed glass bottles and take to the laboratory chemical disposal cabinet. DO NOT DISPOSE OF IT DOWN THE DRAIN!

I. Principle

C-banding of lymphocyte slides is required to further investigate a questionable variation that occurs within the centromeric region of a chromosome upon cytogenetic analysis. The principle of the C-banding technique is that when slide preparations of metaphases are denatured by heat or alkali, a marked loss of affinity for stain results. This is associated with the unpairing of the complementary DNA strands that form the chromatids. Conditions that are conducive to reassociation of the strands cause reappearance of the stain affinity. The resulting darkly stained areas along the chromatids correspond to the areas of highly repetitive DNA. In addition, chromosomes in early stages of condensation show variation in their susceptibility to denaturation compared to those in the later stages of condensation. Excess denaturation results in uniform, very faint staining and melting together of the chromosomes. Chromatids are reassociated by immersing the slide in 2× SSC at 60 °C. In general, after reassociation, only the repetitive DNA at the centromeres will be reassociated and darkly stained.

II. Materials

Supplies

1. Sodium citrate, 500 g (Fisher #S-279-500)
2. Sodium chloride (NaCl) crystal, 500 g (Fisher #S-271B)
3. Barium hydroxide [Ba(OH)₂] anhydrous, Fisher #B-47
4. Leishman's stain, BDH, 1 L (VWR #CA R03087-76)
5. Giemsa stain, Ricca, 500 mL (VWR #RC325016)
6. PO₄ Buffer solution, Harleco, pH 7.0, 1 L (VWR #CA 1219-75)
7. Sterile water (Laboratory Stores)

Equipment

1. Fume hood, Labconco Basic 47 (VWR Model #22473)
2. Water bath, isotemp (Fisher Model #10L-M)
3. Coplin jars (VWR #25457-006)
4. Forceps

Solutions

1. NaCitrate solution, 0.15 M: 4.4 g in 100 mL sterile water
2. NaCl solution, 1.5 M: 8.8 g in 100 mL sterile water
3. Stock solution, 10× SSC: combine 100 mL NaCitrate solution and 100 mL NaCl solution.
4. Working solution, 2× SSC: combine 10 mL 10× SSC stock solution and 40 mL sterile water.
5. Phosphate buffer working solution (PO₄): Combine 200 mL PO₄ buffer (Harleco) and 800 mL sterile water in empty labeled buffer bottle.
6. Stain solution; combine:
 - 6 mL of Leishman's stain
 - 3 mL of Giemsa stain, Ricca
 - 50 mL of PO₄ buffer solution
 Filter stain into Coplin jar using Nalgene filter set.

III. Method

1. Heat water bath to 60 °C.
2. Heat a Coplin jar filled with 2× SSC working solution and three Coplin jars filled with sterile water in water bath.
3. Add Ba(OH)₂ to one of the Coplin jars with 60 °C water with a spatula and mix until saturated.
4. Put slides into Ba(OH)₂ for 5–20 seconds or 1–2 minutes, depending on age of slides. Use small increments of 30 seconds when adjusting times.
5. Rinse thoroughly three times with sterile water (one dip in one of the two Coplin jars with 60 °C water and two dips in the other).
6. Incubate slides in 2× SSC working solution for 30 minutes in 60 °C H₂O bath.

7. Rinse thoroughly once with sterile water in a Coplin jar at room temperature.
8. Agitate slide in stain solution for 2–5 minutes.

Analysis

1. Scan slide to select at least two good metaphases with no crossovers in the chromosomes of interest.
2. Capture two metaphases. Do not enhance, otherwise G-banding will appear. However, if C-banding is too light, then image can be darkened.
3. Annotate prints with “C-banding” and print images.
4. Record information in the “C-banding QC logbook” table.

IV. Notes (technique tips)

1. Dry slides as you would for G-Banding.
2. Ba(OH)₂ solution is made up fresh for each batch of slides.
3. If there is a thick film at the surface of the Ba(OH)₂ Coplin jar, try removing the film with a filter paper or a clean slide.
4. The critical step is the time in Ba(OH)₂. Too short a time and all of the chromosomes will re-anneal and give a complete banding pattern (G or R bands). Too long a time and the chromosomes will be too damaged to re-anneal even at the centromeres. If this is the case, try decreasing time in Ba(OH)₂, or try lowering the temperature of water bath to 40–50 °C, or use older slides.
5. If you have extra slides to use, it may save you time if you process two or three slides at different times in Ba(OH)₂. They can then be incubated with 2× SSC together.
6. If you are using a circulating water bath, TURN IT OFF before the 2× SSC step. The motion will cause the weakened chromosomes to lift off the slide.

Protocol 6.8 Giemsa-11 staining technique

Contributed by Oregon Health & Science Center, Knight Diagnostic Cytogenetics Laboratory, Portland, Oregon

Safety warning

Azure B, eosin Y and methanol are toxic chemicals and should be handled accordingly. Avoid contact with skin.

I. Principle

This technique differentiates the centromeres of certain chromosomes, which is useful for the identification of some marker chromosomes and rearrangements. A subset of constitutive heterochromatin within C-bands is not present on all chromosomes. This Giemsa-11 (G-11) heterochromatin is carried on chromosomes 1, 4, 5, 7, 9, 10, 13, 14, 15, 17, 20, 21, 22, and Y, and sometimes on chromosomes 2 and 3. Euchromatin of chromosomes properly stained with this technique appears pale blue, while certain C-bands or portions thereof stain intensely red. The mechanism is not entirely clear. It is known that the active ingredients in Giemsa, responsible for the staining, are azure B and eosin Y. It is believed that removal of certain acidic chromosomal proteins under alkaline conditions facilitates precipitation of a red azure/eosin complex into the G-11 positive regions.

II. Materials

Specimen

Well-labeled, well-prepared, well-spread unbaked metaphase chromosome slides.
Slides more than six months old usually do not stain well.

Supplies

1. Unstained metaphase prepared slide, unbaked
2. 1 mL pipettes (Lab Stores)
3. Coplin jars

4. 37 °C water bath
5. KimWipes (Lab Stores)

Reagents

1. pHydron buffer, pH 11.0
2. Azure B
3. Eosin Y
4. Methanol Propar

III. Method

1. Prepare 100 mL of pH 11.0 buffer immediately before use. (pHydron buffer 10.99 Micro Essential Laboratory; 1 capsule makes 100 mL of buffer). Adjust pH of buffer by adding several drops of 5 M NaOH to 100 mL of pH 11.0 buffer, bringing the pH up to 11.3–11.4.
2. Divide 100 mL of buffer between two Coplin jars. Save one jar for use if needed (stain is only good for two or three slides). Place jars in 37 °C water bath for 10–15 minutes.
3. To one Coplin jar of buffer, add 0.6 mL of azure and 0.5 mL of eosin Y (1.0% azure B aqueous solution; 0.25 eosin Y in methanol). Once stains are added to buffer, this mixture has a short stain life (approximately 10 minutes).
4. Wet slide with distilled water. Remove surface scum that results with tissue. Drop slide in stain for approximately 2 minutes. Agitate occasionally.
5. Rinse slide immediately in distilled water. Blow dry with air jet. Uniformly pale blue chromosomes are undertreated. An understained slide (blue in appearance) may be further treated if done very quickly. Uniformly pink-red chromosomes are over treated. Stain time will need to be decreased and a new slide will have to be used. It is also recommended to mix new batch of stains and buffer.

IV. Notes

Chromosomes stained with G-11 are 1, ±2, ±3, 4, 5, 7, 9, 10, 13, 14, 15, 17, 20, 21, 22, and Y.

To use G-banded slides for G-11 remove any oil by rinsing slide in clean Propar. Two rinses are preferred. Air dry the slide using air jet. Destain the slide for 10 minutes in 100% methanol. Use two rinses. Stain slide according to above procedure.

V. References

1. Bobrow M, Madan K, Pearson PL. Staining of some specific regions of human chromosomes, particularly the secondary constriction of no. 9. *Nature New Biol* 1972; 238:122–124.
2. Wyandt HE, Wysham DG, Minden SK, Anderson RS, Hecht F. Mechanisms of Giemsa banding of chromosomes. I. Giemsa-11 banding with azure and eosin. *Exp Cell Res* 1976; 102:85–94.
3. Donlon T. Cytologic characterization of human constitutive heterochromatin. Master of Science in Biology Thesis. Portland State University, 1981.

Protocol 6.9 Distamycin A/DAPI staining

Contributed by Oregon Health & Science University, Knight Diagnostic Cytogenetics Laboratory, Portland, Oregon

Safety warning

Distamycin A and DAPI are both very toxic chemicals and should be handled accordingly. Avoid contact with skin and inhalation.

I. Principle

In some cases it is necessary to differentially detect the short arm of chromosome 15, particularly when there is a small satellite marker chromosome. The distamycin A/DAPI (DA-DAPI) technique is one of only a few techniques that distinguish the short arm of the acrocentric chromosome 15 from the other acrocentrics. It is particularly used when there is a small satellite

marker chromosome that requires identification. Distamycin A (DA) is an oligopeptide antibiotic. DAPI, or 4''-6-diamidino-2-phenylindole, is a fluorochrome dye. Both bind to A-T-rich DNA. DAPI used alone produces Q-bands on all chromosomes and bright C-band regions on chromosomes 1 and 16, and probably less visible C-bands on the 9, Yq, and 15p. When preparations are treated with nonfluorescent Distamycin A and then DAPI, the DAPI Q-bands are quenched and brightly fluorescent heterochromatic blocks on chromosomes 1, 9, 16, distal Y, and 15p are produced, with dull nonbanded arms on them and the other chromosomes. Distamycin A apparently binds to A-T-rich regions on the chromosome arms, suppressing DAPI fluorescence in those regions. It has been known that the two dyes are competing for similar but not identical sites on DNA. Distamycin A binds to the minor groove of DNA and the binding is strongly dependent upon DNA conformation, binding to the "B" but not the "A" or "Z" forms of DNA. The DA-DAPI-bright regions of DNA at the centromeres have been shown to be in a left-handed helix or "Z" form, which has no minor groove. Distamycin A thus cannot bind to these regions. The DA-DAPI-bright regions are known to be sites containing 5-methylcytosine and highly repetitive DNAs. DAPI seems to bind in these regions and is not quenched by Distamycin A.

II. Materials

Supplies

1. Unstained metaphase prepared slides, baked 20 minutes at 90 °C
2. Pasteur pipettes, 5¾ inches
3. Coverslips (Baxter #M6045-6)
4. Paper towels

Reagents

1. McIlvane's Buffer recipe
2. Distamycin A (0.2 mg/mL): 2 mg Distamycin A in 10 mL of McIlvane's buffer (pH 7.0). Store in the dark in the refrigerator in 2-mL plastic freezing vials.
3. DAPI (0.2 µg/mL): 2×10^{-5} g DAPI; dissolve in a few drops of methanol.

III. Method

1. Place 5–6 drops Distamycin A on slide; coverslip. Set in dark for 15 minutes.
2. Shake off coverslip and rinse with McIlvane's buffer, pH 7.0.
3. Place slide in a Coplin jar of DAPI for 15 minutes (in dark).
4. Rinse with McIlvane's buffer as above. Air dry with jet.
5. Mount in 1 : 1 glycerol/buffer.
6. Refrigeration of the slide overnight may improve quality.

Microscopy and photography

1. Use a neofluor objective and these filters: 365 exciter, 395 mirror, and 470 barrier.
2. The stained chromosomes become brighter and brighter as exposure to UV causes photodecomposition of the bound Distamycin A. Bound DAPI then fluoresces and free DAPI can bind.
3. Methyl green, a common nonfluorescent dye, has binding affinity to DNA very similar to that of Distamycin A. Identical results can be achieved using methyl green and then DAPI.

IV. References

1. Bobrow M, Madan K, Pearson, PL. Staining of some specific regions of human chromosomes, particularly the secondary constriction of no. 9. *Nature New Biol* 1972; 238:122–124.
2. Wyandt HE, Wysham DG, Minden SK, Anderson RS, Hecht F. Mechanisms of Giemsa banding of chromosomes. I. Giemsa-11 banding with azure and eosin. *Exp Cell Res* 1976; 102:85–94.
3. Donlon T. Cytologic characterization of human constitutive heterochromatin. Master of Science in Biology Thesis. Portland State University, 1981.

R-band methods

Protocol 6.10 Chromomycin/methyl green and chromomycin/distamycin fluorescent R-banding method

Contributed by Oregon Health & Science University, Knight Diagnostic Cytogenetics Laboratory, Portland, Oregon

Safety warning

Chromomycin A3 is highly toxic. May cause birth defects; do not inhale, contact with skin, or swallow. Possible mutagen. Use gloves, mask, and laboratory coat when weighing powder. Use gloves and lab coat while staining slides.

Distamycin A is toxic. Avoid skin contact and inhalation.

I. Principle

To provide an alternate banding procedure which enhances different chromosome regions than Q-banding and allows still further differentiation of certain regions of the chromosomes.

In general, Q-band pale regions of the chromosomes are heavily fluorescent with R-banding, and vice versa. Sahar and Latt demonstrated clear fluorescent R-bands by the use of chromomycin A3 and subsequent methyl green staining. The band differentiation was a result of electron energy transfer from the fluorescent chromomycin A3 to the nonfluorescent methyl green.

In this particular method, the donor, or primary stain, which is fluorescent by definition, is chromomycin A3. This dye is a GC-specific antibiotic, which, when complexed to calf thymus DNA, has an excitation (absorption) maximum at 430 nm and an emission (fluorescence) maximum at 570 nm. The use of chromomycin A3 alone results in a faint R-band pattern. Methyl green serves as the nonfluorescent acceptor counterstain in this technique. In an adaptation, distamycin A may be substituted for the methyl green. The advantage is that the fluorescent bands are quicker to "develop" and show on the microscope. Distamycin A (DA) is a nonfluorescent basic oligopeptide antibiotic that preferentially forms stable bonds to AT-rich double-stranded DNA. It does not intercalate, but it binds by hydrogen bonding and hydrophobic interaction. Distamycin A mainly binds to the chromosome arms, possibly with preference for Q-band regions. Using calf thymus DNA, the UV absorption spectrum of the distamycin A-DNA complex peaks at 340 nm.

II. Materials

Supplies

1. Unstained metaphase prepared slides, baked 20 minutes at 90–95 °C.
2. Coverslips
3. Pasteur pipettes
4. Paper towels

Reagents

1. Preparation of McIlvaine's buffer, pH 7.0
 - a. Stock solution:
 - A: 28.38 g/1000 mL of water of sodium phosphate *dibasic*. Make fresh every 1–2 months.
 - B: 21.01 g/1,000 mL of water of citric acid. Make fresh every 1–2 months.
 - b. Working solution:

For pH 7.0, add 164.7 mL stock A plus 35.3 mL stock B. Make fresh every 2 weeks. Dilute 50 mL of this McIlvaine's buffer, pH 7.0 with 50 mL distilled water. Add 0.102 g MgCl₂·6H₂O for a final concentration of 5 mm.
2. Chromomycin A3: 0.5 mg/mL chromomycin A3 (Sigma) dissolved in a few drops of methanol and then made up in the above buffer.
3. Distamycin A: 0.1 mg/mL distamycin A (Sigma) dissolved in a few drops of methanol and then made up in above buffer.
Or Methyl green (Eastman #C1767), 0.11 g/25 mL Gurr's buffer, pH 6.8 (Biomedical Specialties, Santa Monica). Wrap tightly in foil to prevent contact with light. See Protocol B.
4. Glycerol.

III. Method

A. Chromomycin/distamycin

1. Place 5 drops of chromomycin A3 on slide, add coverslip. Keep slide in the dark for 20 minutes.
2. Rinse slide with buffer and shake off excess.
3. Place 5 drops of distamycin A onto slide and a coverslip. Keep in the dark for 5 minutes.
4. Again, rinse slide, blow dry with air jet and mount the coverslip with glycerol. Be careful not to move the coverslip, as the glycerol is slippery.
5. Examine the slide on a fluorescent microscope using a 490 nm exciter, a 510 reflector, and 520 nm barrier filter or equivalent.

B. Chromomycin/Methyl Green

1. Protocol B Modified buffer for chromomycin:

0.15 M NaCl: 8.77 g/L
 0.0025 M MgCl₂: 0.24 g/L
 0.03 M KCl: 2.24 g/L
 0.01 M Na₂HPO₄: 1.42 g/L

Add all chemicals together in a liter of water and pH to 7.0.

2. Place slide on staining tray. Flood with Chromomycin solution, coverslip, and stain in dark for 20 minutes.
3. Rinse in distilled water.
4. In staining jar, add 2 mL stock methyl green to 50 mL Gurr's buffer, pH 6.8. Place slide in solution for 6 minutes. Rinse quickly in distilled water.
5. Drain and blow slide dry. Mount with a small drop of glycerin and a coverslip.
 Squeeze out excess gently.
6. For best results, wait overnight to several days for stain to "develop," keeping slide at room temperature in the dark.

The orange color is the result of the methyl green, and the yellow is the chromomycin.

The staining may need to be adjusted (time) accordingly. As the slide is exposed to the UV light, the bands will burn in and then fade out.

Tip: If the buffer is not at pH 7.0, the yellow color will appear mottled and ugly.

IV. References and suggested readings

1. Sahar E, Latt SA. Enhancement of banding patterns in human metaphase chromosomes by energy transfer. *Proc Natl Acad Sci USA* 1978; 75 (11):5650–5654.
2. Sahar E, Latt SA. Energy transfer and binding competition between dyes used to enhance staining differentiation in metaphase chromosomes. *Chromosoma* 1980;79:1–28.
3. Schweizer D. Simultaneous fluorescent staining of R-bands and specific heterochromatic regions (DA-DAPI bands) in human chromosomes. *Cytogenet Cell Genet* 1980; 27:190–193.
4. Prantera G, Bonaccorsi S, Pimpinelli S. Simultaneous production of W and R bands after staining with chromomycin A3 or oligomycin. *Science* 1979; 204:79–80.

G-band methods

Protocol 6.11 Bone marrow and cancer blood G-banding

Contributed by the Pittsburgh Cytogenetics Laboratory, Magee-Womens Hospital of UPMC, Pittsburgh, Pennsylvania

I. Principle

Aged slides containing cells fixed in the metaphase or prometaphase stage of cell division are pretreated with trypsin and stained with Giemsa. The trypsin digests certain proteins on the chromosomes and produces a characteristic pattern of light and dark bands on each chromosome that is the same in all humans. Certain chromosomes contain polymorphic regions when G-banded (i.e., satellites and qh+ regions) that may be useful in determining parental origin of chromosomes. Slides stained by the G-banding method can be analyzed for abnormalities in chromosome number or structure.

II. Reagents

Supplies

1. Four glass Coplin jars
2. Forceps
3. Scale for weighing out trypsin
4. 5 mL trypsin (1 : 250) 0.25% 1× solution (Fisher #25-050-C1] in HBBS (w/o calcium and magnesium salts and w/o sodium bicarbonate: Fisher #MT21-021-CV)
5. Diluent 2 (Hematol; Cardinal Health #B3157-12B, or equivalent, stored at room temperature)
6. Fetal bovine serum (Irvine Scientific #3003)
7. Gurr's buffer (pH 6.8 buffer tablet dissolved in 1 liter deionized water, Biomedical Specialties #33199)
8. Giemsa stain (Sigma #GS-500)
9. Deionized water
10. Hanks' Balanced Salt Solution (Fisher #MT21-021-CV or equivalent)
11. Timer

III. Method

1. Set up the Coplin jars as follows:

Jar #1: 5 mL of trypsin / 45 mL diluent 2 solution

Jar #2: 3 mL of thawed fetal bovine serum added to 47 mL of HBSS

Jar #3: 50 mL Gurr's buffer

Jar #4: 7 mL Giemsa stain added to 70 mL Gurr's buffer, mixed thoroughly

Note: Clean jars and precise attention to time exposures will give the best results.

2. Begin banding by making one or more initial test runs in order to determine the optimum trypsin and staining times. Slides on cases that have already been completed should be used for the test run(s), if available. If none are available, use a slide from a case or cases on which several slides are available for staining on that day. Prior to banding, all slides should be dried in a 60 °C oven overnight or a 90 °C oven for about 1 hour and 15 minute to 1 hour and 30 minutes prior to G-banding.
3. Trypsin time should be in the vicinity of 45 seconds, however, consult the banding log from the previous day to determine optimal amount of time in trypsin solution. Place a slide into jar #1.
4. Using forceps, remove slide from jar #1 and blot end on paper towel. Place into jar #2 for a minimum of 1 minute and blot end on paper towel.
5. Place slide into jar #3 for a minimum of 1 minute. Remove and blot end on paper towel.
6. Place slide into jar #4 for 2–7 minutes (check previous day data in logbook). Blot end on paper towel.
7. Rinse slide under running tap water.
8. Promptly blow the slides dry with the in-house air supply. Carefully evaluate the slides under the microscope noting conditions of under- or overtrypsinization and under- or overstaining.
9. Adjust the amount of time in trypsin solution or Giemsa stain solution as necessary (see troubleshooting notes) until a test slide produces acceptable banding results. Run the remaining slides through the banding protocol using times determined by test run(s). Record the trypsin and Giemsa times in the appropriate log.

Note: Each case must be carefully checked and the banding and staining times individually adjusted. Also note if a case has a suboptimal number or quality of metaphase spreads. If necessary, ask for slides to be redropped.

IV. Notes (troubleshooting)

1. If there are a large number of slides, make a fresh jar #2 part of the way through the banding.
2. If slides appear fuzzy, decrease amount of time in trypsin solution, or make a fresh jar #2 to inactivate the trypsin completely.
3. If slides appear under banded (solid stained), increase time in trypsin solution.
4. If slides appear banded but too light/dark, increase/decrease amount of time in Giemsa stain. Alternately, more/less Giemsa may be added to the staining solution and a test slide run through at the original time.

Protocol 6.12 Trypsin G-banding

Contributed by Oregon Health & Science Center, Knight Diagnostic Cytogenetics Laboratory, Portland, Oregon

I. Principle

Trypsin treatment and Giemsa staining are applied to unstained metaphases to obtain high quality chromosome banding patterns. Trypsin is postulated to cleave proteins (presumably nuclear matrix proteins), which slightly disperses the chromatin in some regions to increase Giemsa banding. The G-bands correspond to the naturally occurring chromomere pattern and are related to DNA density differences as shown by Feulgen staining. Phase microscopy and electron microscopy also suggest that G-band positive regions contain more heterochromatin than G-banded negative regions.

II. Materials

Specimen

Well-labeled, well-spread metaphase chromosome slides, baked at 90–95 °C for 20 minutes

Supplies

1. Coplin jars
2. Graduated cylinder
3. 1 mL and 5 mL pipettes

Reagents

1. Trypsin Solution
 - a. Stock solution (Gibco® 0.25%, with phenol red, without Ca²⁺, Mg²⁺ (#25200-056)
 - b. Working solution: 5 mL 0.25% trypsin and 45 mL 0.9% NaCl
2. Buffer Solution
 - a. Stock solution (100%): 1 capsule pHydron buffer in 100 mL distilled water; expiration: 6 months
 - b. Working solution (5%): 5 mL stock solution in 95 mL distilled water; expiration: 6 months
3. Wright Stain
 - a. Stock solution: 0.3–0.34 g Wright stain (Sigma 25 g, #W3000) in 200 mL methanol (0.15–0.17%). Amount to add will depend upon how dark the stain must be, and is dependent upon tissue type (usually bone marrow chromosomes take the darker stain and amniotic fluid and blood chromosomes have better banding with the lighter stain). Solution should age for 10 minutes: agitate for first minute, then let stand the remaining 9 minutes. Filter with Whatman #1 filter paper and funnel into a dark bottle to decrease exposure to light.
 - b. Working solution: Mix 1 part of stock Wright stain with 4 parts of 5% working buffer just prior to staining. Expiration: indefinite

III. Method

1. Make slides by applying about 3 drops of cell suspension to an alcohol-cleaned slide which has been covered with a thin, even film of water. After application of the cells, flood the slide with fixative and allow to air dry (see slide-making method in Chapter 2).
2. Heat slides in oven for 20 minutes at 90–95 °C.
3. Mix 5 mL 0.25% stock trypsin with 45 mL 0.9% NaCl. The pH of trypsin can be adjusted in order to control the activity of trypsin. Increase pH with 0.1 M NaOH to increase activity of trypsin, or decrease pH with 0.1 M HCl to decrease activity.
4. Dip room temperature, oven-baked slide in trypsin for 30–90 seconds. Time in trypsin is dependent on the conditions and the material being banded.
5. Rinse briefly in two changes of 0.9% NaCl.
6. Stain IMMEDIATELY for 1–3 minutes in freshly made Wright stain: 5% pHydron buffer pH 6.8: 1 part to 4 parts (1 mL: 4 mL per slide). Mix stain and buffer just prior to staining and apply to a horizontally positioned slide.
7. Rinse with distilled water and air dry with compressed air.

IV. References

- Seabright M. A rapid banding technique for human chromosomes. *Lancet* 1971; 2:971–972.
- Comings DE, Avelino E. Mechanisms of chromosome banding. VII. Interaction of methylene blue with DNA and chromatin. *Chromosoma* 1975; 51:365–381.
- Comings DE. Mechanisms of chromosome banding and implications for chromosome structure. *Ann Rev Genet* 1978; 12:25–46.

Protocol 6.13 Giemsa-trypsin banding with Wright stain (GTW) for suspension culture slides and in situ culture coverslips

Anonymous Contributor

I. Principle

The purpose of this protocol is to obtain banding patterns on chromosomes. In this procedure slides are pretreated with the protease trypsin before being stained with Wright or Giemsa stain. This technique produces a shading variation of the crossbands from light to gray to dark. These crossbands remain consistent and allow precise recognition of the individual chromosomes.

II. Materials

Specimen

All specimens received for routine chromosome analysis.

Equipment and reagents

- Dulbecco's phosphate buffered saline (1×) without calcium and magnesium (PBS), Gibco®/Invitrogen #114200-075.
Add 50 mL of PBS to a 500 mL volumetric flask. Bring to 500 mL with bottled sterile water. Mix. Label volumetric flask with date made, reagents, expiration date (see label) and initial. Store at room temperature.
- Hanks' balanced salt solution (Gibco®/Invitrogen #14170-021)
Store at room temperature. See label for expiration date.
- Fetal bovine serum (FBS) (Gibco®/Invitrogen #261140-012)
Defrost a 100 mL bottle of FBS and dispense in 3 mL aliquots in snap top tubes. Label tubes with amount, reagent, lot #, and expiration date (see label). Store in freezer.
- Trypsin-EDTA-lyophilized (Gibco®/Invitrogen #15405-012)
Reconstitute with 20 mL of sterile bottled water. Allow to stand 20 minutes before dispensing into 1 mL and 4 mL aliquots in snap top tubes. Label tubes with amount, reagent, lot #, and expiration date (from original bottle). Store lyophilized form in refrigerator, aliquots in freezer.
- Gurr's buffer solution, pH 6.8 (Gallard-Schlesing #331932)
Add 1 Gurr's tablet to a labeled 100-mL bottle and add 100 mL sterile bottled water.
Label with preparation date and expiration date (see label). Do not use until completely dissolved. Store at room temperature. Discard unused portion daily for slide procedure. Expiration of stock solution: 1 week.
- Wright stain (Sigma #W-3000)
Add 500 mL methanol to a light-tight bottle and begin stirring. Slowly add 1.5 g Wright stain to the methanol. Continue stirring overnight. Using 2 layers of filter paper, gravity-filter the stain into a clean, dry, light-tight bottle. Allow stain to age for a minimum of 10 days before testing, but it can take 3–6 months before reaching its ideal quality. Run test slides of each tissue type being banded prior to using at a staining session. Filter every month, or as needed, and allow to settle overnight before using. Store at room temperature, away from light. Expiration of stock is provided by manufacturer. Once dissolved with methanol, reagent must be kept in a flammable storage cabinet and used under the fume hood. Waste must be collected, safely stored, and discarded via flammable waste procedures. Because this stain generally improves with age, expiration may be indefinite; therefore, careful monitoring is required to determine when the quality is starting to deteriorate, generally 12–18 months from date of preparation, unless the manufacturer's expiration of the stock reagent supersedes this date.

Quality control

Each new lot of all reagents must be QC tested. A record of this testing is kept on the stain record log.

III. Method

Note: Banding should be set up daily.

Procedure for slides

Slide preparations should be aged overnight on a 60 °C slide warmer. Cells used for this procedure were harvested with a hypotonic solution that consisted of 1 part 0.8% sodium citrate to 4 parts 0.5% KCl or 1 part 0.8% sodium citrate to 3 parts 0.38% KCl.

1. Defrost 4 mL of trypsin and 3 mL of FBS at room temperature.
2. To five Coplin jars (a-e below) and one 50 mL beaker (f), add the following (see Note 1, Horizontal Slide Banding):
 - a. 50 mL phosphate buffer solution (PBS)
 - b. 50 mL PBS and 4 mL trypsin. Mix well.
 - c. 50 mL PBS and 3 mL FBS. Mix well. (see Note 2, Stock FBS Substitute)
 - d. 50 mL Gurr's buffer solution (for adding stain later)
 - e. 50 mL Gurr's buffer solution for a rinse
 - f. Sterile distilled water for a second rinse
3. Label each jar accordingly and date the first jar.
4. Pass a slide through each of the five jars as follows:
 - a. PBS: 1 minute; drain before moving to trypsin jar. Make sure timer is set for trypsin time.
 - b. Trypsin in PBS: Times in both trypsin and Wright stain depend on many variables, which require establishing with every new stock solution prepared and also verifying at every staining session. Suggested starting times are: 30 seconds for slides of cultured amniotic fluid cells, products of conception, and stimulated, routine peripheral blood slides; 25–35 seconds for unstimulated bone marrow and lymph node slides; 45–75 seconds for synchronized prometaphase cultures (see Note 3, Trypsin over/underdigested).
 - c. PBS with FBS: 1 minute, drain before placing in PBS rinse.
 - d. PBS rinse: 1 minute, drain before placing in stain.
 - e. Wright stain: Add 0.5 mL (10%) Wright stain to 49.5 mL Gurr's buffer (0.1:1 solution) just prior to adding slides. Stain slides for 1–2 minutes, depending upon test-slide results (see Note 4, Determining staining time; Note 5, Uneven staining; Note 6, Stain too dark/light; Note 7, Stain debris; and Note 8, Using Giemsa stain).
 - f. Gurr's Buffer rinse: very quick dip in Gurr's buffer.
 - g. Sterile Water: very quick dip in sterile water.
5. Lean slides against the Coplin jars in a slanted position until all slides have been removed.
6. Dry back, use forced air to remove rinse, and place on a 60 °C hotplate for 3–5 minutes until the slide appears dry. Remove slides to benchtop to cool to room temperature.
7. Slides can now be checked with a 10× dry lens before covering with a 24 × 50 mm coverslip over mounting medium.
8. Wait at least 20 minutes after mounting slides before examining with an oil lens.

Procedure for coverslips

1. Defrost 1 mL of trypsin and 3 mL of FBS at room temperature.
2. To three 50 mL centrifuge tubes add the following:
 - a. 35 mL Hanks' balanced salt solution and 1 mL trypsin warmed to 37 °C in water bath or incubator (10 day expiration – store in refrigerator)
 - b. 47 mL Hanks' balanced salt solution (HBSS) and 3 mL FBS (10-day expiration-store in refrigerator)
 - c. 8 mL Gurr's buffer solution and 0.8 mL (1 : 0.1 solution) Wright stain
(Make fresh just prior to each batch of coverslips stained; discard after each use)
3. To five small Coplin jars and one beaker add the following:
 - a. 8 mL Hanks' BSS and trypsin solution from step (a) above
 - b. 8 mL Hanks' BSS and FBS solution from step (b) above

- c. 8 mL HBSS rinse
 - d. 8 mL freshly made Gurr's buffer and Wright solution from step (c) above
 - e. 8 mL Gurr's buffer solution rinse
 - f. Fill a clean 50 mL glass beaker with sterile bottled water.
4. Label each jar and date the first.
5. Each coverslip (removed from Petri dish) should be aged on the 60 °C hotplate for 15 minutes, then baked in a large glass Petri dish lined with lens paper for 25–30 minutes at 95–96.5 °C (allow to cool at room temperature before proceeding to step 6).
6. Pass the coverslips in each of the five Coplin jars as follows:
- a. Trypsin solution: 1 minute 15 seconds
 - b. Hanks' with FBS: dip and leave 30 seconds
 - c. Hanks': 30 seconds
 - d. Wright stain: 1 minute 15 seconds (this may vary according to stain strength)
 - e. Quick dip in Gurr's buffer solution.
 - f. Quick dip in sterile distilled water.
7. Air dry coverslips vertically to avoid water stains – dab off droplets of water using a paper towel.
8. Coverslips are to be mounted immediately after they are dry with cell side against glass slide; slides are labeled with proper patient information.

IV. Notes

1. Horizontal slide banding
 - a. Lay slide flat on a staining tray.
 - b. Pipet trypsin/PBS solution onto slide, filling the surface but not overflowing off the slide; allow to digest according to the times suggested for vertical positions (time is an estimate and will need adjusting with a test slide at each session)
 - c. Tip trypsin off slide and rinse with FBS/PBS, followed with PBS.
 - d. For each slide to be stained, freshly prepare 2.5 mL Gurr's buffer solution and 0.4 mL Wright stain per slide. Mix and pipet this Gurr's/Wright stain solution onto the trypsinized slide that is laying flat on a staining rack, filling the surface but not overflowing off the slide. Stain for 50 seconds (start timer after all stain is applied to slide) to 1 minute.
 - e. Rinse with sterile water and blow off water with forced air. Allow to dry on hotplate and cool down before covering.
2. Stock FBS substitute
 - a. Sterile media that contains FBS or FCS can also be used.
 - b. FBS can also be added to Hanks' BSS, instead of PBS.
3. Trypsin over/undertreated

After banding, chromosomes should appear crisp with distinct light, gray, and dark bands.

 - a. A fuzzy appearance with too much contrast between bands indicates that the chromosomes have been over-trypsinized. Adjust time in trypsin accordingly.
 - b. Indistinct bands with too little contrast indicate that the chromosomes have been under-trypsinized. Adjust time in trypsin accordingly.
4. Determining staining time

Stain immersion time will vary per Wright stain lot and batch prepared; therefore, the optimum time range must be established before the stain can be placed into production. Time will also vary between culture methods and tissue types. Time range between lots is usually 1–2 minutes, but they have also been longer.
5. Uneven staining

If staining appears granular or uneven, gently and slowly pipette the stain solution up and down during the stain immersion step. This movement can sometimes improve stain uptake when slides or coverslips are in a vertical position.
6. Stain too dark/light
 - a. If staining is too dark, decrease amount of stain used in mixture (by ~10%) (preferred) or decrease staining time.
 - b. If staining is too light, increase the amount of stain used or increase staining time (preferred).
7. Stain debris
 - a. Discard after two batches of slides have been processed or a film appears on the surface;
 - b. Wright stain should be mixed with the buffer just prior to staining. Failure to do so could result in pervasive stain debris. Pipet from the top of the stain so as to prevent mixing up the stain sediment.
 - c. Use clean pipettes to withdraw Wright stain from the stock jar; contaminating with buffer may produce debris.

- d. If stain debris becomes a problem, filter stain and recheck stain strength. Allow stain to settle overnight after filtering to prevent stain precipitate on slides and recheck stain strength before using.
8. Using Giemsa stain

Giemsa stain (Gurr's Giemsa stain (Gallard-Schlesing #350862M) can be used instead of Wright stain; however, Wright stain seemed to work better with *in situ* coverslips of POC tissue and any other cell type that may have dense colony growth with a high degree of cytoplasm. Mixing two different brands of Giemsa, e.g., Fisher and Harleco, is another alternative that may enhance Giemsa staining results.

V. References

1. Babu A, Verma S. *Human Chromosomes Principles and Techniques*, 2nd ed., 1995; New York: McGraw-Hill, Inc.
2. Kilner HP. Rapid processing of primary embryonic tissue for chromosome banding pattern analysis. *Cytogenetics* 1972; 378:1–11.

Protocol 6.14 G-banding blood lymphocyte slides

Contributed by the Department of Pathology and Laboratory Medicine, Vancouver General Hospital, Vancouver, BC, Canada

I. Principle

The purpose of this protocol is to provide a method for G-banding cytogenetic blood lymphocyte slides. Slide preparation is important for optimal G-banding. Under phase contrast microscopy, the metaphases should be well spread and the chromosomes should appear medium gray in contrast. Before banding, slides are aged using various methods of heating (slide warmer, hot plate, etc.) in order to achieve sharp banding patterns. After aging, slides are put through a series of solutions to provide permanently stained slides with high-resolution bands for analysis.

II. Materials

Supplies

1. Trypsin EDTA 1×, 100 mL (INVITROGEN #25300-054)
2. Saline 0.9% NaCl irrigation, 500 mL
3. Leishman Stain, BDH, 1 L (VWR #CA R03087-76)
4. Giemsa Stain, BDH, 1 L (VWR #R03055-76)
5. Sterile Water
6. Nalgene filter holder set, filter 250 mL, 0.2 µm filter (Fisher #09-740-23A)
7. Pipettes, 5 mL (VWR sterile serological)
8. Transfer pipettes
9. Beaker, VWR Tripour, plastic 50 mL (VWR #13915-602)
10. Plastic slide holder
11. Coplin jar, glass (VWR #25457-006)
12. Harleco phosphate buffer (VWR #CA1219-75)

Solutions

1. Trypsin, 40 mL full-strength trypsin EDTA 1×
Dispense into a Coplin jar/plastic slide holder.
2. Saline, 40 mL full-strength saline 0.9% NaCl irrigation
Dispense into a Coplin jar.
3. Harleco Buffer Working Solution #1:
32 mL Harleco phosphate buffer
448 mL distilled water
Mix into an empty bottle labeled "Buffer." Proportion may change at the discretion of the technologist.

4. Stain Solution #1 (make fresh before use):
 - 50 mL Harleco phosphate buffer working solution #1
 - 5–6 mL Leishman stain
 - 2.5–3 mL Giemsa stain
 - Filter stain using Nalgene filter set into a Coplin jar.
5. Harleco buffer working solution #2:
 - 200 mL Harleco phosphate buffer
 - 800 mL dH₂O
 - Mix into an empty bottle labeled “Buffer.”
6. Stain Solution #2 (make fresh before use):
 - 30 mL Harleco Phosphate Buffer Working Solution #2
 - 20 mL dH₂O
 - 5–6 mL Leishman stain
 - 2.5–3 mL Giemsa stain
 - Filter stain using Nalgene filter set into a Coplin jar.

III. Method

Drying/aging lymphocyte slides

1. Slides made same day: heat on hotplate at 140 °C for approximately 1–2 minutes.
2. Slides dried overnight or longer at room temperature: heat on hotplate at 140 °C for approximately 1 minute.
3. Slides dried overnight or longer at room temperature: do not heat.

G-banding

1. To G-band, place a test slide into a series of solutions using method below.
2. Use bibulous paper to remove any film as it appears on the surface of the stain to prevent film from adhering to slide.
3. Drain and use compressed air to remove excess water.
4. Scan slide under microscope to assess the quality of G-banding.
5. Adjust trypsin/stain time accordingly for next slide.

G-banding method

1. Trypsin: 45–90 seconds
2. Saline: rinse quickly
3. Stain solution #1 or #2: agitate 15–30 seconds
4. Sterile water: rinse quickly
5. Sterile water: rinse quickly

Protocol 6.15 Cd staining

Adapted from Gustashaw K. Chromosome Stains. In: Barch M, Knutsen T, Spurbeck J, eds., The AGT Cytogenetics Laboratory Manual, 3rd ed. New York, Lippincott-Raven, 1997, pp. 283–284.

I. Principle

The Cd staining technique, as described by Eiberg, reveals two identical dots, one on each chromatid at the location of the centromere. These dots are found only on active, or functional centromeres, and are not secondary constructions. The absence of the dots indicates that the kinetochore is lost or altered and there is therefore no place for attachment of spindle fibers. This technique is useful for resolving active centromeres in Robertsonian translocations, dicentrics, ring chromosomes, and other abnormal marker chromosomes.

II. Materials

Solutions

1. Earle's balanced salt solution, pH 8.5–9.0
2. Giemsa stain (Merck #R03055)
3. Buffered phosphate, pH 6.5

III. Method

1. Store slides at room temperature for 7–9 days.
2. Incubate in Earle's BSS pH 8.5–9.0 at 85 °C for 10–45 minutes.
3. Stain in 4% Giemsa in 3×10^{-3} M buffered phosphate, pH 6.5 for 10 minutes.

IV. Reference

1. Eiberg H. New selective Giemsa technique for human chromosomes, Cd staining. *Nature* 1974; 248:55.

Protocol 6.16 CREST/CENP antibody staining

Adapted from Gustashaw K. Chromosome Stains. In: Barch M, Knutsen T, Spurbeck J, eds. The AGT Cytogenetics Laboratory Manual, 3rd ed. New York, Lippincott-Raven, 1997, pp. 283–284.

I. Principle

Sera from patients with scleroderma contain several autoantibodies that recognize nuclear antigens. Patients with the CREST variant of scleroderma show a high frequency of anticentromeric antibodies that are highly specific for centromeric proteins (CENPs) of which there are several (CENP A-F). CREST/CENP staining by immunofluorescence is used to distinguish between active and inactive centromeres in dicentric, pseudodicentric, and Robertsonian translocations. Mitotically stable dicentric chromosomes originating from translocations are functionally monocentric and the inactive centromeres lack those proteins specifically recognized by CREST antibodies. Of note is that double minutes consistently lack CENPs, evidence that they do not contain centromeres. On the other hand, minute chromosomes that persist in tumor cells and supernumerary microchromosomes in constitutional karyotypes possess fully functional centromeres.

These techniques can also be used to identify proteins associated with cell division. Cytocentrifugation is the preferred method for producing metaphase spreads when using these techniques since acidic fixation destroys or masks the protein components of chromosomes.

II. Materials

Solutions

1. Colcemid®, 10 µg/mL
2. Hypotonic solution, 0.075 M KCl
3. Potassium chromosome medium (KCM):
 - a. 120 mm KCl
 - b. 20 mm NaCl
 - c. 10 mm Tris-HCl, pH 8.0
 - d. 0.5 mm EDTA
 - e. 0.1% Triton X-100
4. Antibodies: primary (CREST/CENP) and secondary (Fluorescein isothiocyanate, FITC; or tetramethylrhodamine isothiocyanate, TRITC) conjugated, affinity-isolated goat anti-human immunoglobulin
5. Formaldehyde solution: 40% (w/v), diluted 1 : 10 in KCM just before use (final concentration 4%)
6. Hoechst 33258 or DAPI (4',6-diamidino-2-phenylindole) stock solutions: 50 µg/mL in distilled water

III. Method

Slide preparation

1. Harvest cells (peripheral blood lymphocytes, suspension cultures, or monolayer cultures) in the standard way (using Colcemid®), through 0.075 M KCl hypotonic treatment for 10 minutes at 37 °C. Transfer the cells to ice; determine the cell density of the KCl/cell suspension by counting on a hemocytometer. Adjust the concentration to a $1-2 \times 10^5$ cells/mL.
2. Using a volume of cells that contains approximately 10^5 cells, cytocentrifuge the cells onto clean slides, following the centrifuge manufacturer's instructions.
3. After cytocentrifugation, dry briefly, then immerse in a Coplin jar containing KCM at room temperature for at least 10 minutes before proceeding with the first antibody incubation.

Antibody reactions

NOTE: Do not allow the slides to dry at any stage until final fixation in formaldehyde.

1. Remove one slide from KCM and drain briefly. Using KimWipes, dry the back of the slide and the edges of the slide around the sample area, leaving approximately 5 mm wet margin around the sample.
2. Keeping the slide flat, apply 40 µL of the primary antibody (CREST/CENP) to the sample area. Cover with a small piece (1 cm^2) of Parafilm or plastic. Repeat with the other slides. Incubate in a moist chamber at room temperature for 1–2 hours.
3. Remove Parafilm with forceps. Rinse with a few milliliters of KCM, using a Pasteur pipette. Transfer the slides to a Coplin jar containing KCM for 5 minutes. Repeat with a second 5-minute wash.
4. Apply the dye-conjugated (FITC or TRITC) secondary antibody as in steps 1 and 2. Incubate for 30 minutes; repeat washes as in step 3.
5. Fix slides in a Coplin jar containing KCM/4% formaldehyde for 15 minutes at room temperature.
6. Transfer to distilled water for 5 minutes; drain and allow to dry.

Counterstaining and microscopy

1. Stain slides in a Coplin jar containing Hoechst 33258 or DAPI at 0.5 µg/mL in distilled water.
2. Rinse 5 minutes in distilled water; drain and dry.
3. Apply coverslip with a small amount of antifade/glycerol. Press out the excess.
4. Examine by fluorescence microscopy using filters appropriate for the reporter dye and counterstain chosen.

IV. Reference

1. Jeppesen P. Immunofluorescence techniques applied to mitotic chromosome preparations. In J Gosden, ed. *Methods in Molecular Biology*. Totowa, NJ, Humana Press, 1994; 253–285.

Protocol 6.17 AgNOR (silver staining)

Contributed by Oregon Health & Science Center, Knight Diagnostic Cytogenetics Laboratory, Portland, Oregon

Safety warning

Silver nitrate is toxic and can stain anything it comes in contact with, i.e., clothing, fingers, etc. Use gloves throughout the procedure.

I. Principle

The purpose of this protocol is to provide a means to identify the nucleolar organizer regions (stalk regions/NORs) of the acrocentric chromosomes, especially when involved in translocations or in marker chromosomes. NORs contain the genes for the production of 18S and 28S rRNA. Their location on the five pairs of acrocentric chromosomes (chromosomes 13,

14, 15, 21, and 22) is demonstrated by *in situ* hybridization using tritiated rRNA. The amount of hybridization, and therefore the number of rDNA genes per NOR, is not constant. These variations are heritable and are inherited in a Mendelian fashion. In the human, a theoretical maximum of 10 NORs per cell can be visualized, although six to eight is more likely.

It has been shown that the silver-staining method stains protein and reflects the activity, and not merely the presence, of rRNA genes. Using mouse-human somatic cell hybrids in which human rRNA genes were suppressed, it was determined that the absence of silver stain indicated that the human acrocentrics were not acting as nucleolar organizers in the preceding interphase in those particular cells. Howell, Denton, and Diamond [1] were the first to report the use of silver nitrate to stain the NOR regions of acrocentric chromosomes. They called their technique the Ag-SAT (satellite) technique. It involved pretreating the slides in a 10% formalin solution, staining them in ammoniacal silver stain (two different solutions), and developing them in % formalin.

Until the mid-1970s, there was controversy over whether the NOR regions were located at the satellites (p13) or the stalks (p12) of the acrocentric chromosomes. Goodpasture et al. [2] demonstrated by combined brightfield and phase microscopy that NORs are actually located at the stalks and not at the satellites of human chromosomes. This was confirmed along with the equivalency of N bands and Ag-positive NORs. Many modifications of AgNOR staining have been published. The technique used by us is a modification of Howell and Black, 1980 [3].

II. Materials

Supplies

1. Unstained metaphase prepared slides, unbaked or baked at 90 °C for 20 minutes
2. Pasteur pipettes, 5¾ inches.
3. Coverslips (Baxter #M6045-6)
4. Paper towels

Reagents

1. 50% (w/v) aqueous silver nitrate
2. Colloidal developer: Dissolve 2 g of powdered gelatin and 1 mL of formic acid into 100 mL of deionized water. Filter the silver nitrate every week or so and make up fresh colloidal developed every two weeks.
3. 0.15% Wright stain: 0.3 g of Wright (MCB) in 200 mL methanol, absolute

III. Method

1. Place 2 drops of colloidal developed on slide followed by 2 drops of the silver nitrate solution and mix carefully.
2. Add a coverslip and incubate at 65–70 °C on a slide warmer or in an oven for 1–2 minutes, or until the slide starts to turn amber.
3. Rinse slide in deionized water.

NOTE: Slides may be stained afterwards by most techniques or may simply be counterstained with 0.15% Wright stain for 1 minute.

IV. References

1. Howell WM, Denton TE, Diamond JR. Differential staining of satellite regions of human acrocentric chromosomes. *Experientia* 1975; 31:260–262.
2. Goodpasture C, Bloom SE, Hsu TC, Arrighi FE. Human nucleolus organizers: the satellites or the stalks? *Am J Hum Genet* 1976; 28:559–556.
3. Howell WM, Black DA. Controlled silver staining of nucleolus organizer regions with a protective colloidal developed: a 1-step method. *Experientia* 1980; 36:1014–1015.

Protocol 6.18 Sister chromatid exchange blood culture and staining

Contributed by Oregon Health & Science Center, Knight Diagnostic Cytogenetics Laboratory, Portland, Oregon

Safety warning

5-bromo-2'-deoxyuridine (BrdU) and acridine orange are toxic chemicals and should be handled accordingly. BrdU is a strong teratogen and mutagen. Exposure may induce genetic abnormalities and cause birth defects and/or other heritable genetic mutations. Primary routes of occupational exposure to BrdU including aerosol inhalation, ingestion, skin absorption, or accidental injection and may produce serious subacute and chronic effects including skin lesions, anemia, leukocytopenia, thrombocytopenia, and inhibition of cell growth. Acridine orange is known to be an eye and skin irritant; no information is available regarding mutagenic, carcinogenic, or teratogenic effects.

For both of these agents, avoid contact with skin, eyes, or inhalation. In case of eye contact, flush eyes with copious amounts of water for 15 minutes. For skin contact, wash skin with soap and copious amounts of water for 15 minutes. Seek medical attention if swallowed. Use gloves, lab coat, and goggles at all times.

I. Principle

Sister chromatid exchange (SCE) testing is performed to assist in the diagnosis of inherited diseases that are characterized by genomic instability and predisposition to the development of neoplasia. Patients with Bloom syndrome, for instance, have a very high frequency of exchange between sister chromatids compared to normal controls and to other chromosome breakage syndromes in the differential. The purpose of this protocol is to culture blood lymphocytes in a manner that will allow direct microscopic visualization of the interchange of DNA between sister chromatids.

Blood lymphocytes are stimulated by the mitogen phytohemagglutinin (PHA) to rapidly grow and divide such that three days of culturing is sufficient to yield material of acceptable quantity. The thymidine analog BrdU (5-bromo-2'-deoxyuridine) is added two complete cell cycles prior to harvest. The DNA that has incorporated BrdU during replication will partially quench the fluorescence of the acridine orange after the staining process. This should produce differentially stained sister chromatid under fluorescence microscopy.

II. Materials

Specimen

Lymphocytes are obtained through procurement of anticoagulated peripheral blood. Three to five mL sodium heparinized whole blood is required for optimum results. Samples should be kept at room temperature and sent to the laboratory as soon as possible for optimum viability. Samples sent by overnight mail generally yield adequate results.

Supplies

1. 15 mL Corning polypropylene tube (Laboratory Stores #64.2005)
2. Pasteur pipettes, 53/4 inches (Laboratory Stores #63.1614)
3. Pipette bulbs, 2 mL (Laboratory Stores #63.0202)
4. 5 mL sterile pipettes (Falcon #7543)
5. 10 mL sterile pipettes (Falcon #7551)
6. 1 mL sterile pipettes (Falcon #7521)
7. 25 mL sterile pipettes (Falcon #7525)
8. T-12.5 blue-capped flasks (Falcon #353018)
9. Micropipettors and sterile tips (Eppendorf)
10. Sterilizing filter unit, 115 mL, 0.2 µm (Nalgene #124-0020)

Culture media and stock reagents

1. RPMI 1640 (Gibco® #11875-051)
2. Fetal Bovine Serum (Irvine #3000)
3. Glutamine 200 mm (Gibco® #250030-016)
4. Gentamicin 50 mg/mL (BioWhittaker #17-5282)
5. Incomplete media
 - a. 50 mL fetal bovine serum
 - b. 10 mL glutamine
 - c. 0.5 mL gentamicin
 - d. 439.5 mL RPMI 1640

Mix together for a total of 500 mL. Aliquot into 250-mL sterile flask or bottle and label as incomplete media (also known as Bone Marrow Media). Store at 4 °C. Shelf life is 6 weeks.

6. Phytohemagglutinin (PHA) M-Form (Gibco® #10576-015)

As of 3/29/05, PHA comes reconstituted and frozen. To use, thaw a 10-mL vial and dispense into four 2.5 mL aliquots. Refreeze unused aliquots and thaw just before making the next batch of complete media. Aliquot the next 10 mL vial when the last 2.5-mL aliquot is used.

Add 2.5 mL of PHA-M to 250 mL of incomplete media (same as Bone Marrow media). This is now complete medium used to grow peripheral blood T- lymphocytes. Store at 4 °C. Shelf life is 4 weeks.

7. Hypotonic Solution: KCl (Mallinckrodt #6838)

Hypotonic KCl (0.075 M): add 5.59 g KCl to 1 L of deionized H₂O.

Aliquot into 100-mL bottles and store at 4 °C. Shelf life is 4 weeks. Warm to 37 °C before using.

8. Fixative

Methanol, absolute (Mallinckrodt #3016)

Glacial acetic acid (EM Science #AX0073-9)

Add 1 part glacial acetic acid to 3 parts methanol. Make fresh before each use.

9. Colcemid® 10 µg/mL (Gibco® #15210-016). Shelf life is 12 months.

10. Stock BrdU: Add 12.5 mg of BrdU to 1 mL of sterile H₂O.

11. Sorenson buffer (pH 6.8)

Combine 1000 mL of Na₂HPO₄ M/15 (9.51 g/1000 mL diH₂O) and 1000 mL of KH₂PO₄ M/15 (9.12g/1000 mL deionized water) and refrigerate. Adjust pH to 6.8. Use within 6 months.

12. Acridine orange

Mix 0.1 g of acridine orange into 1000 mL of diH₂O to produce 0.01% stain. Acridine orange is photosensitive and will not be effective if exposed to light. Always wrap containers of stain in foil to remove from light.

III. Method

1. Set up two 72-hour cultures for the patient using 5 mL Complete Media with PHA. One culture should be labeled "SCE" and one "B." A normal control should also be set up.
2. After 24 hours, add 10 µL of the stock BrdU to the "SCE" culture and wrap in foil for the remaining 48 hours.
3. Add 20 µL of Colcemid® one hour before harvest.
Harvest cells as usual (see Chapter 3, Protocol 3.1, Blood culture and harvest procedure).
Drop slides as usual (see Chapter 2, Protocol 2.1, Slide-making).
4. Immediately stain
 - a. 5 minutes acridine orange 0.01% (light sensitive).
 - b. Rinse in deionized water.
 - c. 1 minute Sorenson buffer (pH 6.8).
 - d. Apply extra Sorenson buffer to slide as coverslip media and apply coverslip.
5. Score 25 cells using the FITC fluorescent filter, counting the number of exchanges on every chromosome and recording as the total number of exchanges per metaphase (see later).

Protocol 6.19 Sister chromatid exchange fibroblast culture and staining

Contributed by Oregon Health & Science Center, Knight Diagnostic Cytogenetics Laboratory, Portland, Oregon

Safety warning

Acridine orange is known to be an eye and skin irritant. No information is available regarding mutagenic, carcinogenic, or teratogenic effects. Wear gloves and lab coat at all times when handling. Avoid contact with skin, eyes, or inhalation. In case of contact, flush eyes with copious amounts of water for 15 minutes. For skin contact, wash skin with soap and copious amounts of water for 15 minutes. Seek medical attention if swallowed.

I. Principle

Sister chromatid exchange (SCE) testing is performed to assist in the diagnosis of inherited diseases that are characterized by genomic instability and predisposition to the development of neoplasia. Patients with Bloom syndrome, for instance, have a very high frequency of exchange between sister chromatids compared to normal controls and to other chromosome breakage syndromes in the differential. The purpose of this protocol is to culture blood fibroblasts in a manner that will allow direct microscopic visualization of the interchange of DNA between sister chromatids.

Fibroblasts are cultured to yield material of acceptable quantity. The thymidine analog BrdU (5-bromo-2'-deoxyuridine) is added two complete cell cycles prior to harvest. The DNA that has incorporated BrdU during replication will partially quench the fluorescence of the acridine orange after the staining process. This should produce differentially stained sister chromatid under fluorescence microscopy. Colcemid® inhibits the formation of the spindle fibers. When added to the culture it causes mitotically active cells to be arrested in early metaphase.

II. Materials

Specimen

Ideally, tissue specimens of at least 0.5–1 mL are collected with sterile methods into closable containers with sterile culture medium supplemented with serum and antibiotics (transport medium). If transport medium is unavailable, sterile Ringer's solution (lactated or nonlactated) or sterile isotonic saline are acceptable. Specimens should be delivered to the laboratory as soon as possible (up to 2 days after collection), and should be well protected from temperature extremes. Samples sent by overnight mail generally yield adequate results. Skin samples should be deep enough to include the dermis layer.

Unacceptable specimens include dried-out, frozen, and over-heated specimens, or specimens fixed in formalin, which all cause the death of the cells and preclude culturing. In case of receipt of unacceptable specimens, notify referring physician immediately.

Supplies

1. Sterile 35 cm² Corning plastic Petri dishes
2. Sterile forceps
3. Sterile scissors
4. Sterile scalpel handles with sterile disposable blades
5. Sterile T25 tissue culture flasks (Corning #25100)
6. Sterile cotton-plugged Pasteur pipettes
7. Pipette bulbs 2 mL (Lab Stores #63.0202)
8. Sterile graduated pipettes, 1 mL, 5 mL, and 10 mL
9. Inverted microscope
10. CO₂ controlled incubator at 37 °C

Culture media and stock reagents

1. Collagenase (Worthington Type I Collagenase #LS004196), 210 units/mg. Dilute with incomplete medium (*see below*) to a final concentration of about 1 unit per mL, rounding up to the closest multiple of 10 (e.g., for 142 mg/mg, use 150 mL medium). Filter through a vacuum filtration unit with 0.2 μm pore size. Aliquot into 4 mL snap cap sterile tubes and freeze. Shelf life is one year.
2. MEM Alpha complete medium
 - a. MEM Alpha (Gibco #12561)
 - b. 20% fetal bovine serum (Irvine #3000)
 - c. 0.1% gentamicin (BioWhittaker #17-5282)
 - d. 1% L-Glutamine (Gibco #250030-016). Shelf life 14 days. Store at 4 °C.
3. Gentamicin 50 mg/mL (BioWhittaker #17-5282)
4. Incomplete media
 - a. 50 mL fetal bovine serum
 - b. 10 mL glutamine
 - c. 0.5 mL gentamicin
 - d. 439.5 mL RPMI 1640 (Gibco® #11875-051)

Mix together for a total of 500 mL. Aliquot into a 250-mL sterile flask or bottle and label as “incomplete media” (also known as bone marrow media). Store at 4 °C. Shelf life is 6 weeks.
5. Hypotonic solution: KCl (Mallinckrodt #6838)
Hypotonic KCl (0.075 M): Add 5.59 g KCl to 1 L of deionized H₂O. Aliquot into 100-mL bottles and store at 4 °C. Shelf life is 4 weeks. Warm to 37 °C before using.
6. Fixative
Methanol, absolute (Mallinckrodt #3016)
Glacial acetic acid (EM Science #AX0073-9)
Add 1 part glacial acetic acid to 3 parts methanol. Make fresh before each use.
7. Colcemid® 10 $\mu\text{g}/\text{mL}$ (Gibco #15210-016). Shelf life is 12 months.
8. Stock BrdU: add 12.5 mg of BrdU to 1 mL of sterile water.
9. Sorenson buffer (pH 6.8): Add together 1000 mL of Na₂HPO₄ M/15 (9.51 g/1000 mL diH₂O) and 1000 mL of KH₂PO₄ M/15 (9.12 g/1000 mL distilled water) and refrigerate. Adjust pH to 6.8. Use within 6 months.
10. Acridine orange: Mix 0.1 g of acridine orange into 1000 mL of distilled water to produce 0.01% stain. Acridine orange is photosensitive and will not be effective if exposed to light. Always wrap containers of stain in foil to remove from light.
11. Gas: 5% CO₂, 5% O₂, 90% N₂
12. Trypsin-EDTA 1X: Combine 2 mL Trypsin-EDTA 10X (Gibco #610-5400AG) and 18 mL HBSS 1X.

III. Method

SCE fibroblast setup and culture

There are two methods for establishing solid-tissue cultures: the *collagenase method* which dissolves the collagen and elastin fiber matrix of the sample thereby releasing the cells from the tissue, and the *explant method* in which very small minced up fragments of tissue are allowed to attach to the flask and grow from the edges out. *The collagenase method is always preferred.*

Tissue preparation

Rinse tissue in HBSS 1×. Remove any excess adipose tissue. Go to step 1 for the collagenase method, and step 2 for the explant method.

1. Collagenase method
 - a. Place tissue in one sterile 35 mm² Petri dish. Use about 5 mm² of tissue per dish. Add 4 mL collagenase to dish. Mince the tissue as much as possible to increase the total surface area of tissue being digested by the collagenase. Note time.
 - b. Incubate at 37 °C with 5% CO₂; check the sample every half hour. The smaller the pieces, the less time is required. Check tissue dissociation on an inverted scanning microscope. Tissues should appear translucent and cellular. If not, incubate further.

- c. Add cell-medium suspension to a sterile centrifuge tube and pipette vigorously to dissociate any clumps. Cap tubes and centrifuge at 1000 RPM for 10 minutes at room temperature.
 - d. Label flasks with GL#, flask #, date, tissue type, and patient name.
 - e. Decant supernatant and resuspend cell pellet in 1–2 mL of MEM Alpha complete medium, depending on the pellet size. Seed 2 mL of suspension in a T25 flask. Gas with 5% CO₂, 5% O₂, and 90% N₂ for 5–10 seconds, close tightly, and incubate at 37 °C. Check for growth, and feed with 2 mL of media at 3–5 days.
2. Explant method
- a. Transfer cleaned, appropriate tissue into Petri dish. Moisten with 1 mL of medium, and mince each tissue with scissors and/or scalpel into fragments as small as possible.
 - b. Transfer fragments with the 1 mL of medium into T25 flasks and rotate flask to allow cell suspension to cover the surface of the growth area. If there is more than 1 mL of medium, cells will not be in contact with growth surface, and may not attach. Remove excess medium if necessary.
 - c. Label flasks with GL #, flask #, date, tissue type, and patient name.
 - d. Gas with 5% CO₂, 5% O₂, 90% N₂, for 5–10 seconds; tighten cap.
 - e. Leave undisturbed in incubator for 3–5 days. Then check cultures for growth and feed as follows: if good growth is observed, pour off medium and feed with 3 mL of fresh medium. If no growth is observed, pipet off the medium and gently replace with 1 mL of fresh medium. Re-gas cultures that are not growing.
 - f. Cultures will yield better harvests if subcultured first. Subculture when 3 or more colonies are seen per flask that fill the lower-power field during microscopy and appear mitotic.

Subculturing SCE fibroblast cultures from solid tissues methods

1. Fibroblast cultures are subcultured when growth is such that when transferred, the subcultured flask reaches confluence in 3–5 days.
2. Pour off medium into biohazard waste container.
3. Rinse cells in flask twice with 2 mL HBSS 1× solution and pipet off into waste container. Add 1 mL of trypsin-EDTA 1× solution and incubate on the warming plate or in the 37 °C incubator for 1–5 minutes, until cells are rounded up and floating. Rap flask sharply on the counter top to encourage cell detachment.
4. Depending upon the mitotic activity of the cultures, select a T25 flask for rapidly dividing cells.
5. Pipet cells a few times against the culture flask to encourage single cell suspension. Pipet an appropriate volume of cells into the new T25 flask. Volume to be transferred depends upon cell density and activity, and varies from about 0.1 mL to 0.9 mL.
6. Label flask with culture designation (A, B, C, etc.), GL#, patient's name, and date, plus tissue of origin. Feed both flasks with fresh medium to bring volume to a total of 5 mL in the T25 flasks. Gas cultures and incubate at 37 °C.
7. After the study has been reported out and no further clinical work is necessary, freeze down the cultured cells (in MEM Alpha complete with DMSO at 10%) for long term storage in liquid nitrogen (see Protocol 4.28, Freezing tissue cultures (cryopreservation) in Chapter 4).

Treatment and harvest of SCE fibroblasts

1. Two fibroblast cultures (5 mL of complete MEM Alpha in T25 flask), each at 25–40% confluency, are needed to perform the testing:
 - a. SCE culture = BrdU treated
 - b. B culture = untreated control

Note: a negative and a positive control, when possible, should be run.
2. 10 µL of the stock BrdU is added 48 hours before harvest. The SCE (BrdU treated) cultures are wrapped in aluminum foil to protect against light induced degradation.
3. All cultures are grown for 48 hours at 37 °C.
4. Colcemid® (15 µL per culture) is added about 6 hours prior to harvest. Continue to incubate at 37 °C.
5. After a sufficient number of cells are rounded up and floating (visible with an inverted microscope), begin harvest.
6. Label one 15-mL centrifuge per culture: SCE, B.
7. Decant media from each culture into its own labeled 15-mL centrifuge tube.
8. Rinse each culture flask with 2 mL of HBSS 1× twice. Decant each rinse to its corresponding 15-mL centrifuge tube.

9. Add 1 mL of trypsin-EDTA solution to each culture flask. Incubate 3–5 minutes at 37 °C. Tap culture flasks to help loosen cells, if necessary.
10. Aspirate trypsinized cell suspension with pipette and transfer to corresponding 15-mL centrifuge tube.
11. Rinse each culture flask with 2 mL of HBSS 1× and transfer to corresponding 15-mL centrifuge tube.
12. Centrifuge tubes at 1000 RPM for 10 minutes at room temperature.
13. Aspirate supernatant down to approximately 0.1 mL above cell pellet.
14. Resuspend cells gently with pipet.
15. Slowly add 10 drops of hypotonic solution and mix. Slowly bring volume of hypotonic solution to 2–4 mL (depending upon the original size of the cell pellet), mix as before and let sit at room temperature for 15 minutes.
16. Add 1 mL of fresh fixative drop-wise and mix as before.
17. Centrifuge as before.
18. Aspirate supernatant down to approximately 0.1 mL above cell pellet.
19. Resuspend cells as before taking care to leave no clumps. Slowly add 1 mL of fresh fixative dropwise and mix. Bring volume to 2–4 mL (depending upon pellet size) and let sit at room temperature for 20 minutes.
20. Centrifuge as before.
21. Aspirate supernatant avoiding pellet. Add fresh fix and make slides for SCE analysis (see Slide-Making, Protocol 2.1 in Chapter 2).
22. Immediately stain
 - a. 5 minutes acridine orange 0.01% (light sensitive).
 - b. Rinse in deionized water.
 - c. 1 minute Sorenson buffer (pH 6.8).
 - d. Apply extra Sorenson buffer to slide as coverslip media and apply coverslip.
23. Score 25 cells using the FITC fluorescent filter, counting the number of exchanges on every chromosome and recording as the total number of exchanges per metaphase (see later).

Protocol 6.20 T-banding by thermal denaturation

Adapted from Gustashaw K. Chromosome Stains. In: Barch M, Knutsen T, Spurbeck J, eds., The AGT Cytogenetics Laboratory Manual, 3rd ed. New York, Lippincott-Raven, 1997, pp. 310–311.

I. Principle

Telomeric (or terminal) bands apparently represent a subset of R-bands because they are smaller than the corresponding R-bands and are more strictly telomeric. T-banding is useful for analyzing deletions or translocations that involve the terminal ends of chromosomes. The techniques use controlled thermal denaturation to produce C- and T-bands. The kinds of banding produced depend on both the pH and the type of salt employed.

II. Method

Method 1

1. Heat 94 mL of distilled water and 3 mL of phosphate buffer (pH 6.7) to 87 °C in a Coplin jar.
2. Add 3 mL of Giemsa stain.
3. Add slides to jar; stain for 5–30 minutes.
4. Rinse in distilled water, air-dry, and examine.

For fluorescent observation

1. Destain, rehydrate through a series of alcohols, rinse in distilled water.
2. Stain in acridine orange (5 mg/100 mL) for 20 minutes.
3. Rinse in phosphate buffer, mount and examine with fluorescence microscope (excitation: 450–490 nm; suppression: 515 nm).

Method 2

1. Heat a Coplin jar containing Earle's BSS, PB, or phosphate buffer to 87 °C. The pH must be adjusted to 5.1.
2. Stain with Giemsa or acridine orange as in Method 1, steps 2–7.

III. Note

Giemsa and acridine orange both show T-bands, which appear only at pH 5.1.

IV. Reference

1. Dutrillaux B. Obtention simultanee de plusieurs marquages chromosomiques sur les memes preparartions, apres traitement par le BrdU. *Humangenetik* 1975; 30:297–306.

Protocol 6.21 CT-banding

Adapted from Gustashaw K. Chromosome Stains. In: Barch M, Knutsen T, Spurbeck J, eds., The AGT Cytogenetics Laboratory Manual, 3rd ed. New York, Lippincott-Raven, 1997, pp. 310–312.

I. Principle

This method produces both C- and T-bands (CT-bands) after heat treatment at high pH using various alkaline solutions such as barium hydroxide [1]. Staining with the cationic dye “Stains-all” (4,5,4',5'-dibenzo-3,3'-diethyl-9-methyl-thiacarbocyanine-bromide) stains RNA purple, DNA blue, and proteins red. The kinds of banding that appear (G, C, CT, or heterochromatin of acrocentric short arms) depend on both the pH and the type of salt used.

II. Materials

1. Ba(OH)₂: saturated solution, pH approximately 13
2. 2× SSC (0.3 M NaCl, 0.03 M sodium citrate), pH 7.2
3. Giemsa stain in Sorensen's buffer (0.07 M KH₂PO₄, 0.07 M Na₂HPO₄)
4. Stains-All (Kodak #114-8675 or Sigma #E-9379): 0.005% in 1 : 1 formamide-water mixture, pH 8.2)

III. Method

1. Place slides in saturated Ba(OH)₂ solution at 60 °C for 10 minutes.
2. Rinse in running distilled water.
3. Incubate in 2× SSC, pH 7.2, at 60 °C for 30 minutes.
4. Rinse in running distilled water.
5. Stain with Stains-all for 10 minutes.
6. Rinse and dry.

IV. Notes

Slides should be aged for 2–3 days prior to banding. Multiple techniques can be carried out following Q banding or GTG bandings. With Q banding, fluorescent light exposure should be kept to a minimum. Trypsin-treated chromosomes require a shorter incubation period in Ba(OH)₂.

V. Reference

1. Scheres JMJC. CT banding of human chromosomes. Description of the banding techniques and some of its modifications. *Hum Genet* 1976; 33:167–174.

Protocol 6.22 Lymphocyte culture and staining procedures for late replication analysis

Adapted from Gustashaw K. Chromosome Stains. In: Barch M, Knutsen T, Spurbeck J, eds. The AGT Cytogenetics Laboratory Manual, 3rd ed. New York: Lippincott-Raven, 1997, pp. 307–309.

I. Purpose

Late replicating portions of the chromosomes generally correspond with the G-band dark regions and with the inactive X. This method may be used in research for which it is necessary for chromosomes to have inherent banding patterns that do not require enzymatic pretreatment, such as for gene mapping by FISH, or to determine which of a patient's X chromosomes is inactivated.

MECHANISM: The late replicating chromosome regions may be stained differentially by using a pulse of the thymidine analog 5-bromo-2-deoxyuridine (BrdU) into the cells at defined periods of the cell cycle and then examining the resulting chromosomes with fluorescent dyes that are sensitive to the incorporation of the BrdU and exhibit quenching of the fluorescence produced using Hoechst 33258 or Acridine Orange stains. The pulse can be timed to substitute in the chromosomes early so the early replicating regions are quenched (Terminal Thymidine Pulse method), or the pulse can be timed late in the cell cycle so the late replicating regions are quenched (Terminal BrdU Pulse method).

FudR inhibits thymidine kinase so no dTMP is produced. Uridine and deoxycytidine are required because BrdU slows uridine synthesis and feedback inhibits ribonuclease reductase. After replication banding or sister chromatid staining has been performed, they may be visualized either by staining with fluorescence or by converting the stain to a permanent dye that can be visualized using a transmitted light microscope by this method, the Fluorescence Plus Giemsa method (FPG).

II. Materials (fluorescent method)

1. Media: MEM with 17% fetal bovine serum or RPMI 1640 with 17% fetal bovine serum with antibiotics. Store at 4 °C.
2. Phytohemagglutinin, Gibco (Life Technologies) #10576-015.
3. Bromodeoxyuridine (BrdU): Sigma 5-bromo-2'-deoxyuridine, #B-5002.
Stock solution: prepare a 10^{-2} M solution in distilled water (0.03071 g/10 mL). Sterilize by filtration; aliquot and freeze. Working solution: just before use dilute 1:4 with Hanks' balanced salt solution (HBSS) to obtain a 2.5-mM solution. Store at 4 °C.
4. Fluorodeoxyuridine/uridine solution: Sigma 5-fluoro-2'-deoxyuridine, #F-0503, and Sigma uridine, #U-3750.
 - a. Stock solutions: Prepare a 10^{-3} M solution of fluorodeoxyuridine in distilled water (0.00246 g/10 mL) and a 10^{-2} M solution of uridine in distilled water (0.02442 g/10 mL).
 - b. Working solution: add 1.0 mL of fluorodeoxyuridine and 1.5 mL uridine to 47.5 mL of HBSS to obtain a working solution of 2×10^{-5} M and 3×10^{-4} M respectively. Filter sterilize. Store at 4 °C
5. 2'-deoxycytidine (Sigma #D3897)
Stock solution: Prepare a 10^{-1} M solution of deoxycytidine in distilled water (0.2272 g/10 mL). Store at 4 °C.
Working solution: Dilute 1 : 10 in HBSS to obtain a 10^{-2} M solution. Store at 4 °C.
6. Thymidine (Sigma #T1895)
Stock solution: 10^{-2} M thymidine (227.2 mg/10 mL) in distilled water. Filter sterilize; aliquot and freeze in 1-mL lots.
Working solution: 1 mL of stock thymidine in 49 mL of HBSS.
7. Phosphate buffered saline (PBS)

8.0 g NaCl
1.44 g Na_2HPO_4
0.24 g KH_2PO_4
0.2 g KCl

Add to 800 mL distilled water and adjust pH to 7.0. Bring volume to 1 L with distilled water.
8. 2× SSC sodium chloride/sodium citrate solution:
17.53 g sodium chloride
8.82 g sodium citrate
Add to distilled water and bring volume to 1 L. Adjust pH to 7.0.
9. Hoechst 33258 stain. Sigma bisBenzimide H 33258, #14530
Stock solution: 50 µg/mL. 5 mg stain in 100 mL distilled water. Prepare fresh monthly and store at 4 °C in foil-covered bottle.
Working solution: 0.5 µg/mL Add 0.1 mL of stock solution into 99.9 mL PBS.
10. McIlvaine's buffer pH 7.5:
0.2 M Na_2HPO_4 ; 28.3 g/L (anhydrous)
0.1 M citric acid: 19.2 g/L
Add 8 mL citric acid solution plus 92 mL sodium phosphate solution.
11. Giemsa stain: 4 mL Gurr's Giemsa (Gibco #10092-013) plus 96 mL Gurr's pH 6.8 buffer (Gibco #10582-013, one tablet dissolved in 1 L of distilled water). Use immediately and discard after use.
12. 15 mL polypropylene conical screw cap centrifuge tubes, sterile, Corning #430052.
13. Colcemid, 10 µg/mL (Gibco #15210040).

III. Fluorescent R- or G-bands method

A. Lymphocyte culture initiation for terminal thymidine pulse (R-band pattern, late labeled chromatin stains dark)

1. Initiate cultures

To a 15-mL Corning centrifuge tube, add 5 mL of supplemented medium, 0.1 mL of phytohemagglutinin, and 0.3–0.5 mL of whole blood or buffy coat. To this, add 0.2 mL (200 µL) of working solution of BrdU, 0.1 mL (100 µL) of working fluorodeoxyuridine and uridine solution, and 0.05 mL (50 µL) of working deoxycytidine solution. Cap tubes tightly and cover with foil to protect from light (BrdU substituted chromosomes are light sensitive and prone to breakage). Incubate cultures at 37 °C on a slant rack.

2. At 9:00 a.m. on the morning of the second day of culture (about 40 hours), centrifuge cultures for 5–10 minutes at 1000–1200 RPM.
3. Remove the BrdU medium by aspiration. Add 5 mL of prewarmed supplemented medium and mix.
4. Centrifuge as in step 2.
5. Aspirate supernatant, add 5 mL of prewarmed medium without PHA, add 0.2 mL (200 µL) of working thymidine solution, and mix thoroughly. Cover with foil and incubate cultures at 37 °C for 6 hours.
6. Add 25 µL of Colcemid and mix well. Incubate cultures at 37 °C for 30–60 minutes.
7. Harvest cultures using standard hypotonic and fixation methods. Make slides and check for metaphases on a phase contrast microscope.
8. Stain slides by the 33258 Hoechst procedure or the fluorescence plus Giemsa (FPG) procedure.
9. Examine slides using a fluorescence microscope (Hoechst) or a light microscope (FPG). 33258 Hoechst can be excited by UV light at around 350 nm, and emits blue/cyan fluorescence at around 461 nm.

B. Lymphocyte culture initiation for terminal BrdU pulse (G-band pattern, late labeled chromatin stains light)

(Optional: Thymidine synchronization can be done simultaneously for high-resolution analysis by inclusion of the steps shown in boldface.)

1. Initiate cultures:

To a 15 mL Corning centrifuge tube, add 5 mL of supplemented medium, 0.1 mL of phytohemagglutinin, and 0.3–0.5 mL whole blood or buffy coat. Cap tubes tightly; invert to mix. Incubate cultures at 37 °C on a slant rack.

2. At 5:00 p.m. after the first 48 hours in culture, add 100 µL of working solution of thymidine to each culture. Incubate overnight at 37 °C.
3. At 9:00 a.m. the next morning (approximately 64 hours after culture initiation), remove the cultures from the incubator. Wash cells in two changes of prewarmed supplemented medium (5 mL each) to remove excess thymidine. Resuspend cultures in 5 mL of supplemented medium.
4. At approximately 9:30 a.m., resuspend cells by gentle inversion of the tubes and add the following:
200 µL of working BrdU solution
100 µL of working fluorodeoxyuridine/uridine solution
50 µL of working deoxycytidine solution
5. Cover the tubes with foil. Invert to mix. Incubate cultures at 37 °C on a slant rack.
6. Approximately 4.5 hours later, add 100 µL of ethidium bromide (Sigma #E-1385, 500 µg/mL) to each tube. Return culture to incubator.
7. Add 25 µL of Colcemid to each culture 5.25 hours after the addition of BrdU (or 45 minutes after the addition of ethidium bromide). Return cultures to the incubator.
8. Harvest (see Protocol 3.1 in Chapter 3) 20–30 minutes later for a 5.5- to 6-hour pulse. (The time can be varied for studies of replication kinetics).
9. Stain slides using 33258 Hoechst or the FPG procedure.
10. Examine slides using a fluorescence microscope (Hoechst) or a light microscope (FPG). 33258 Hoechst can be excited by UV light at around 350 nm, and emits blue/cyan fluorescence at around 461 nm.

IV. Solid staining R- or G-banding method

Fluorescence plus Giemsa (FPG) staining

V. Materials

1. Phosphate buffered saline (PBS)

8.0 g NaCl
1.44 g Na₂HPO₄
0.24 g KH₂PO₄
0.2 g KCl

Add to 800 mL of distilled water and adjust pH to 7.0. Bring volume to 1 L with distilled water.
2. 33258 Hoechst:
 Stock: 0.5 µg/mL Hoechst (Sigma #14530) in distilled water
 Working: 1 part stock Hoechst:1 part PBS
3. 2× SSC sodium chloride/sodium citrate solution:
 17.53 g sodium chloride
 8.82 g sodium citrate
 Add to distilled water and bring volume to 1 L. Adjust pH to 7.0.
4. Black light (GE Black Light, F20 T12)
5. Giemsa stain: 4 mL Gurr's Giemsa (Gibco #10092-013) plus 96 mL of Gurr's pH 6.8 buffer (Gibco #10582-013, one tablet dissolved in 1 L distilled water). Use immediately and discard after use
6. Petri dishes, square, VWR #82051-066
7. Filter paper or paper towels
8. Small slide rack or rubber tubing
9. 24 × 50 mm coverslips.

VI. Method (FPG)

1. Make metaphase slides from cells that have been substituted with BrdU during replication as for sister chromatid exchange, replication banding, or late labeling X studies. Soak slides for 5 minutes in PBS.
2. Prepare square culture dishes using moist filter paper and small slide racks or tubing for slide supports.
3. Drain slides, and place them in dishes. Cover each slide with 3–4 drops of working PBS/Hoechst and coverslip them.
4. Cover the dishes, and expose them to black light approximately 4 inches from the surface of the dishes for 15–30 minutes. A cool white fluorescent light may be used overnight if a black light is unavailable.
5. Rinse the coverslips off with distilled water.
6. Place the slides in prewarmed (60–65 °C) 2× SSC for 15 minutes. Agitate the slides periodically.
7. Rinse the slides well in running distilled water.
8. Stain slides in 4% Giemsa for 7 minutes.
9. Examine and photograph as for other brightfield techniques.

Protocol 6.23 Destaining and sequential staining of slides

Adapted from Gustashaw K. Chromosome Stains. In: Barch M, Knutsen T, Spurbeck J, eds., The AGT Cytogenetics Laboratory Manual, 3rd ed. New York, Lippincott-Raven, 1997, pp. 310–312.

I. Principle

Destaining chromosomes is useful for sequential staining methods, such as G-bands to FISH or Q-bands to C-bands. Once the coverslip has been removed, if necessary, most chromosome stains are removed by 3 : 1 methanol–acetic acid fixative. Often dipping the slide in a Coplin jar of fixative 10–20 times is sufficient to remove all traces of stain, and the slide can be rinsed in additional fixative or in alcohols followed by a water rinse, if desired, or just air-dried and used for the next stain.

II. Materials

1. Alcohol prep pads (for coverslipped slides)
2. For permanently mounted coverslipped slides or unmounted stained slides with immersion oil: xylene substitute such as Anatech Ltd. Pro-Par Clearant or Richard-Allan Clear-Rite 3 or Hemo-De from Fisher.
3. Fresh 3 : 1 methanol-acetic acid fixative

III. Method

1. Strip slides of oil, coverslips, and mounting medium.
 - a. Coverslipped slides: Remove all immersion oil from coverslipped slides using one or two alcohol prep pads.
 - b. Slides mounted in water-soluble mounting media, such as DAPI counterstain or glycerin: place slides into distilled water or 2× SSC until the coverslip falls off easily.
 - c. Permanently mounted coverslips: soak off coverslip in xylene substitute. This may take several hours. Exception: if the cells have been cultured on the coverslip and mounted cell side up, do not remove the coverslips.
 - d. Slides without coverslips that have immersion oil on them: 3 changes of fresh xylene substitute 10–20 times each.
NOTE: For G-bands to FISH, time of exposure to xylene substitute must be minimized for best results.
 - e. Dry slides either by air-drying or blow-drying with pressurized air.
2. Soak slide in 3 : 1 methanol-acetic acid fixative for 30 seconds to 5 minutes with agitation until the slide no longer bleeds off stain. Some stains are not visible to the eye, such as quinacrine or 33258 Hoechst, so use the maximum time in fixative, or experiment to see what the best timing is on a test slide.
3. Air-dry or blow dry slide.
4. Proceed to the next stain.

IV. Notes

For sequential staining, the sequence in which various stains are carried out is critical to success with sequential staining. The methods that are least destructive to the chromatin should be carried out first and the most destructive methods should be last.

Least destructive methods:

Conventional Giemsa (unbanded)

Fluorescent stains such as quinacrine or Hoechst 33258

DAPI/distamycin A

BrdU banding/staining methods

More destructive methods:

G-bands by enzymatic pretreatment

R-bands by heat method

Most destructive methods:

C-banding

AgNOR (must not be attempted after trypsin since the silver stains a protein)

Cd staining

G-11 banding

FISH

T-banding

Protocol 6.24 Restaining permanently mounted slides

Adapted from an anonymous contribution in AGT Cytogenetics Laboratory Manual, 3rd ed.

Keywords: removing permanently mounted coverslips, restaining faded slides, conventionally staining previously treated slides

I. Principle

When a previously mounted slide has faded, and the mounting media is soluble in xylene, the coverslip can be removed and treated with conventional stain to restore color.

II. Materials

1. Xylene or xylene substitute (xylene is a known carcinogen; use with caution)
2. Coplin jar or staining dish
3. 5 N HCl
4. pH meter or strips
5. Bibulous paper (optional)
6. Sterile or distilled water, 1 L
7. 100% Methanol (for Leishman stain only)
8. Gurr's buffer tablets, pH 6.8 (Gibco® #10582-013) *or* NaH₂PO₄ and Na₂HPO₄.

Reagents

1. Working pH 6.8 Buffer Solution: Dissolve one Gurr's buffer tablet in 1 L water. If no Gurr's buffer tablets are available, an equivalent buffer can be made by adding 0.469 g NaH₂PO₄ plus 0.937 g Na₂HPO₄, q.s. to a liter with water. Test pH and bring to pH 6.8 for best results.
2. Stock Giemsa Stain (Gibco® #10092-013)
Working Giemsa Stain: Add 3 mL of Stock Giemsa stain to 48.5 mL of *Working pH 6.8 Buffer Solution*.
3. Stock Leishman stain (Sigma Aldrich #L6254). Leishman Stain Solution: 0.15% Stock Leishman stain in 100% methanol.
Working Leishman Stain: Add 1 part Leishman Stain Solution to 4 parts Working pH 6.8 Buffer Solution.

III. Method

Coverslip removal

Caution: Use xylene under a fume hood. Avoid skin contact; wear gloves and use forceps.

1. Check MDS of mounting medium to ensure that it is soluble in xylene. If not, follow manufacturer's procedure for removal.
2. Place mounted slide in fresh xylene (see Note 1, Xylene exposure). Staining and banding quality may be reduced with prolonged exposure to xylene.
3. Gently pry off the coverslip. Once the coverslip has been removed, rinse the slide in two changes of fresh xylene.
4. Air-dry thoroughly before proceeding.

Conventional Giemsa stain (unbanded)

1. Place slides in a Coplin jar or staining dish.
2. Prepare Working Giemsa stain and add it into the jar with the slides.
3. Stain for 7–10 minutes.
4. Decant the stain and rinse slides in two changes of distilled water.
5. Air-dry or blow-dry slides.
6. Optional: coverslip with mounting medium for permanent storage.

Conventional Leishman stain

1. Stain slides 3–5 minutes in Working Leishman Stain.
2. Rinse well with buffer. If stain is too intense, wash longer in buffer. If it is too weak, restain the slides.
3. Blot dry with bibulous paper or air-dry with an air jet.
4. Mount slides if desired in mounting medium.

IV. Note

1. Xylene exposure. The time of exposure will depend upon the freshness of the mounting medium. Coverslips that have been applied the same day should fall off in approximately 10 minutes. Slides that have been covered for one week or more may require from 2 hours to overnight soaking, depending on the mounting medium used.

CHAPTER 7

Human chromosomes: identification and variations

Helen J. Lawce and Luke Boyd

Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA

7.1 Understanding the basics

Learning to identify G-banded human chromosomes is essential for clinical and research cytogenetics technologists. Before banded methods were developed, the chromosomes were identified by their size and arm ratios. They were classified by size and morphology into groups, called the A through G groups. Because these classifications can still be useful for chromosome identification, even with subsequent development of chromosome banding patterns, they have been retained on karyograms as the international standard for karyotyping. It can take several weeks or months to become competent at classifying normal chromosomes correctly, and it can take even longer to be able to consistently identify abnormal chromosomes in many cases. However, it is a skill that most people can learn, and is analogous to learning the faces of 24 new people on sight. Recognizing and identifying rearranged or abnormal chromosomes is similar to trying to guess which person you are seeing by just looking at his glasses or his feet.

It is helpful for those in training to be able to consult with a verbal description, as well as the idiograms of the banding patterns and photographs of the banded chromosomes, while they are classifying chromosomes on a karyogram. The descriptions of the chromosomes and collection of idiograms and photographs of G-banded normal human chromosomes in this chapter are meant to assist those learning these skills. The descriptions are only meant to point out the landmark bands that attract the attention and help identify each homologue at a relatively short banding resolution of 550 bands per haploid set. For learning the banding patterns, it is usually best to concentrate on short chromosomes first, and after these patterns become second nature, the longer chromosome banding patterns will make more sense. For learning the chromosome banding patterns, refer to the photographs of varying chromosome lengths in this chapter.

7.1.1 Chromosome heteromorphisms

Differentiating chromosome variations from chromosomal abnormalities is important, because variants are not generally associated with phenotypic abnormalities. There are several classes of variants that are commonly seen in human chromosomes [1]. One involves the satellite DNA, usually clustered around the centromeric region but also found in the long arm of the Y. Since it consists of repeat DNA, and is not transcribed, there is little selection pressure towards conservation. From individual to individual, it can be present or absent, large or small, single or multipartite, and may have many different characteristics using various chromosome stains, such as DAPI, C-banding, Q-banding [2], etc.

Another type of variation is seen in certain common euchromatic rearrangements, such as the inversions of chromosomes 2 and 10 in the pericentromeric region (see Chapter 9, section 9.2.4, Inversions). Examples of common variations in the pericentric regions of all human chromosomes are portrayed in the idiograms for each chromosome group.

A third variable is the size and intensity of the satellites and the size of the stalks of the acrocentric chromosomes [3] (see D group and G group). Since stalks are the sites of nucleolar organizing regions where mRNA is manufactured, and extra mRNA is not pathogenic, these structures can translocate to almost any other chromosome arm as a normal variant. Therefore, chromosomes other than the acrocentrics (e.g., other autosomes, the Y chromosome) can also acquire satellites as a normal variant. Similarly, the long arm of the Y chromosome (Yq12) contains Q-band bright/G-band dark heterochromatin, which can also be found on other chromosomes without having any phenotypic effect, and has been seen in females as well as males. For example, it can translocate to the short arms of the 15 and 22. For an in-depth discussion of chromosome variation, see Wyandt and Tonk [1].

7.2 Description of human chromosome shapes

The following is a description of the general size and shape for each chromosome, which was essential for identification in the era before banding, but can also be useful as a basic tool for karyotyping banded cells today, especially when the banding pattern is not very distinct, such as in cancer cytogenetics. Banded chromosomes will be described later in the chapter.

7.2.1 Chromosome shapes

Chromosomes are said to be metacentric (centromere in the middle with equal length long and short arms), submetacentric (centromere placed above the middle with a short arm and a long arm), acrocentric (centromere almost at the end of the chromosome with small short arms and usually with satellites), and telocentric (centromere placed at the end, not usually seen in human chromosomes) (see Chapter 2, section 2.6.1, Chromosome abnormalities).

7.2.2 Karyogram arrangement

Submetacentric chromosomes are always arranged with the short arms uppermost, and acrocentrics are placed with the centromere at the top. The chromosomes are grouped and arranged in alphabetical order by size and morphology, with the autosomes comprising the A–G groups and the sex chromosomes separate. The sex chromosomes may be placed together at the end or separated with the autosomal group of similar morphology; either way is acceptable.

Chromosomes are banded using various pretreatments (see Chapter 6, Chromosome stains). The banding patterns are described using an international system for nomenclature [2] (see Chapter 8, ISCN: The universal language of cytogenetics).

7.2.3 A group

The largest chromosomes are in the A group (two each of numbers 1, 2, and 3). Chromosomes 1 and 3 are metacentric chromosomes, 1 being larger than 3, and the number 2 chromosomes are slightly submetacentric.

7.2.4 B group

The B group consists of two pairs of chromosomes that are large and strikingly submetacentric; the short arms are about one fourth the length of the long arms. Without banding treatment, these two chromosome pairs, numbered 4 and 5, cannot be identified individually.

7.2.5 C group

The C group contains the largest number of chromosomes and is the most difficult group to karyotype in both banded and nonbanded spreads. The C-group chromosomes are medium-sized and are all submetacentric; they are numbered from 6 to 12. The X chromosome appears similar to this group in size and shape. In the nonbanded state, these chromosomes cannot be individually identified, so they are arranged roughly in order of decreasing size, with the largest being the pair of chromosome 6 homologues. Chromosomes X and 7 are about the same size, but the X sex chromosome appears slightly more metacentric than chromosome 7, for example, the centromere of the X appears a little closer to the middle of the chromosome. Chromosome 11 is the closest to being metacentric of all the C group chromosomes, and chromosomes 9 are variable in size, depending on the length of the heterochromatic region just below the centromere. Chromosomes 8, 10, and 12 all have the centromere at a higher position on the chromosome, showing the most submetacentric shapes of the C group, with chromosome 12 being the most submetacentric of the group.

7.2.6 D group

The D group consists of three pairs of medium-sized acrocentric chromosomes, numbered 13, 14, and 15. In the nonbanded spread, all D-group chromosomes look alike. Tiny short arms with satellites are often visible and vary greatly in size and intensity of staining.

7.2.7 E group

The E group consists of three pairs of chromosomes that are about the same length as the D group chromosomes but have clearly defined short arms. The chromosome 16 pair are metacentric, while chromosomes 17 and 18 are submetacentric. Chromosome 18 has the shortest short arms in this group, making that pair the most submetacentric of the E group.

7.2.8 F group

The F group contains two pairs of small metacentric chromosomes, 19 and 20, which cannot be individually distinguished without banding treatment.

7.2.9 G Group

The G group contains two pairs of autosomes, numbers 21 and 22, and the Y sex chromosome. Chromosomes 21 and 22 are small acrocentrics that frequently have short arms with satellites, similar to the D group, but smaller. The size of the Y sex chromosome can vary, from being very similar to these two G-group chromosomes, although it does not have satellites, to being larger than chromosome 18. The short arms of the Y sex chromosome are small, but more defined than the acrocentric chromosomes, and its long arms may appear more parallel than those of chromosomes 21 and 22.

A note about satellites: Other chromosomes (autosomes, the Y chromosome) can acquire satellites as a normal variant. In addition, the short arms of the acrocentric chromosomes can acquire the heterochromatic region of the Y sex chromosome (Yqh) without an effect on the phenotype, and can be seen in females, as well as males.

7.2.10 Sex chromosomes

The normal female has two X chromosomes and no Y chromosome; therefore, the nonbanded spread has 16 chromosomes of C-group size and only four G-group chromosomes. The normal male, with only one X chromosome, has only 15 C-group chromosomes but has five G-group chromosomes because of the presence of the Y.

For a description of banded chromosome identifiers, along with both photographic and idiogram representations of each chromosome, see Figures 7.1–7.29.

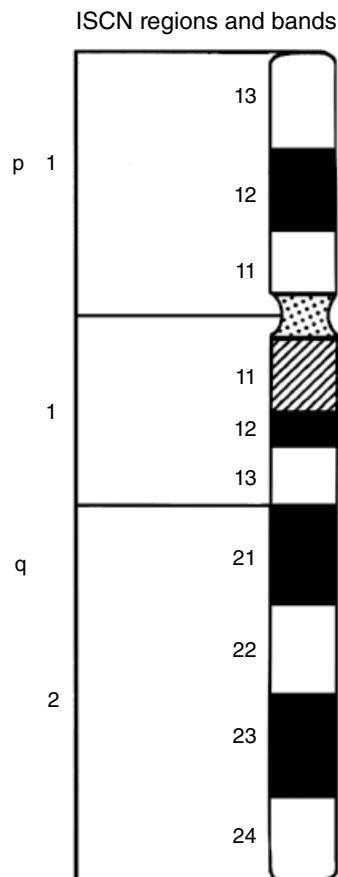


Figure 7.1 ISCN regions and bands – numbering system. This chromosome has one landmark band in the q arm, dividing the arm into two regions. Bands are numbered outward from the centromere. A two-digit code defines the region number, followed by its band number within that region. This numbering system is used when specifying breakpoints. If a break is between bands, the higher numbered band, farther from the centromere, is used. The ISCN numbering system provides a unique language that has for decades provided geneticists with the ability to accurately describe chromosome rearrangements verbally, without requiring photographs.

ISCN regions, bands, and sub-bands

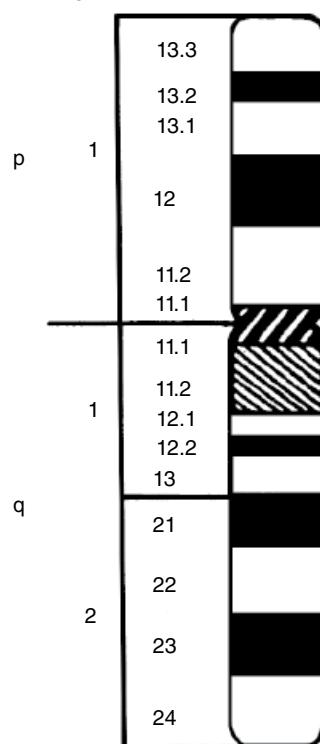


Figure 7.2 ISCN regions, bands, and sub-bands – numbering system. As shown in Figure 7.1, this chromosome is divided into regions that are defined by landmarks, and then further divided into bands. Sub-bands are noted by numbers following the decimal point: for example, 16q12.2 indicates chromosome 16 long arm, region 1, band 2, sub-band 2.

(a)

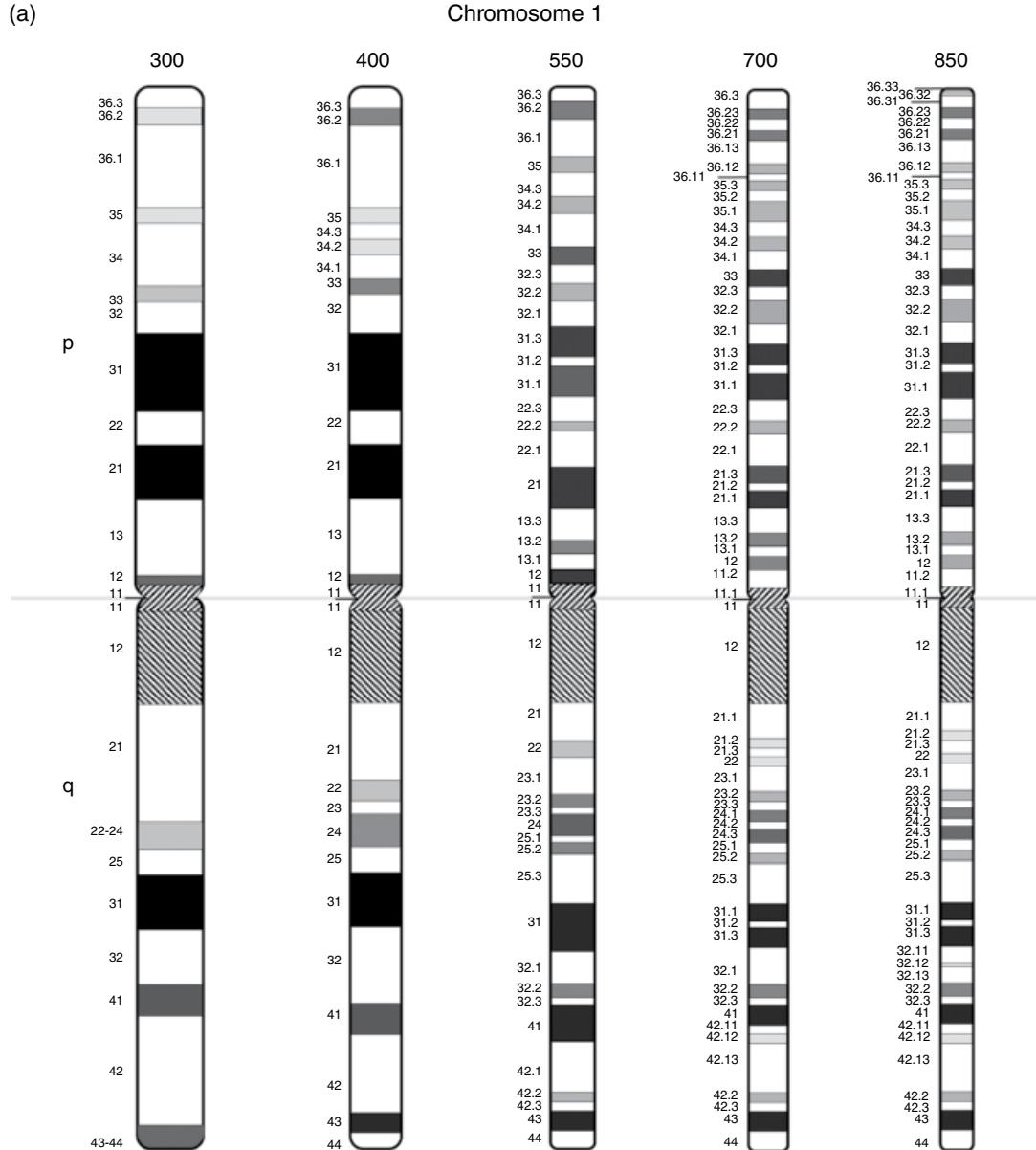
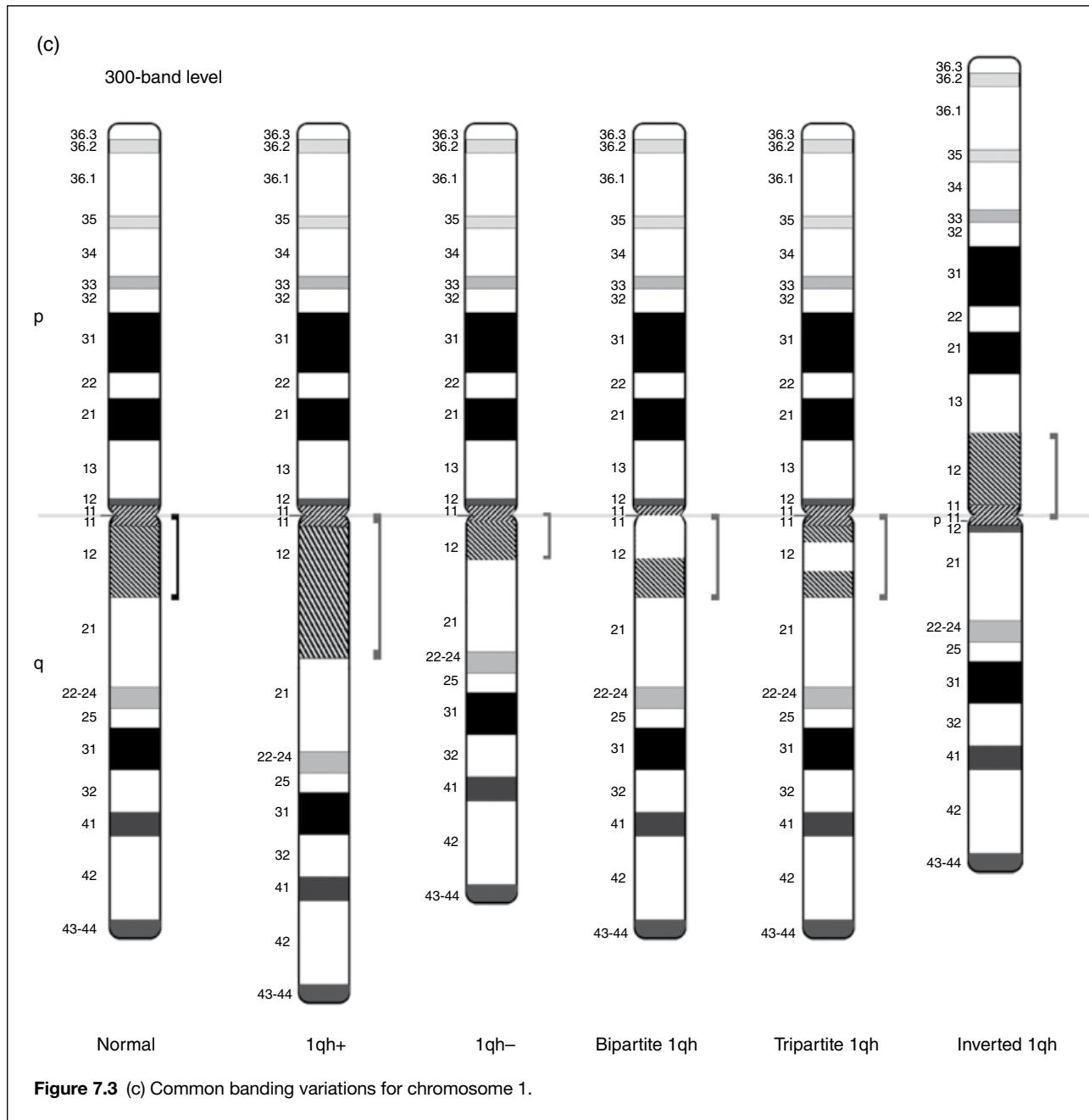


Figure 7.3 (a) ISCN diagrams of chromosome 1 at the 300-, 400-, 550-, 700-, and 850-band levels. Courtesy of Nicole Chia. ISCN diagrams throughout this chapter that show all 23 normal human chromosomes at 300-, 400-, 550-, 700-, and 850-band levels, that also indicate being a courtesy of Nicole Chia, have been provided as a courtesy of the artist. (©2009 Nicole Chia) Reproduced from *ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013*. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

(b)



Figure 7.3 (b) Examples of chromosome 1 at various band levels. Chromosome 1 (A Group): Largest metacentric. Short arm fades out to light gray at the distal end. Long arm has variable heterochromatic region (qh) just below the centromere that is G-band dark. The qh region may have several blocks of light and dark material, may vary greatly in size, or may be located in the short arm instead of the long arm. In the proximal half of the p-arm there are two distinct dark bands. The distal end of the q-arm has three evenly spaced bands, with the most proximal band being the darkest.



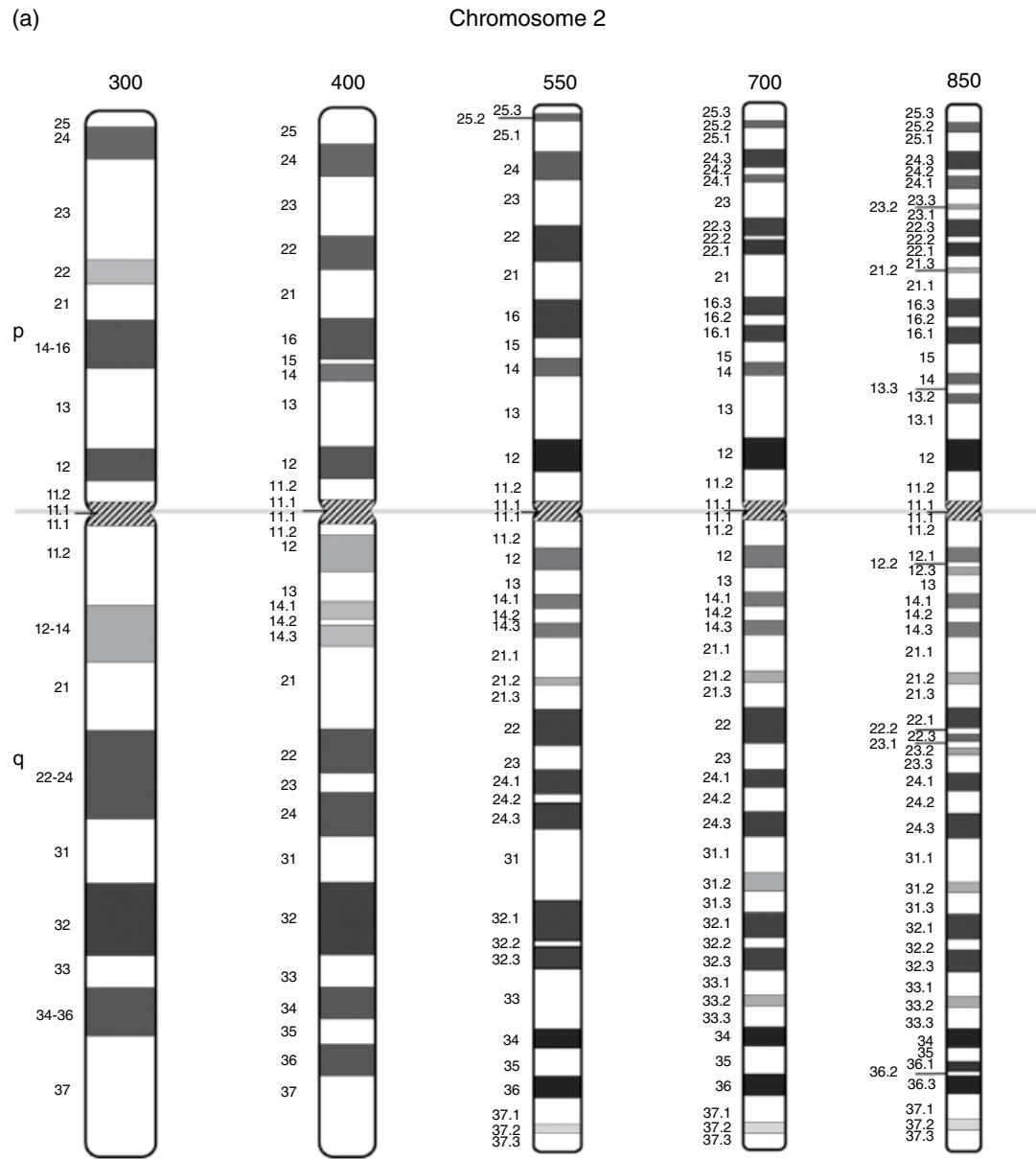


Figure 7.4 (a) ISCN diagrams of chromosome 2 at the 300-, 400-, 550-, 700-, and 850-band levels. Courtesy of Nicole Chia.



Figure 7.4 (b) Examples of chromosome 2 at various band levels. Chromosome 2 (A Group): Largest submetacentric. Larger than number 3. Proximal short arm has characteristic dark band just above the centromere, and there are four distinct dark bands spanning the short arm; proximal long arm starts with a light-staining region containing three low-density dark bands. The distal long arm has two close dark bands that can be imagined as a “window frame.”

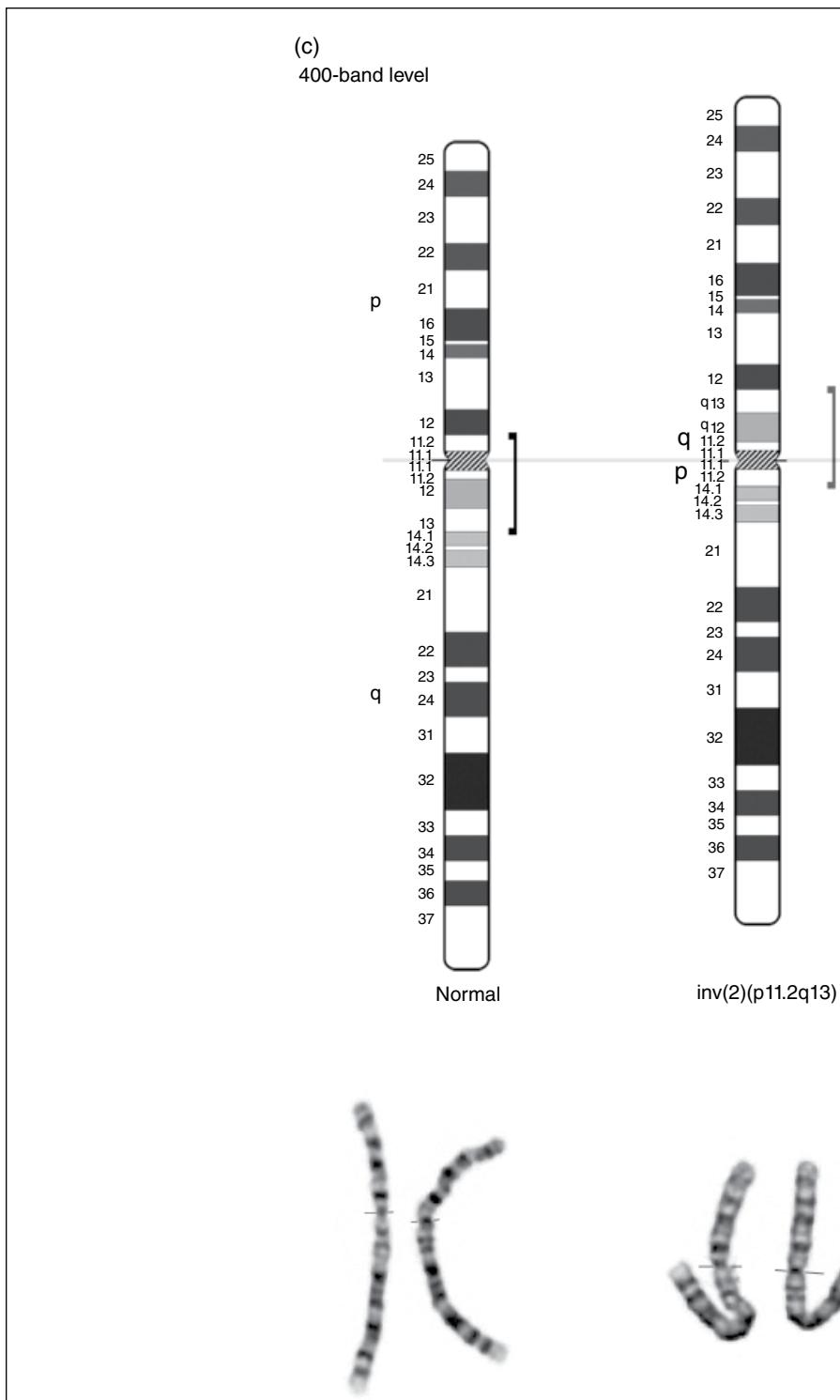


Figure 7.4 (c) The most striking normal variation is a pericentric inversion at p11.2 and q13, illustrated by two pairs of chromosome 2 with the inverted variant on the right in each pair.

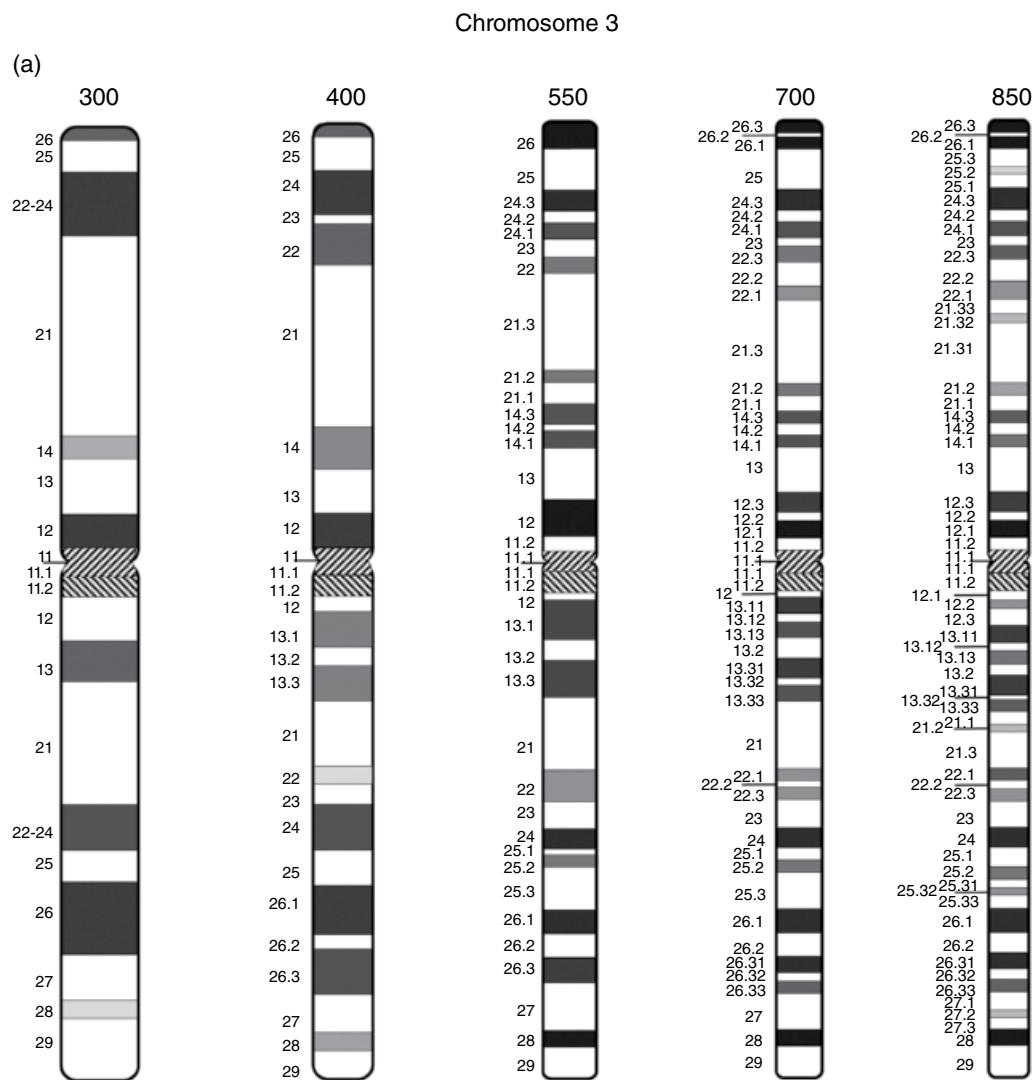


Figure 7.5 (a) ISCN diagrams of chromosome 3 at the 300-, 400-, 550-, 700-, and 850-band levels. Courtesy of Nicole Chia.



Figure 7.5 (b) Examples of chromosome 3 at various band levels. Chromosome 3 (A group): Second largest metacentric. It has a distinct dark band cap at the distal short arm. There are two light band "windows": one is central in the short arm and one is proximal to the center in the long arm.

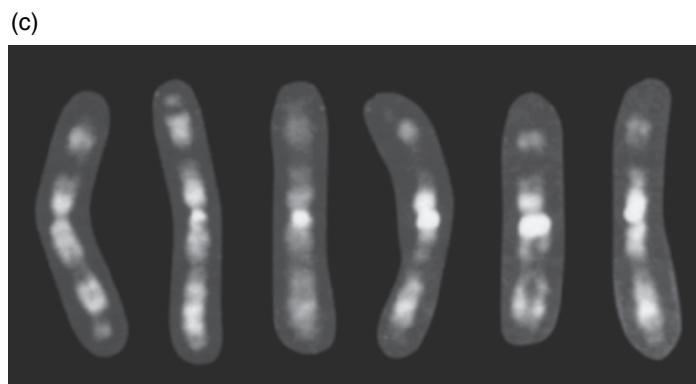


Figure 7.5 (c) Common Q-banding variations for chromosome 3. Chromosome 3 has a bright Q-banded pericentric region that may be absent or present, small or large, present on both arms or present on only the p-arm or only the q-arm. This variant is sometimes visible on G-banded chromosome 3 as a darkly staining region. Reproduced from Olson SB, Magenis RE, and Lovrien EW. Human chromosome variation: the discriminatory power of Q-band heteromorphism (variant) analysis in distinguishing between individuals, with specific application to cases of questionable paternity. *Am J Hum Genet* 1986;38:235–252 (3), with permission from the publisher.

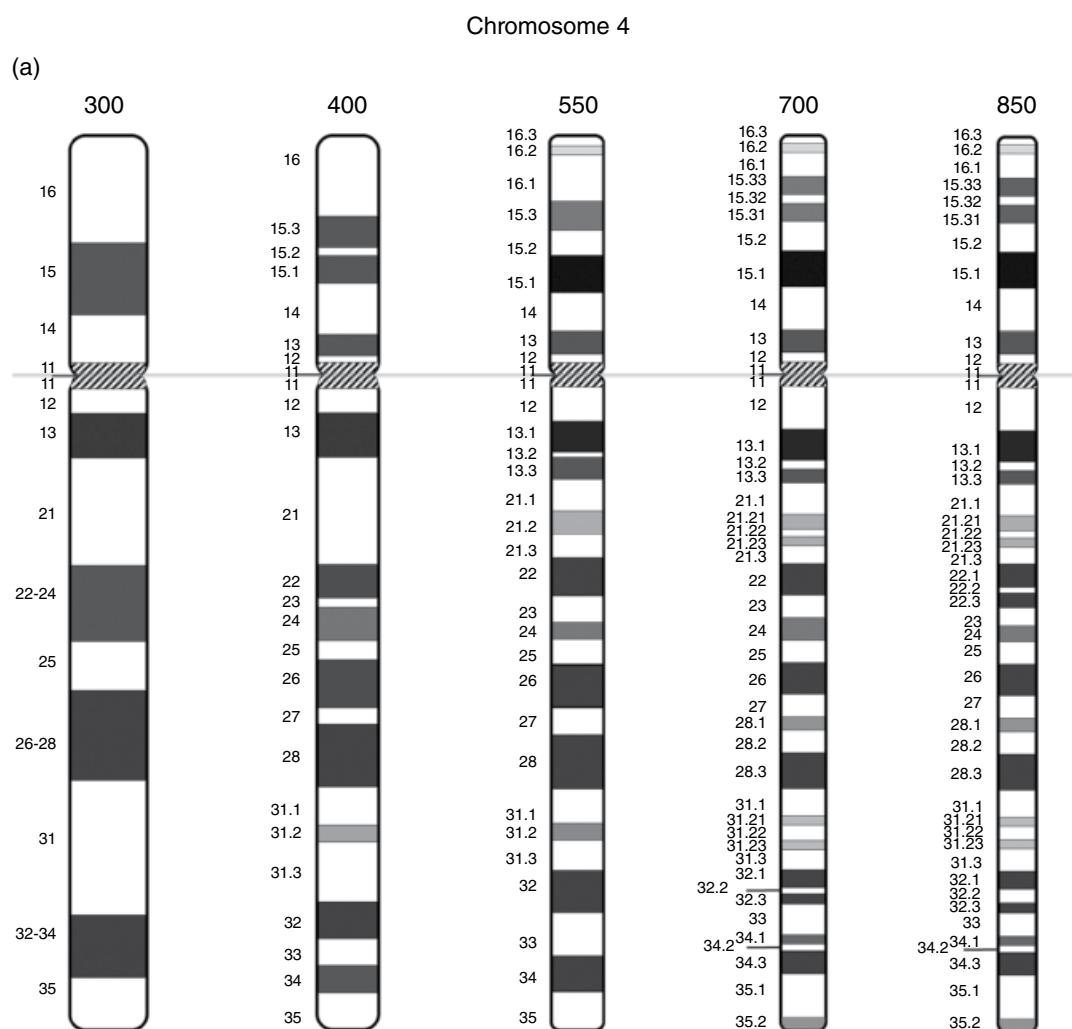


Figure 7.6 (a) ISCN diagrams of chromosome 4 at the 300-, 400-, 550-, 700-, and 850-band levels. Courtesy of Nicole Chia.



Figure 7.6 (b) Examples of chromosome 4 at various band levels. Chromosome 4 (B group): The distal short arm has a characteristic light cap, and there are two characteristic medium-density dark bands centrally located in the short arm. The proximal long arm has a characteristic dark shoulder band, followed by four closely spaced, medium-density dark bands that can blend together in shorter chromosomes. The distal long arm has two dark bands of similar density. Chromosome 4 usually appears of lower contrast overall than chromosome 5.



Figure 7.6 (c) Common Q-banding variations for chromosome 4. Chromosome 4 has a bright Q-banded pericentric region that may be large or small, dull or bright, absent or present on either the short or long arm of the chromosome. This variant is sometimes visible on G-banded chromosome 4 as a darkly staining region. Reproduced from Olson SB, Magenis RE, and Lovrien EW. Human chromosome variation: the discriminatory power of Q-band heteromorphism (variant) analysis in distinguishing between individuals, with specific application to cases of questionable paternity. *Am J Hum Genet* 1986;38:235–252, with permission from the publisher.

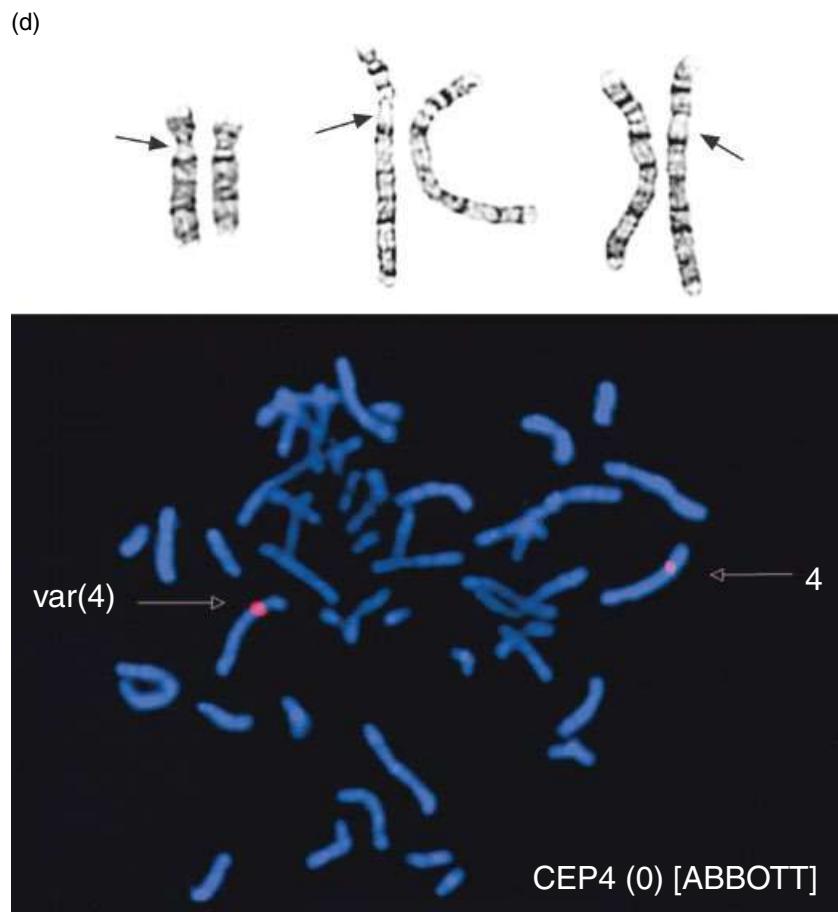


Figure 7.6 (d) Common G-banding and FISH signal variations for chromosome 4. The pericentromeric region of chromosome 4 may show variations with FISH probes for the alpha satellite region. The smaller CEP 4 signal is the more commonly seen variation and the larger signal can be seen as a larger pericentromeric region on the G-banded metaphase chromosomes (the arrowed homologue in each pair). Contributed by Cedars Sinai Medical Center Cytogenetics Laboratory. See insert for color representation of this figure.

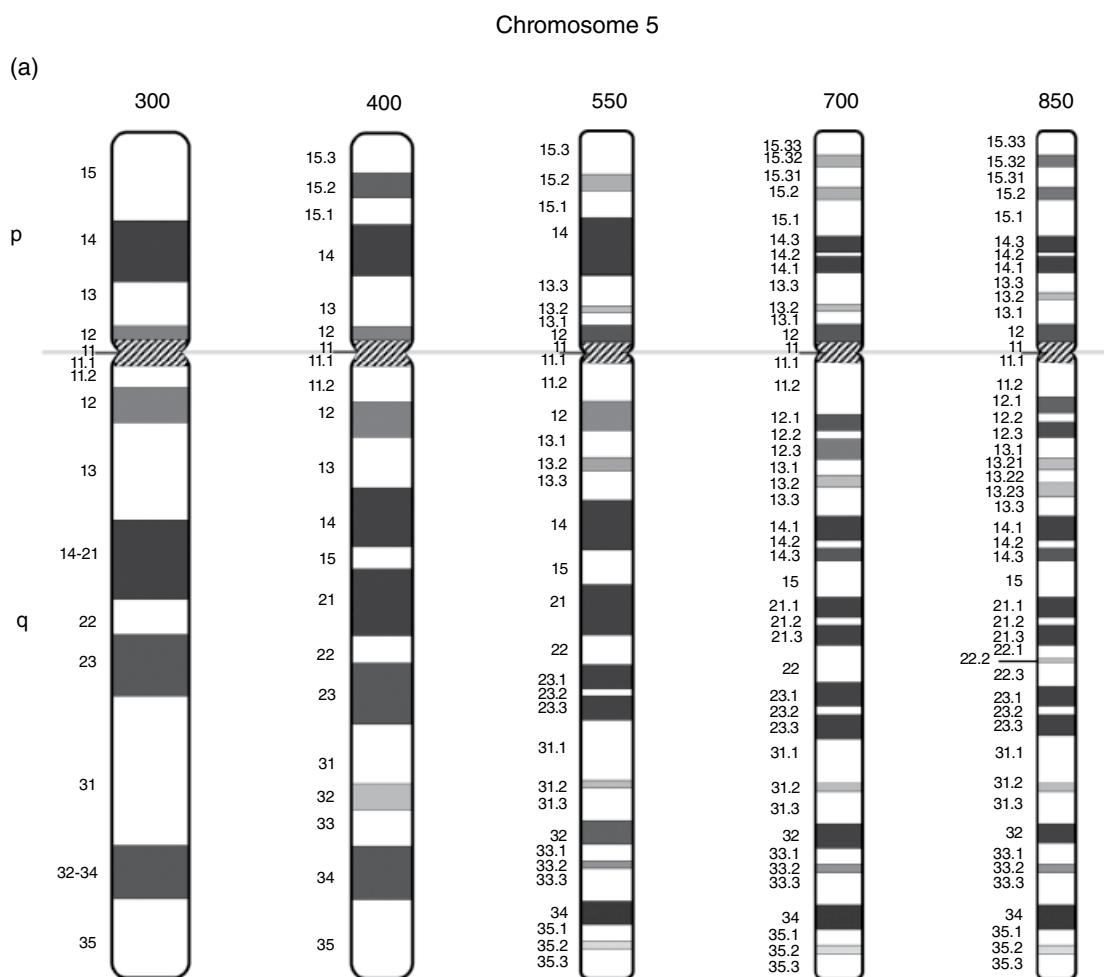


Figure 7.7 (a) ISCN diagrams of chromosome 5 at the 300-, 400-, 550-, 700-, and 850-band levels. Courtesy of Nicole Chia.



Figure 7.7 (b) Examples of chromosome 5 at various band levels. Chromosome 5 (B group): The short arm has a distinct, high-density dark band centrally. The long arm has three, closely spaced, medium-density dark bands that may blend together. The distal long arm has two dark bands of different density, with the most distal being the darkest. While rare, chromosome 5 can be missing all or part of band p14 in normal individuals.

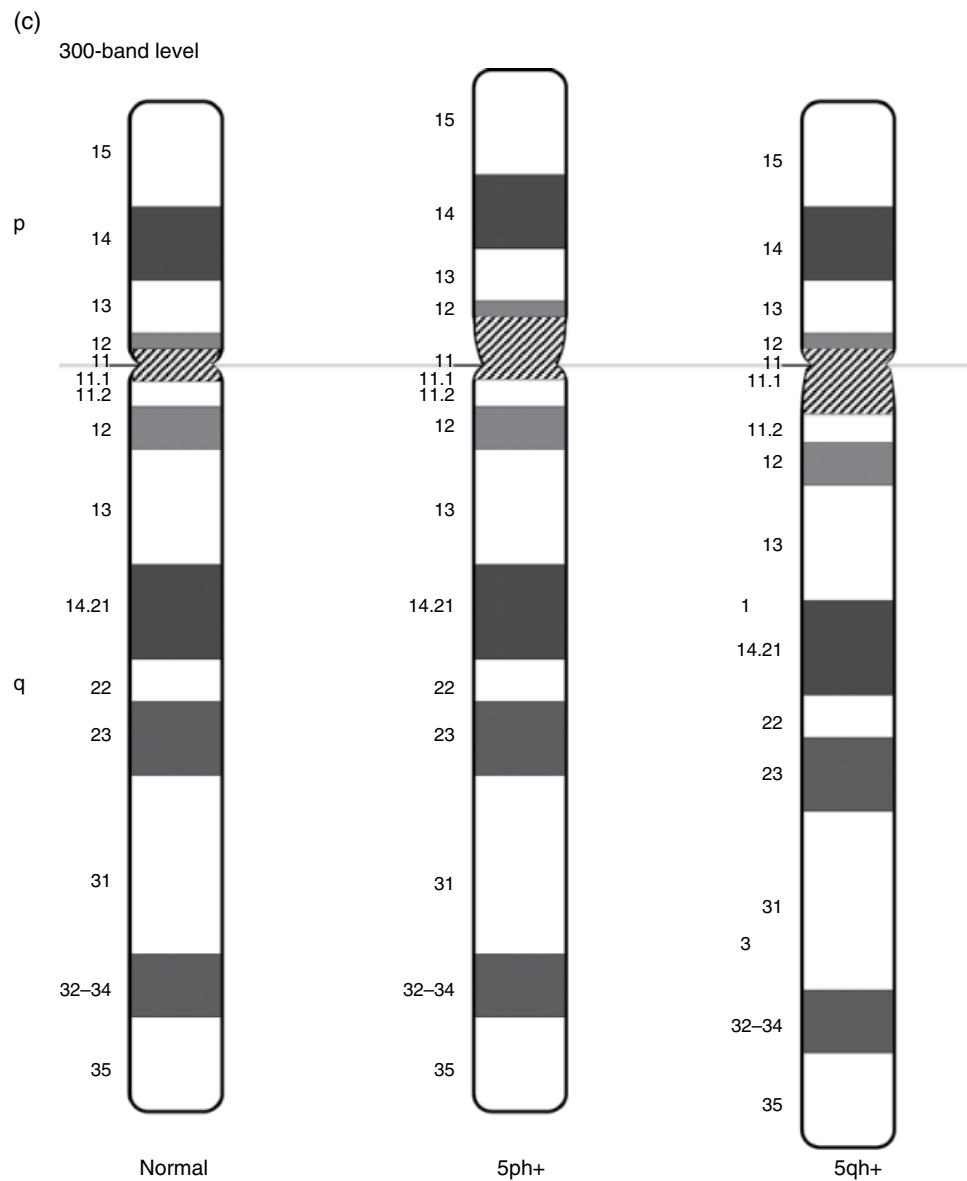


Figure 7.7 (c) Common banding variations for chromosome 5.

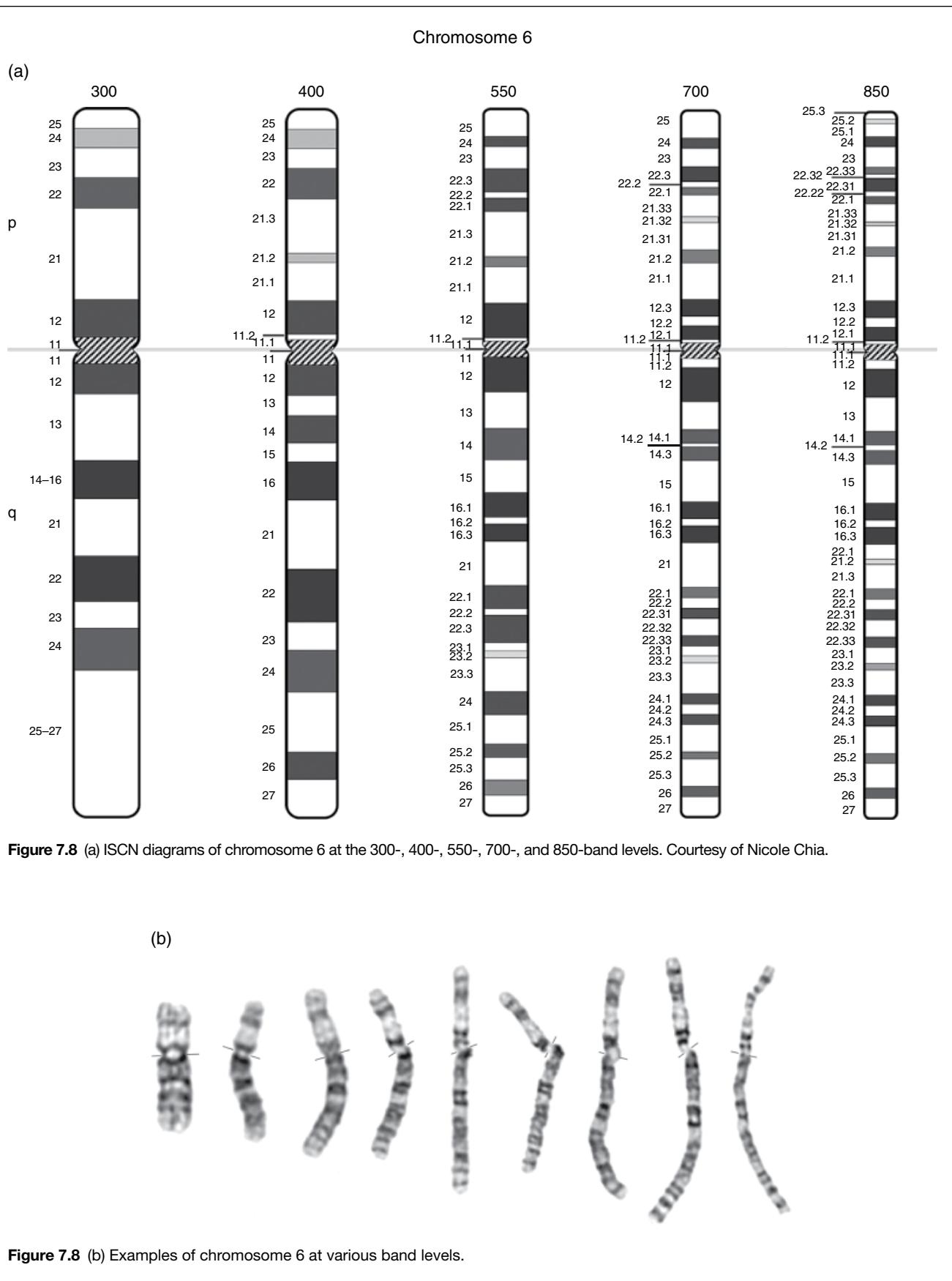


Figure 7.8 (a) ISCN diagrams of chromosome 6 at the 300-, 400-, 550-, 700-, and 850-band levels. Courtesy of Nicole Chia.

Figure 7.8 (b) Examples of chromosome 6 at various band levels.

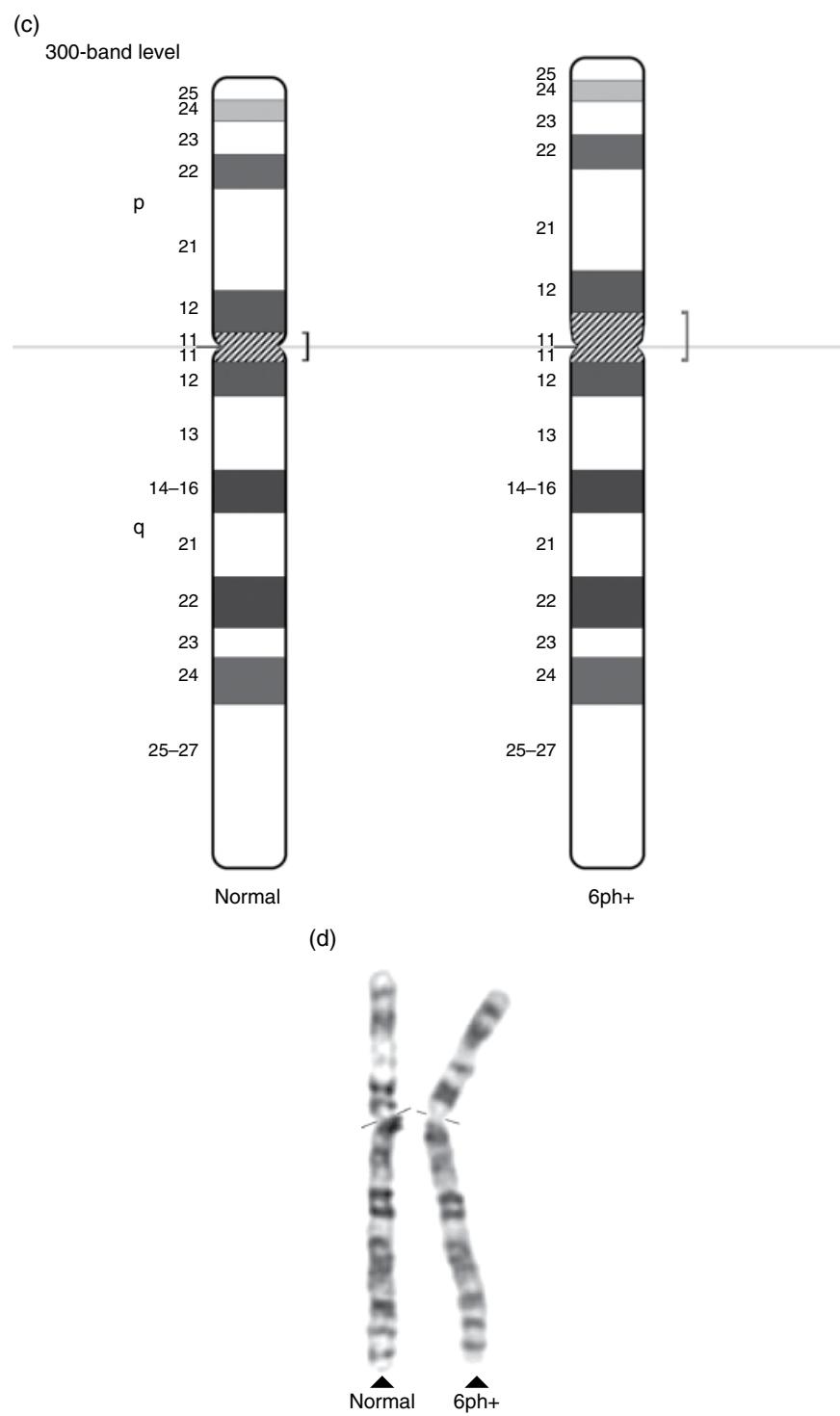


Figure 7.8 (c) Chromosome 6 (C-group): One of the three largest chromosomes in this group, similar in size and morphology (submetacentric) to chromosomes 7 and X. It has a short arm to long arm ratio of 1 : 2. There is a characteristic light gap (window) in the middle of the short arm. The long arm has several dark bands, with two centrally located high-density dark bands and three terminal dark bands that may not be visible in short or poorly banded material. (d) A common banding variation for chromosome 6, light G-band heterochromatin in the proximal short arm.

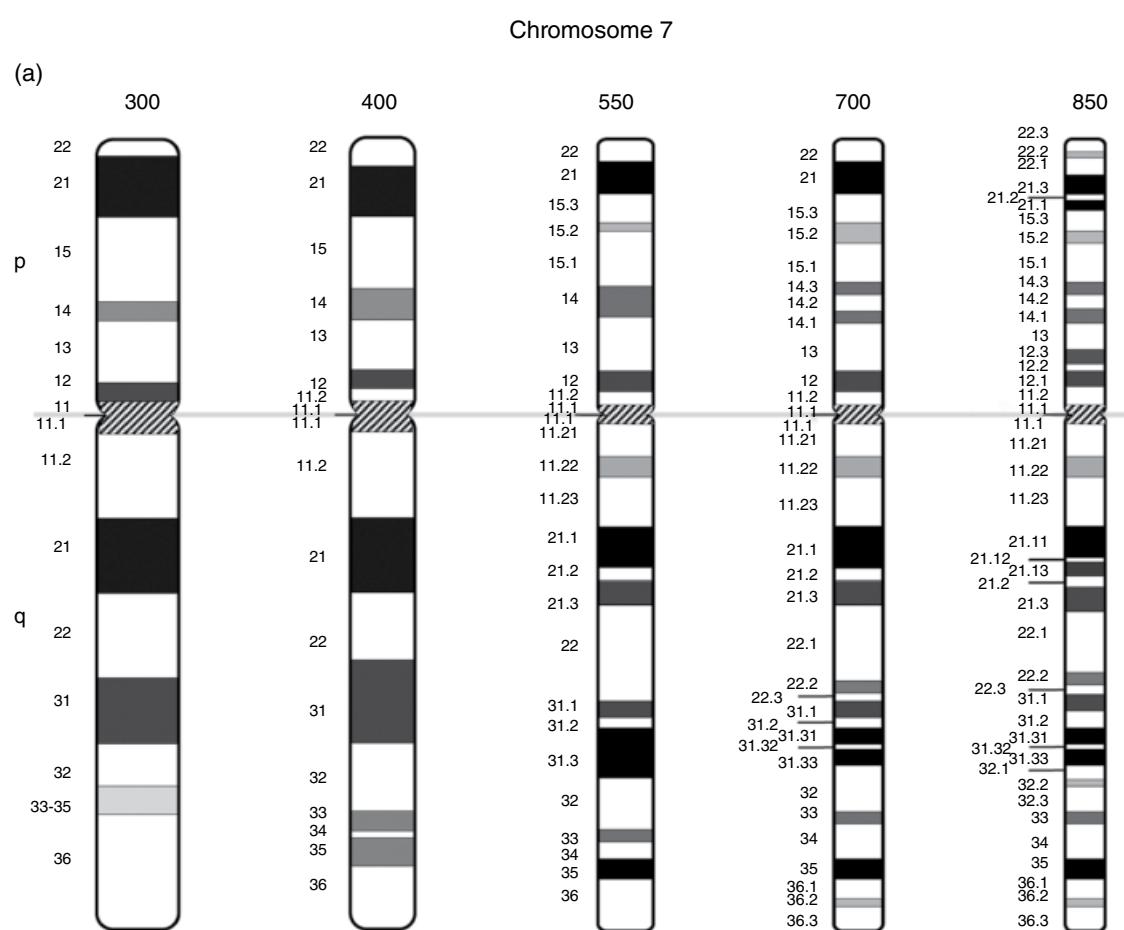


Figure 7.9 (a) ISCN diagrams of chromosome 7 at the 300-, 400-, 550-, 700-, and 850-band levels. Courtesy of Nicole Chia.



Figure 7.9 (b) Examples of chromosome 7 at various band levels. Chromosome 7 (C Group): One of the three largest chromosomes in the C-group. Similar in size and morphology (submetacentric) to the 6 and the X. Because of the size and density of its dark bands, it has a characteristic contrasting appearance. The short arm has a prominent dark, almost-distal band followed by a “white” cap. The long arm has two very prominent large dark bands, one about 1/3 and one about 2/3 of the way down the arm.

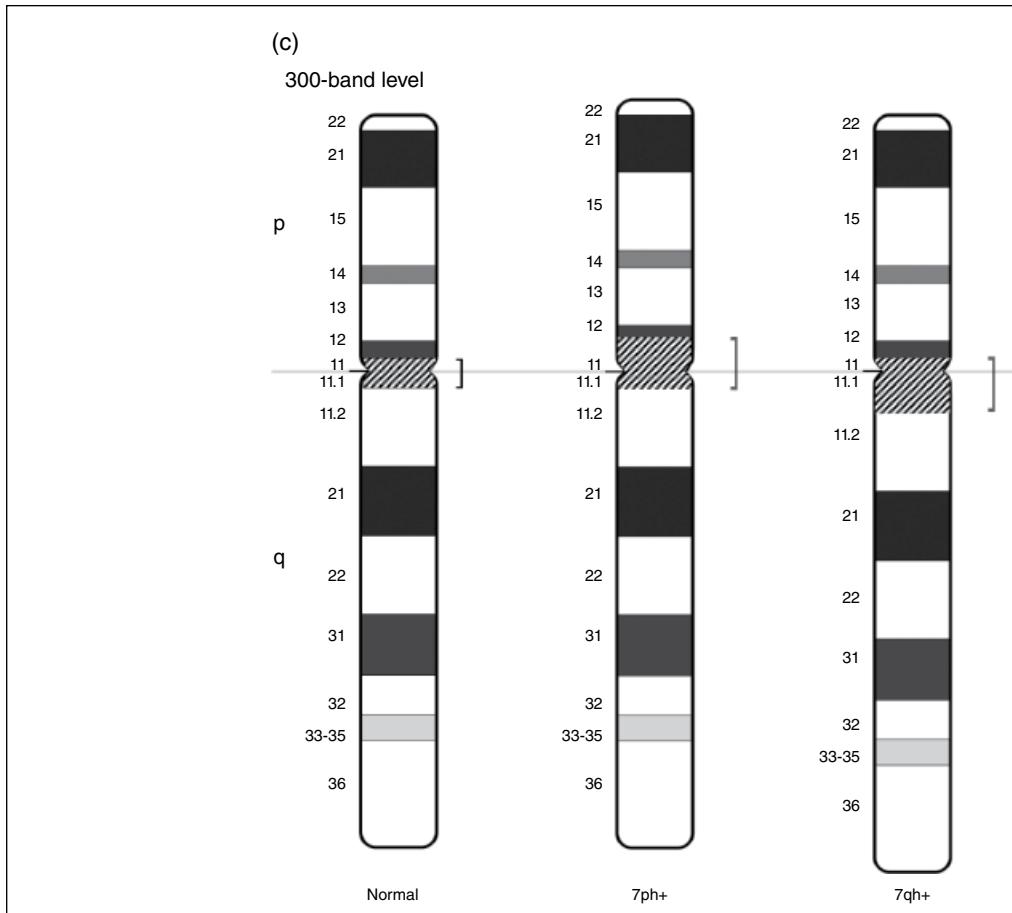
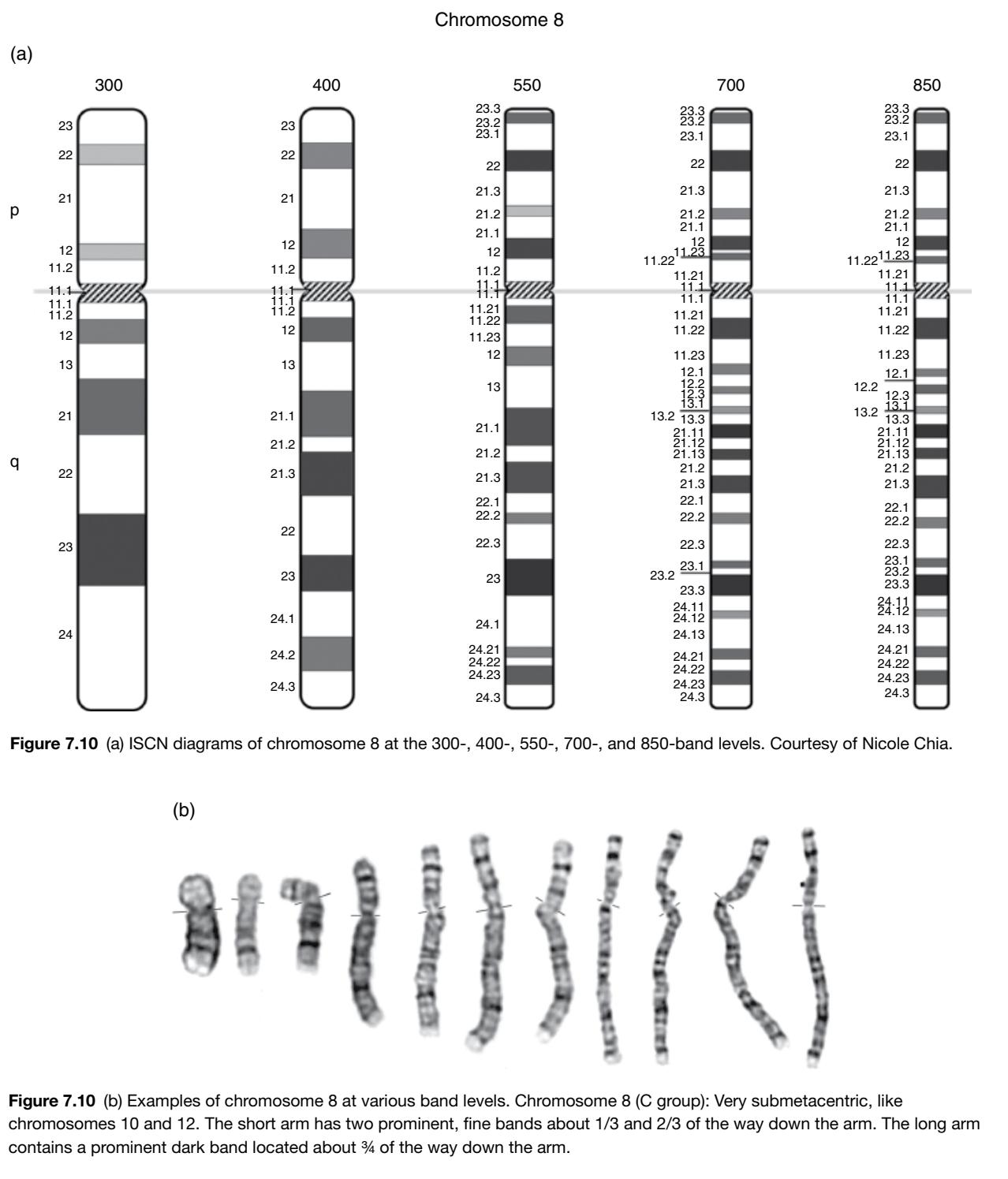


Figure 7.9 (c) Common banding variations for chromosome 7.



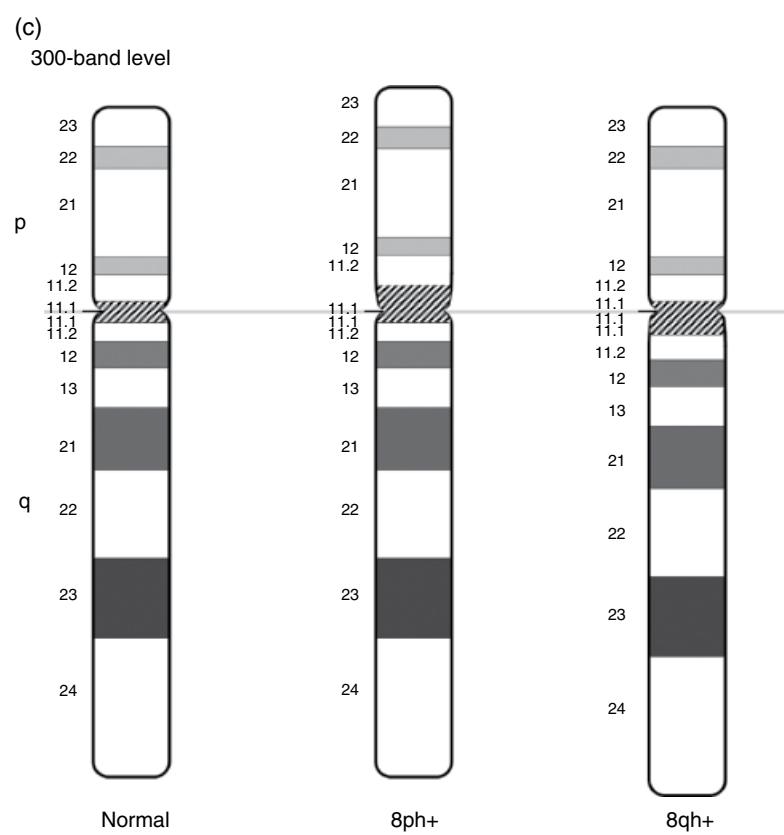
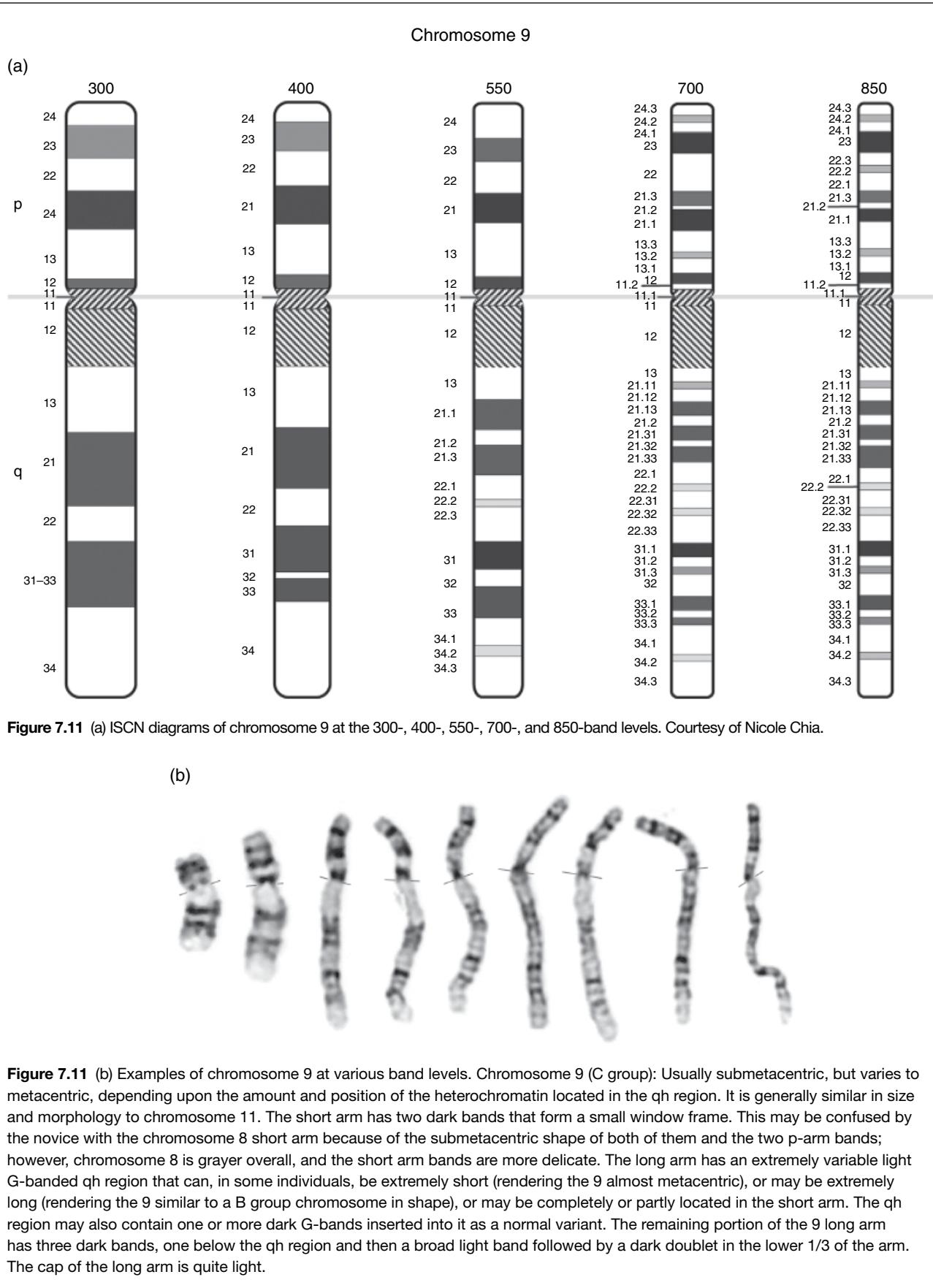


Figure 7.10 (c) Common banding variations for chromosome 8.



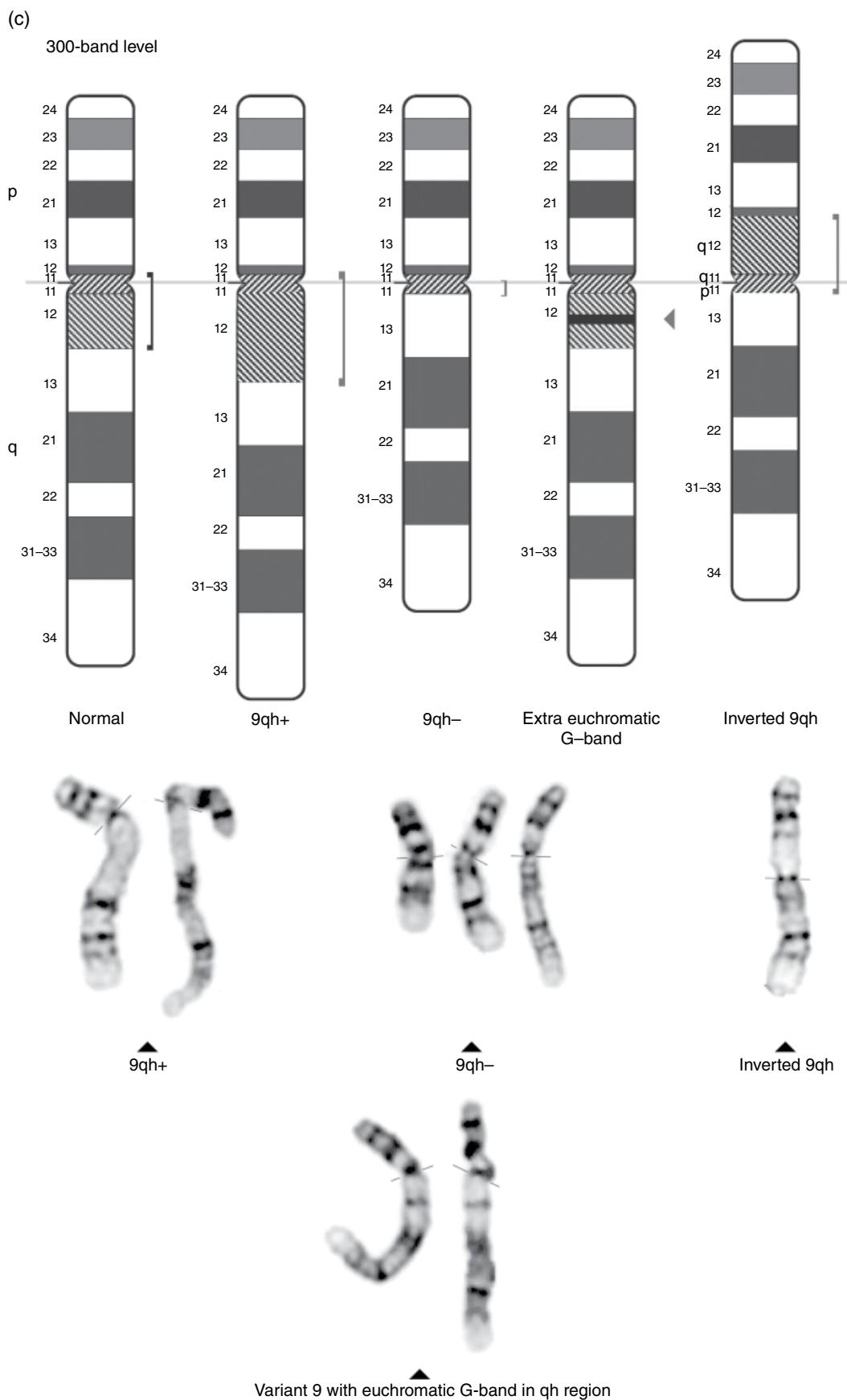
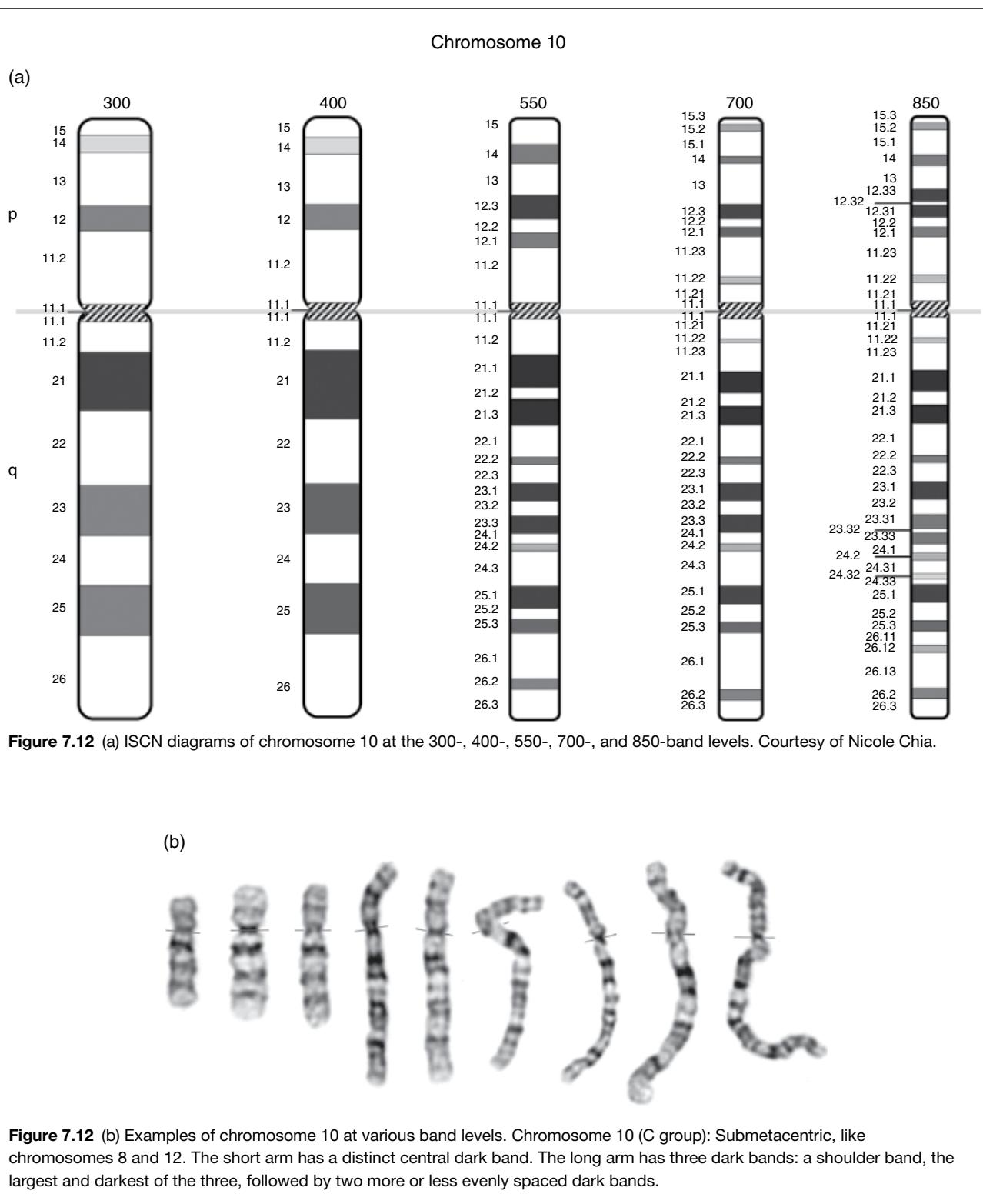


Figure 7.11 (c) Common banding variations for chromosome 9.



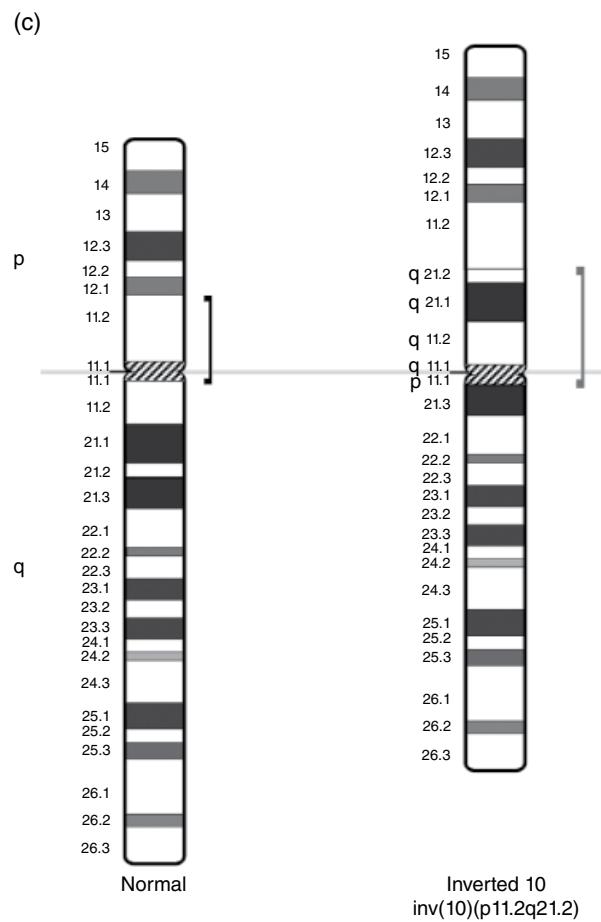


Figure 7.12 (c) A common banding variation for chromosome 10.

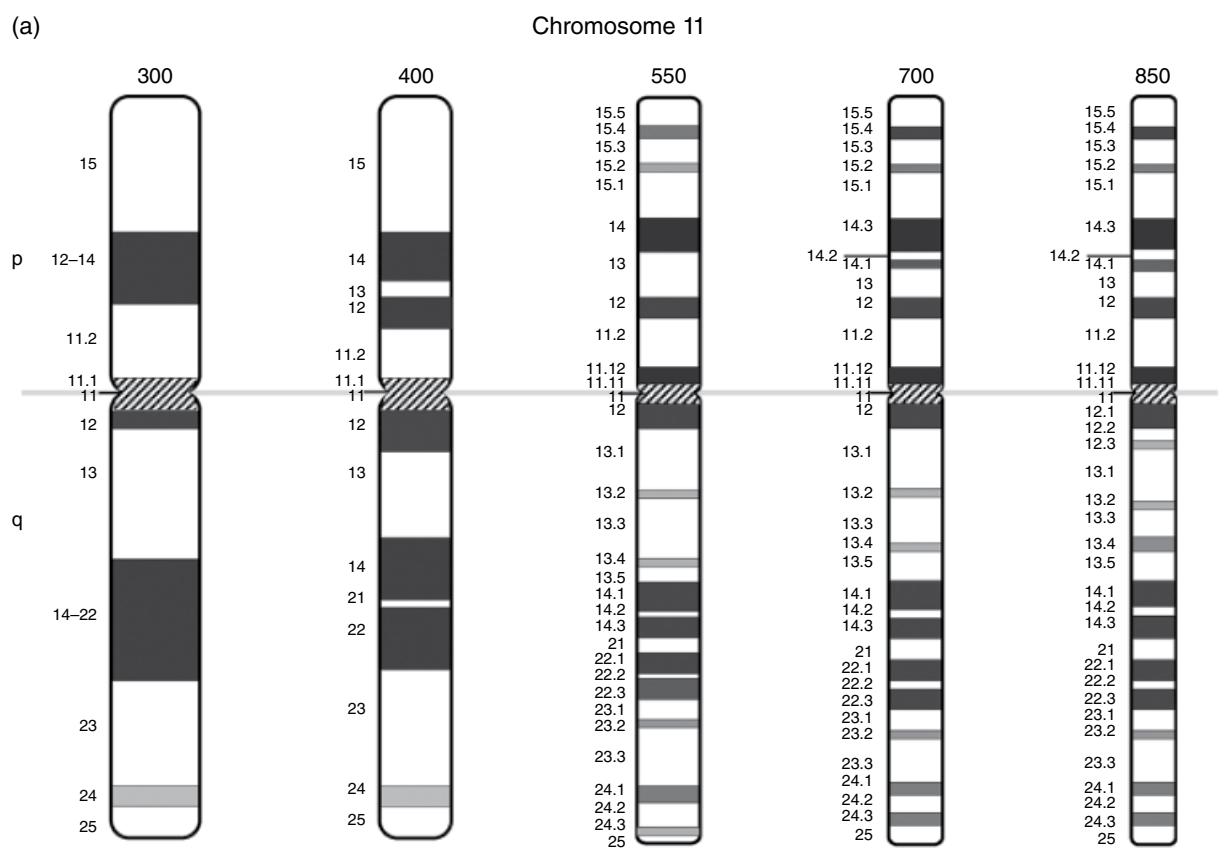


Figure 7.13 (a) ISCN diagrams of chromosome 11 at the 300-, 400-, 550-, 700-, and 850-band levels. Courtesy of Nicole Chia.



Figure 7.13 (b) Examples of chromosome 11 at various band levels. Chromosome 11 (C group): Similar in size and arm ratio to chromosome 9, and has light material just below the centromere like the 9. The short arm has two distinct dark bands in the proximal ½. The long arm has a small dark band just at or below the centromere, followed by two broad, dark bands that may fuse together in very short preparations. The q arm cap has a characteristic gray band.

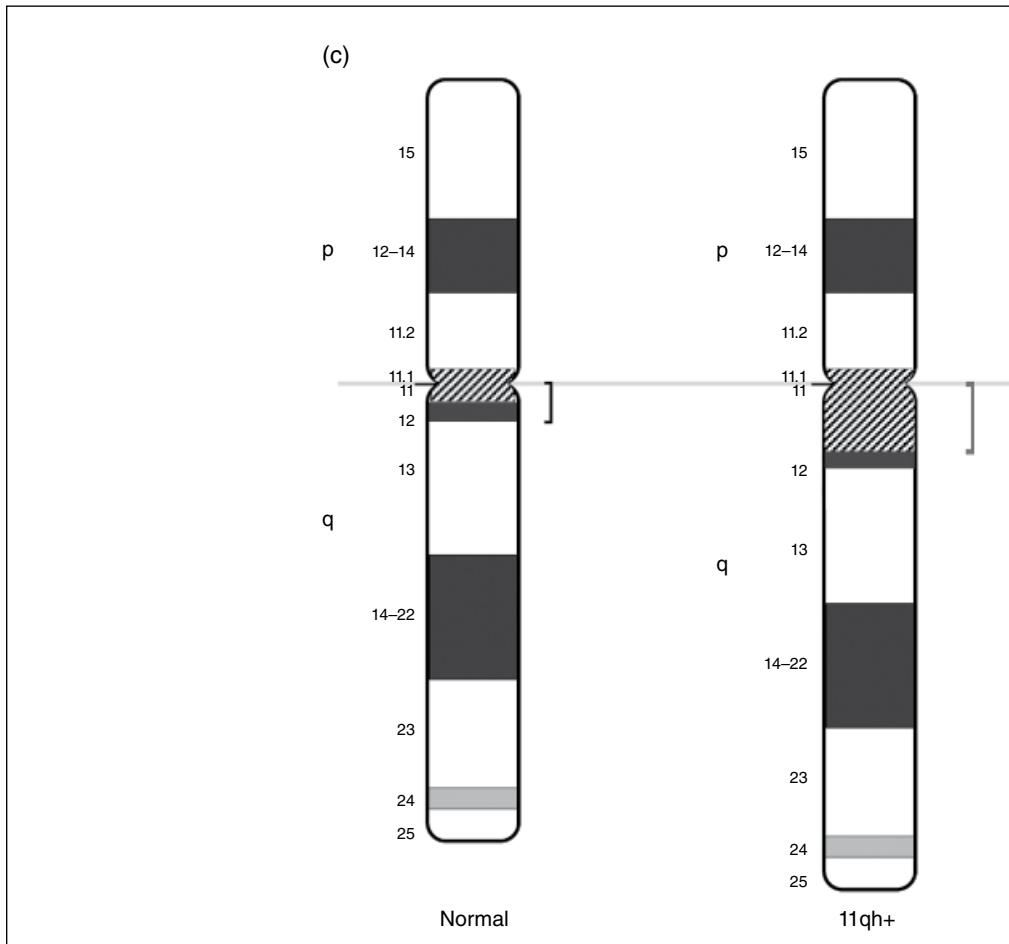


Figure 7.13 (c) A common banding variation for chromosome 11.

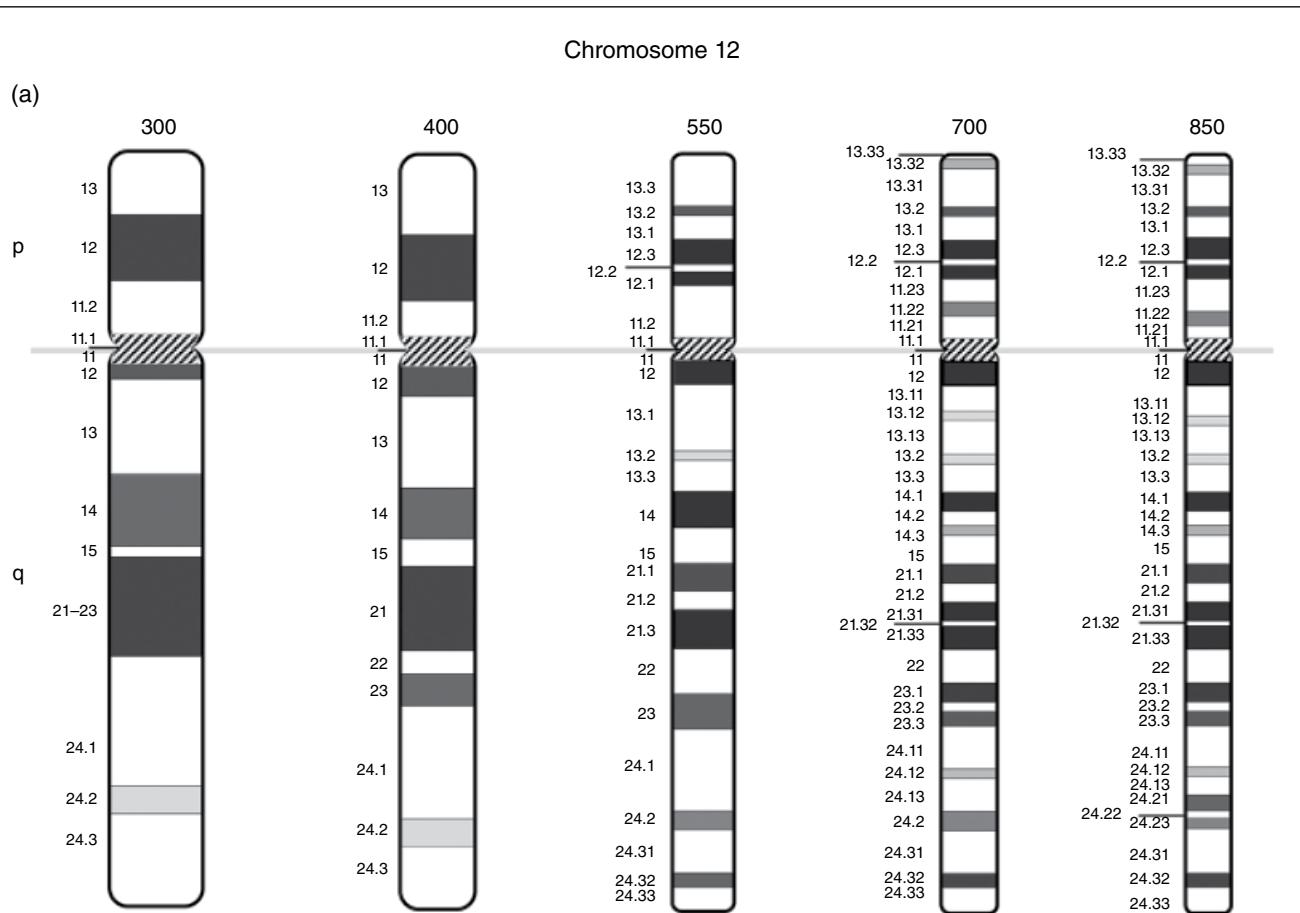


Figure 7.14 (a) ISCN diagrams of chromosome 12 at the 300-, 400-, 550-, 700-, and 850-band levels. Courtesy of Nicole Chia.



Figure 7.14 (b) Examples of chromosome 12 at various band levels. Chromosome 12 (C group): Very submetacentric with a short arm to long arm ratio of 1:3. The short arm is the smallest of the C group, and has a broad central dark band. The long arm has a small dark band just below the centromere followed by a broad light band. Next are the central dark bands (3–4) that can fuse together in very short preparations. The long arm ends with a broad light region.

(c)

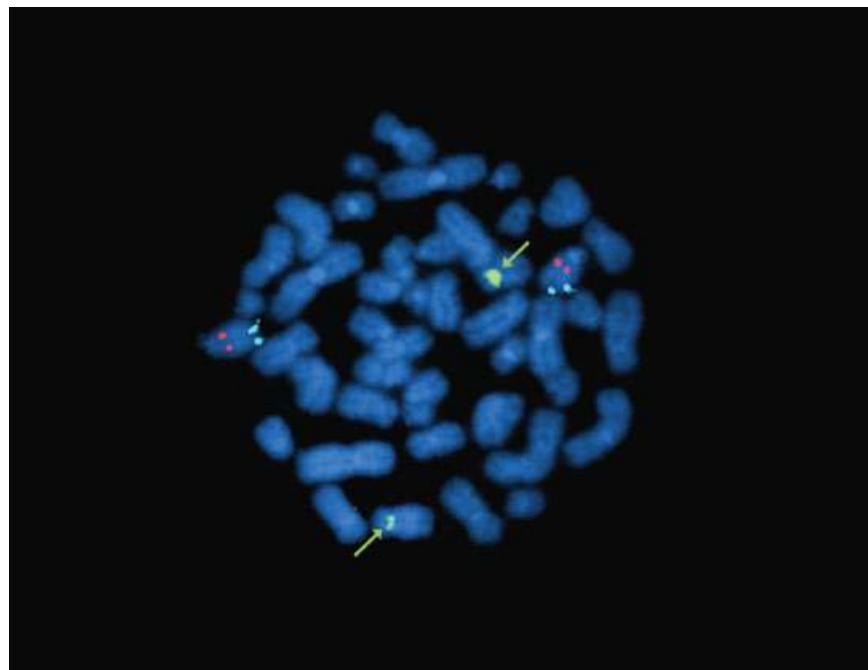


Figure 7.14 (c) Common variants for chromosome 12. Chromosome 12 has few variants. The pericentromeric heterochromatin can be quite variable. Shown is a normal metaphase cell hybridized with probe for chromosome 13 (red, blue) and the centromeric alpha satellite probe for chromosome 12 (green). Note the discrepancy in the size of the two green signals. This is a normal variation for chromosome 12. These centromeric signals may be very small and difficult to observe. See insert for color representation of this figure.

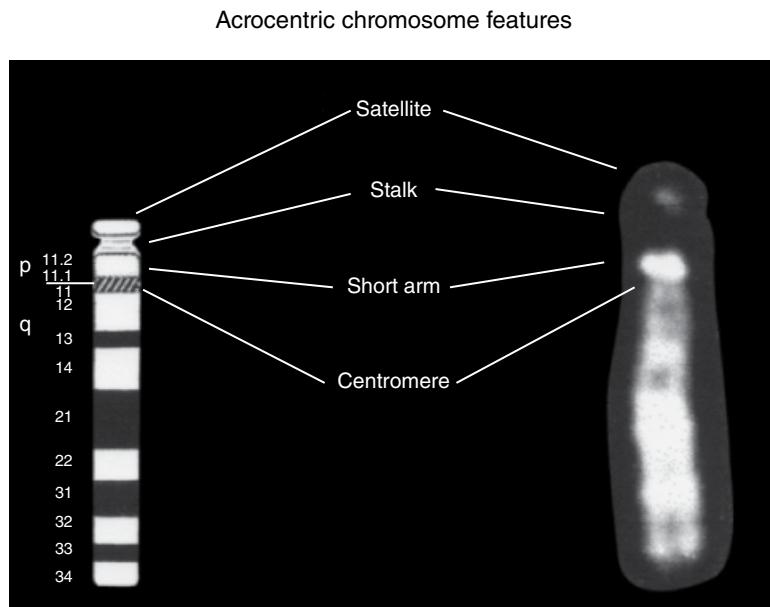


Figure 7.15 Features of autosomal acrocentric chromosomes, demonstrated on a Q-banded chromosome 13. The acrocentric chromosomes (13, 14, 15, 21, and 22) all have variable regions involving the centromeric and short arm regions. This Q-banded chromosome 13 demonstrates the satellite, stalk, short arm, and centromere region, all of which may show variation in presence or absence, size, staining intensity, and in some cases, position. The variable stalk lengths of acrocentric chromosomes correspond with variably sized NOR staining regions. Reproduced from Olson SB, Magenis RE, and Lovrien EW. Human chromosome variation: the discriminatory power of Q-band heteromorphism (variant) analysis in distinguishing between individuals, with specific application to cases of questionable paternity. *Am J Hum Genet* 1986;38:235–252, with permission from the publisher.

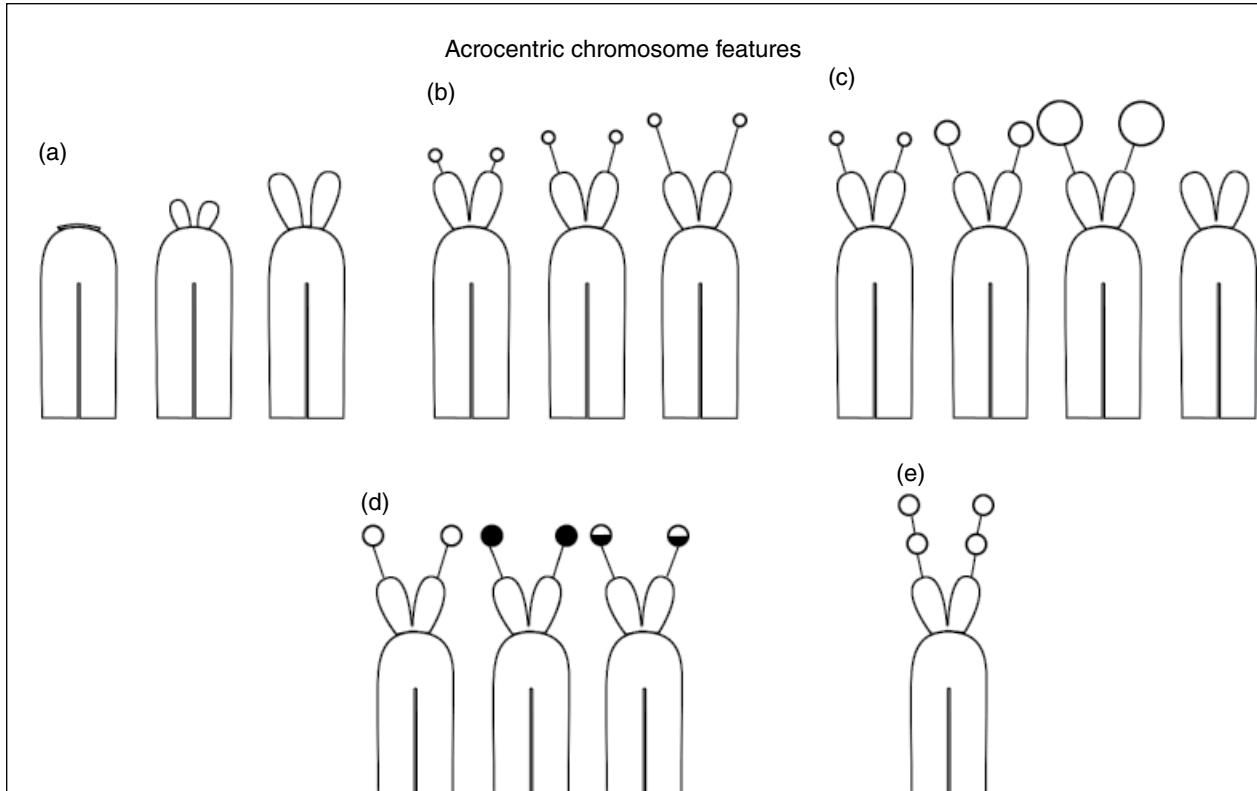
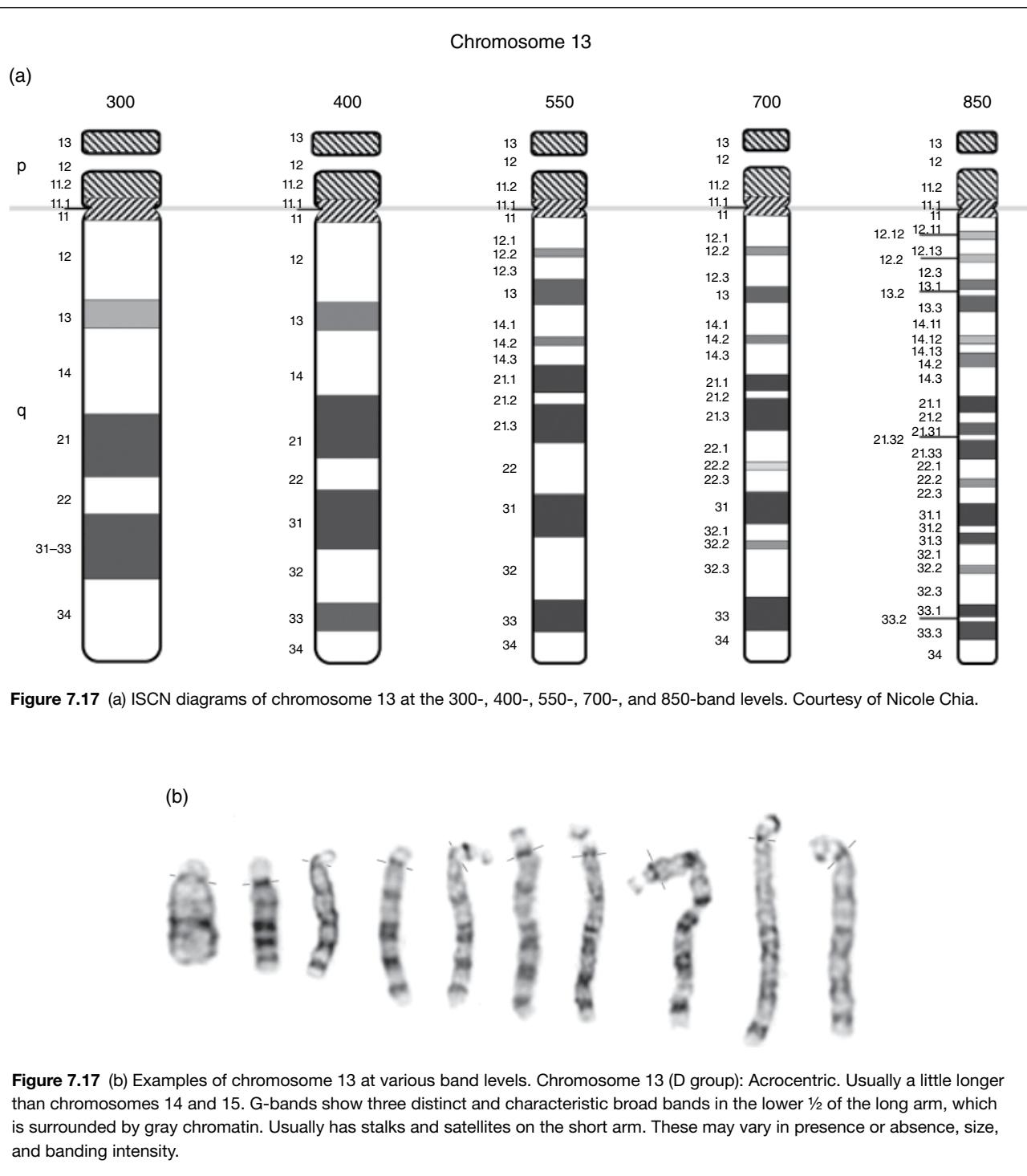


Figure 7.16 Acrocentric chromosome variations. (a) Acrocentric chromosomes 13, 14, 15, 21, and 22 may have no visible short arm (left), or the short arm may be small (middle) or large (right), with or without stalks and satellites. (b) Stalks may be very short (left), moderate length (middle), or long (right). (c) Satellites may be very small (left), medium-sized or large (middle), or absent (right). (d) Satellite staining intensity may vary from light (left) to dark (middle), and may be of uniform staining intensity or show some variation (right). (e) Some acrocentric chromosomes may have more than one satellite.



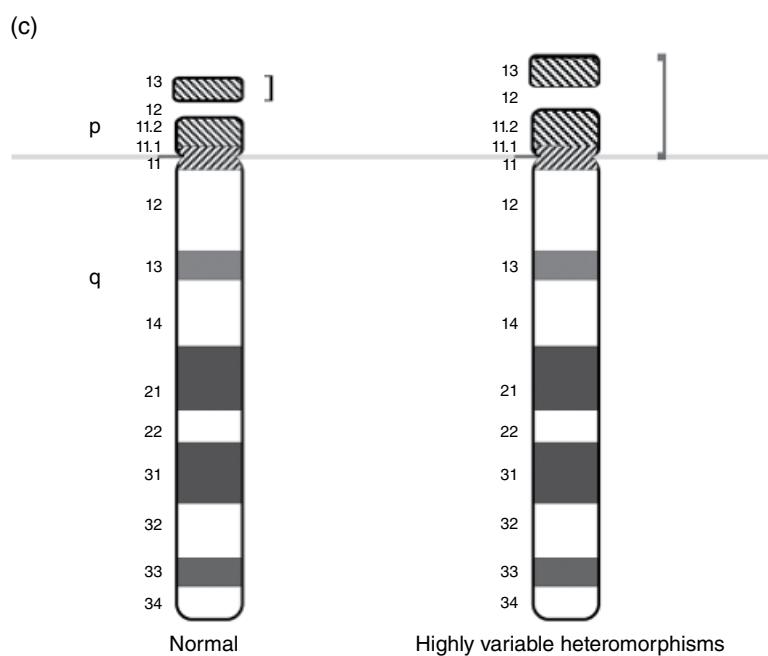
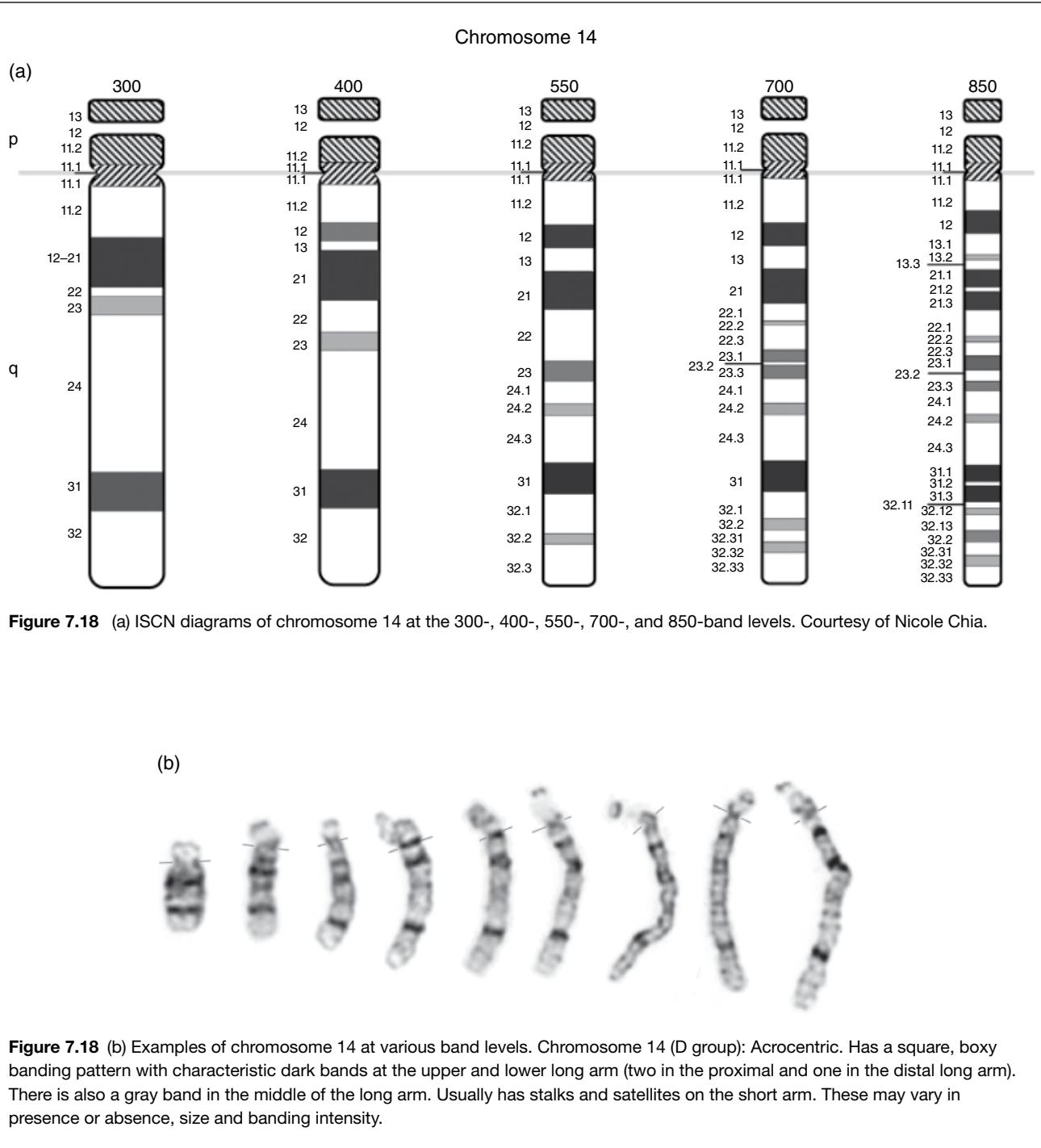


Figure 7.17 (c) Common banding variations for chromosome 13. Chromosome 13 can have a variable brightly stained region in the pericentromeric region of Q-banded cells, and the bright variant can be present or absent, large or small. G-banded chromosomes may show a darkly staining region in the centromere region of chromosome 13 to reflect the Q-banded variant. The short arm, stalks, and satellites are variable in presence or absence, size, and staining intensity. See Acrocentric Chromosome Variations, Figure 7.15.



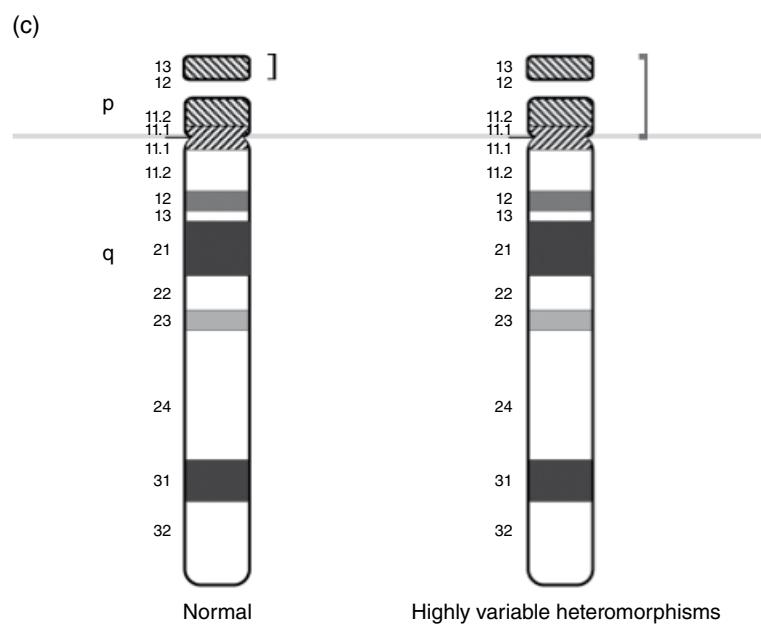
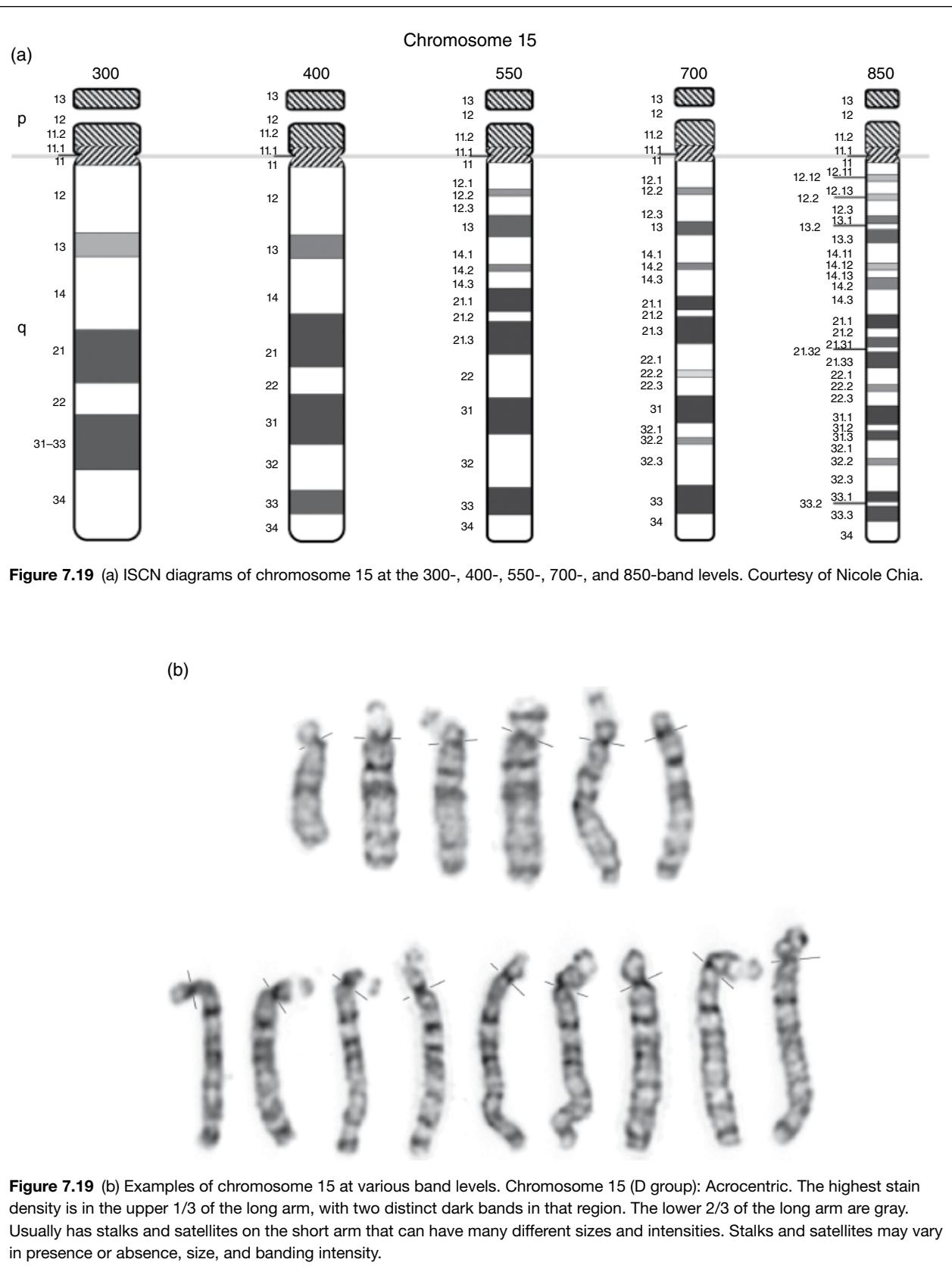


Figure 7.18 (c) Common banding variations for chromosome 14. Chromosome 14 can show short arm, stalks, and satellites that are variable in presence or absence, size, and staining intensity. See Acrocentric chromosome variations, Figure 7.15.



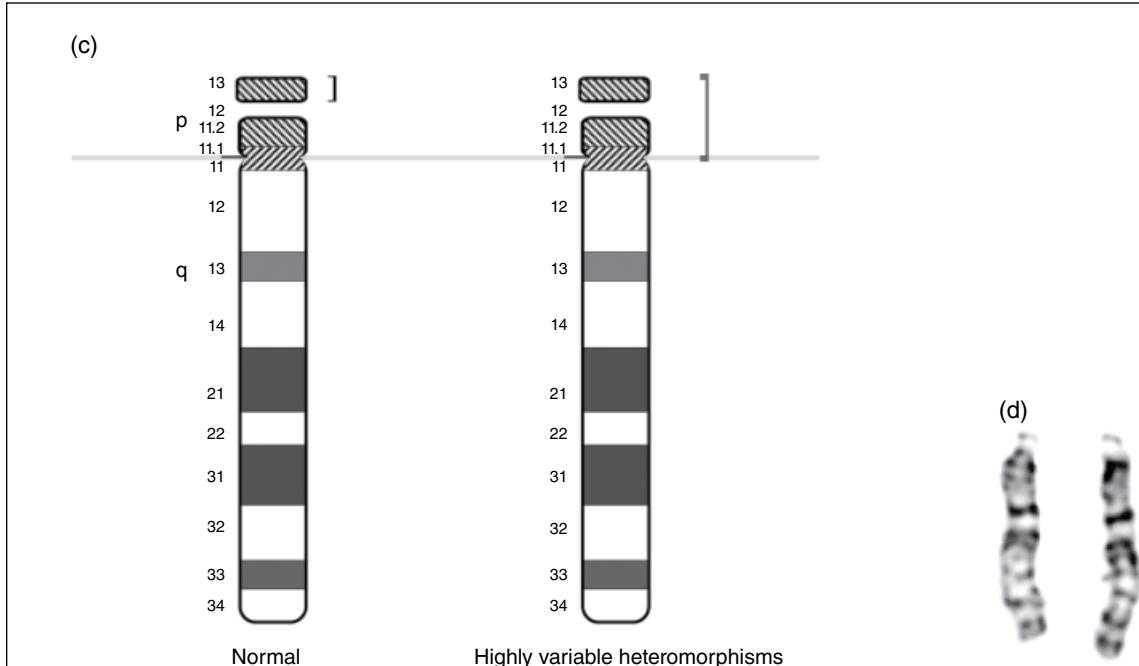
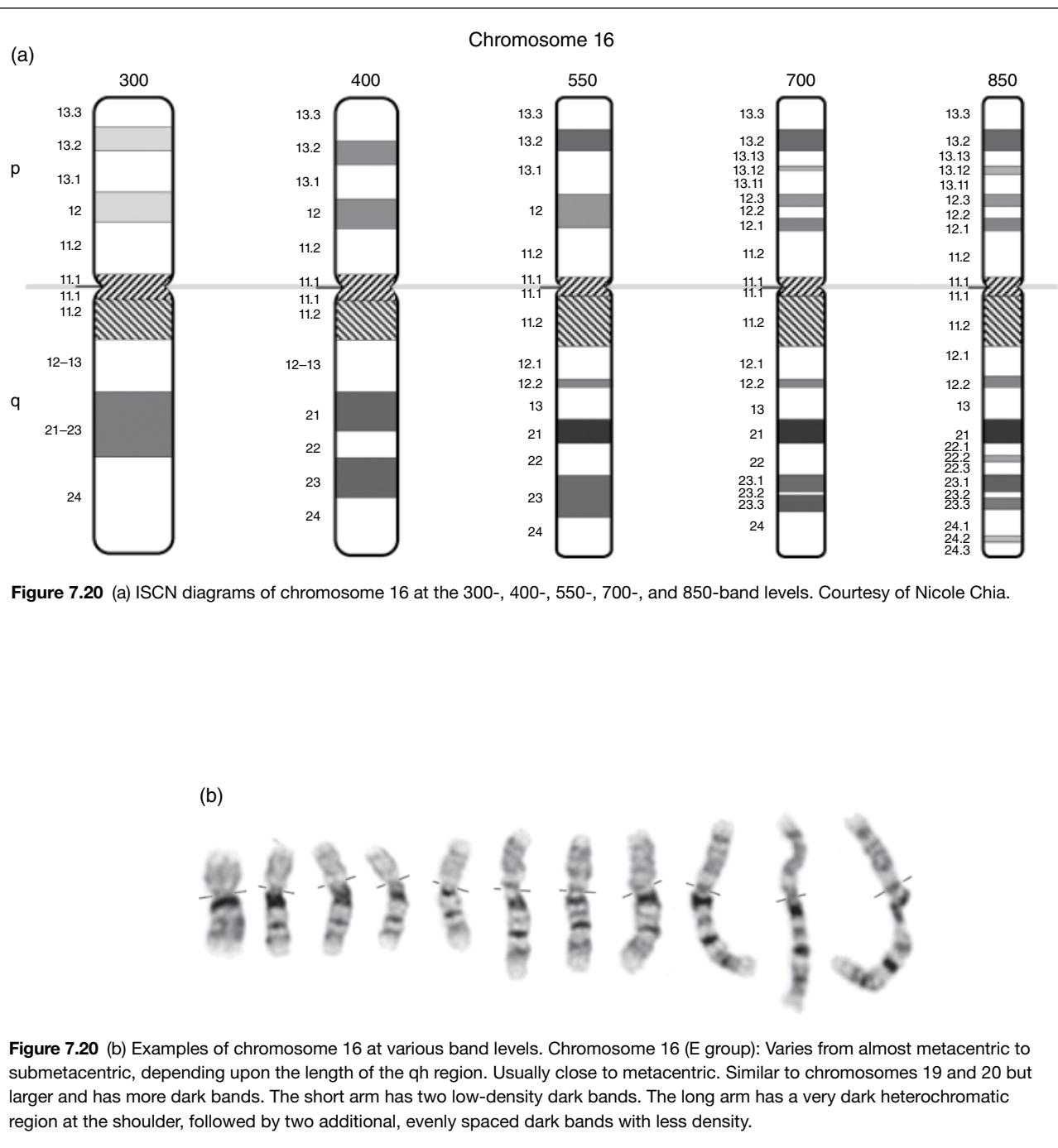


Figure 7.19 (c) Common banding variations for chromosome 15 can show short arm, stalks, and satellites that are variable in presence, absence, size, and staining intensity. See Acrocentric chromosome variations, Figure 7.15. The pericentromeric heterochromatin stained by FISH probes for D15Z1 can be translocated to chromosome 14 in some individuals as a normal variation. It usually manifests as an extra signal for D15Z1 in addition to the signals on chromosome 15. D15Z1 may also be absent from or duplicated on a normal chromosome 15. The heterochromatin of chromosome Yq may be translocated to chromosome 15p (see Y chromosome variant illustration, Figure 7.27c) or to chromosome 22p. It will be G-banded dark, Q-banded bright, and will stain with FISH probes for DYZ1. Chromosome 15 can also exhibit a proximal long arm duplication of band 15q11.2 in normal individuals. However, it is important to use FISH probes to distinguish the normal variant from an abnormal duplication of the region in which the *SNRPN* gene and others may be duplicated. The normal variant will exhibit only normal copy numbers of FISH probe signals in the 15q11.2 region. (d) Two variant chromosomes 15 with a benign duplication of the proximal q arm. FISH results for *SNRPN* and D15S10 were normal, with a single copy for each on the duplicated chromosome. A duplication that includes an extra signal for the *SNRPN* and/or D15S10 probes would not be considered normal, and can cause phenotypic effects, including autism spectrum disorder.



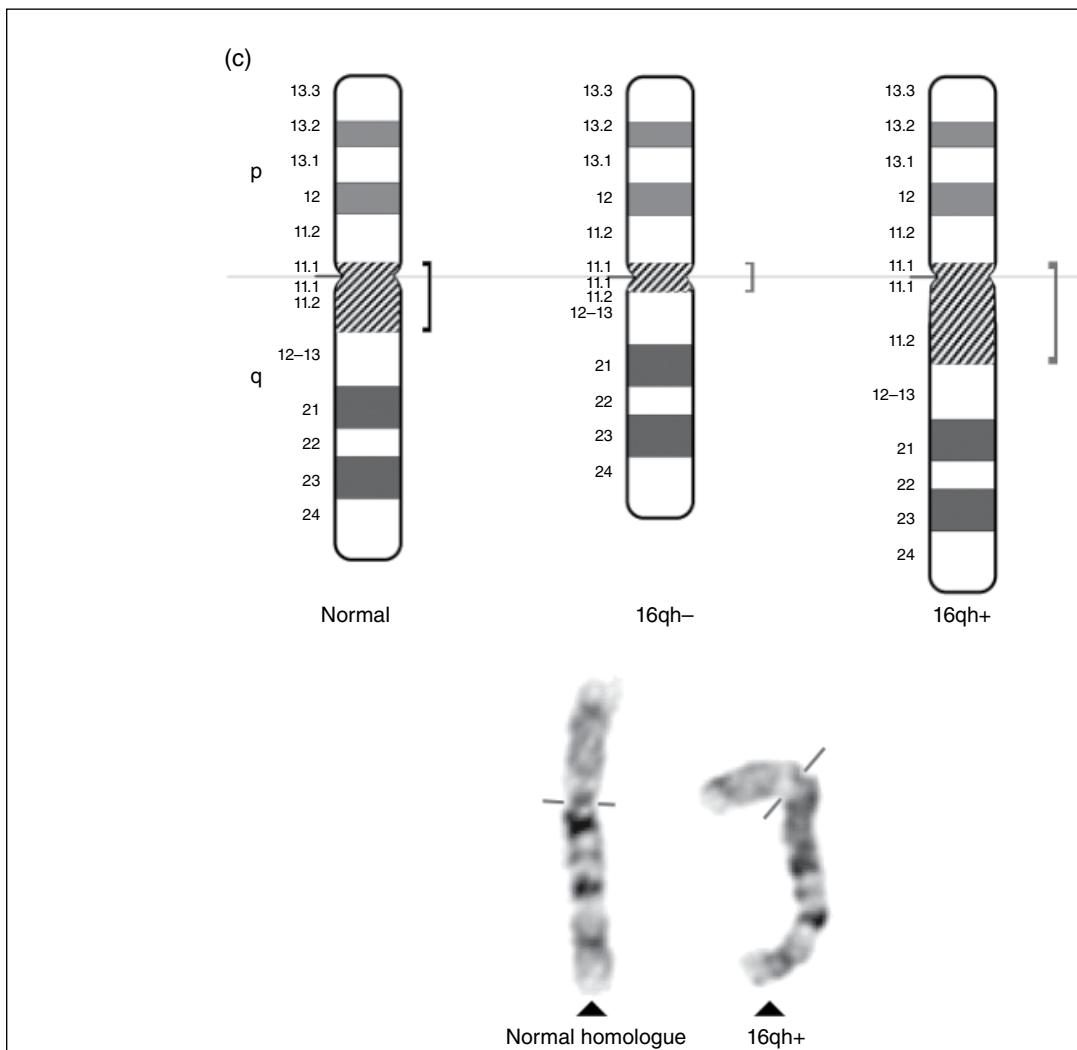
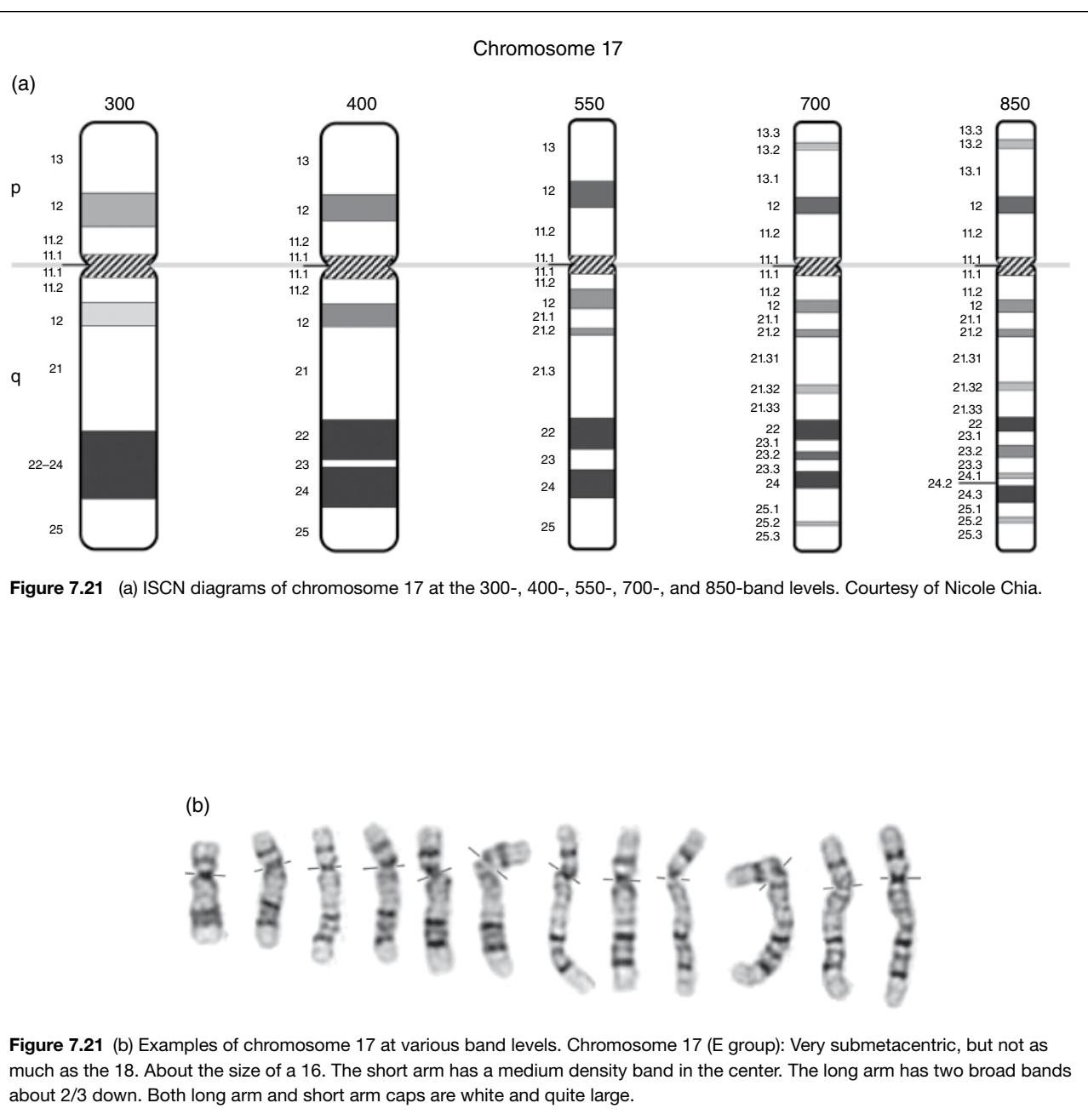


Figure 7.20 (c) Common banding variations for chromosome 16.



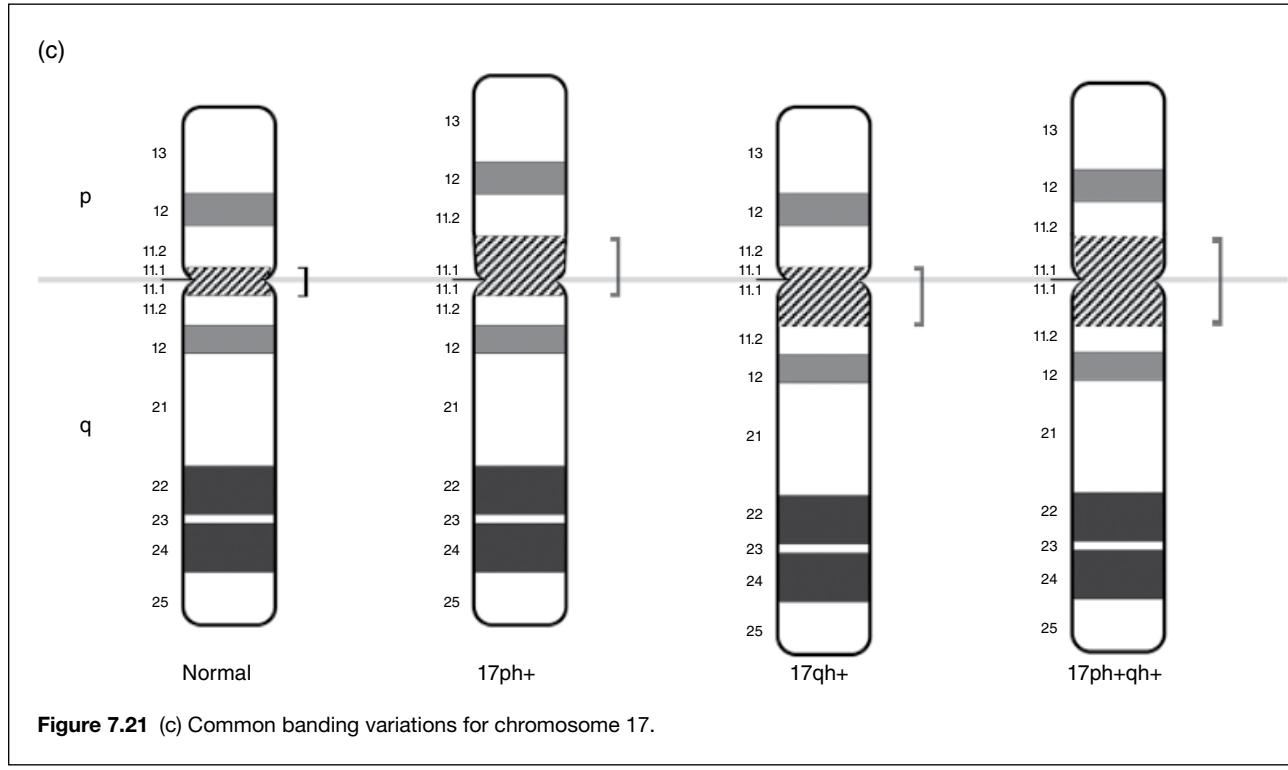
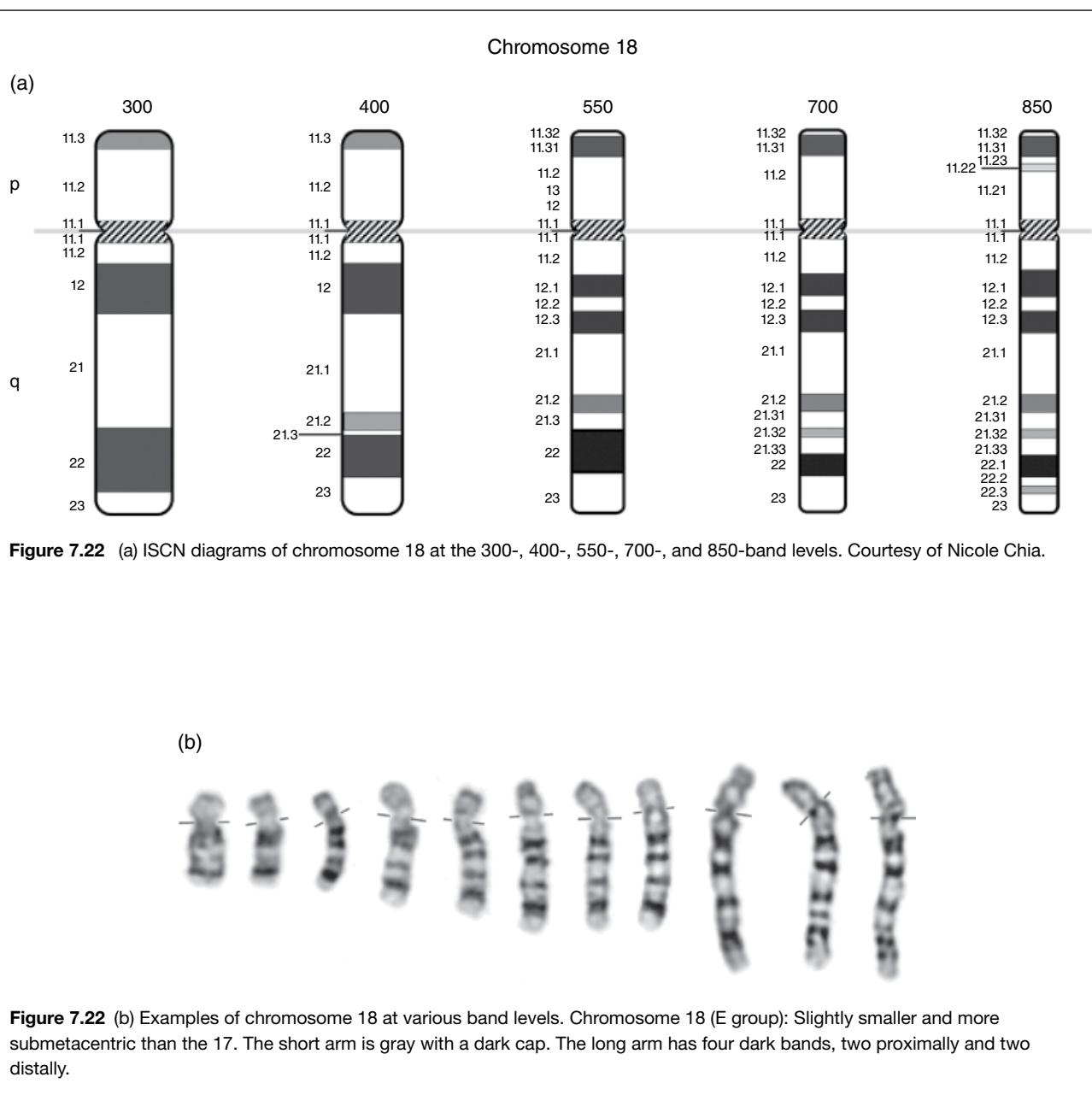


Figure 7.21 (c) Common banding variations for chromosome 17.



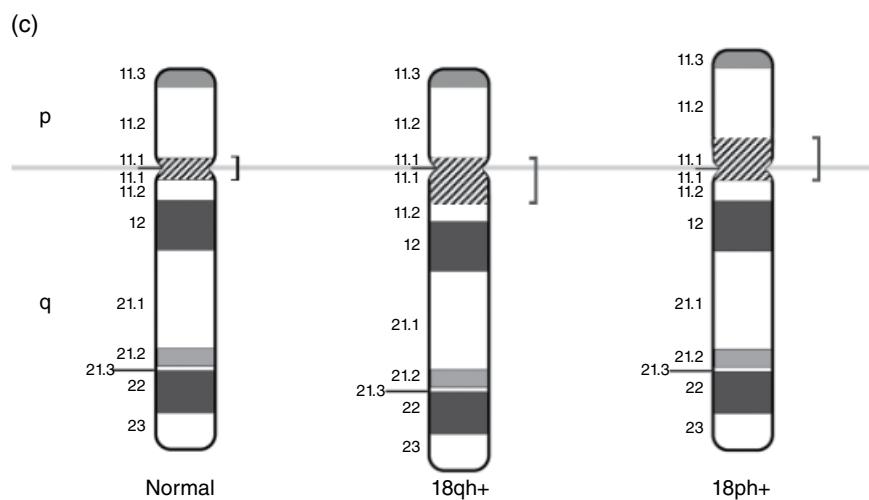


Figure 7.22 (c) Common banding variations for chromosome 18.

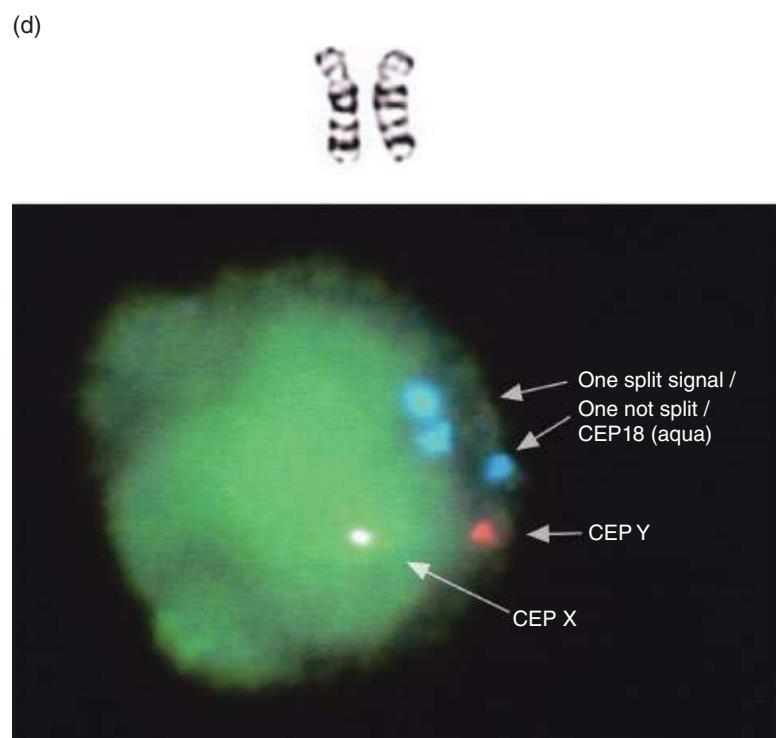
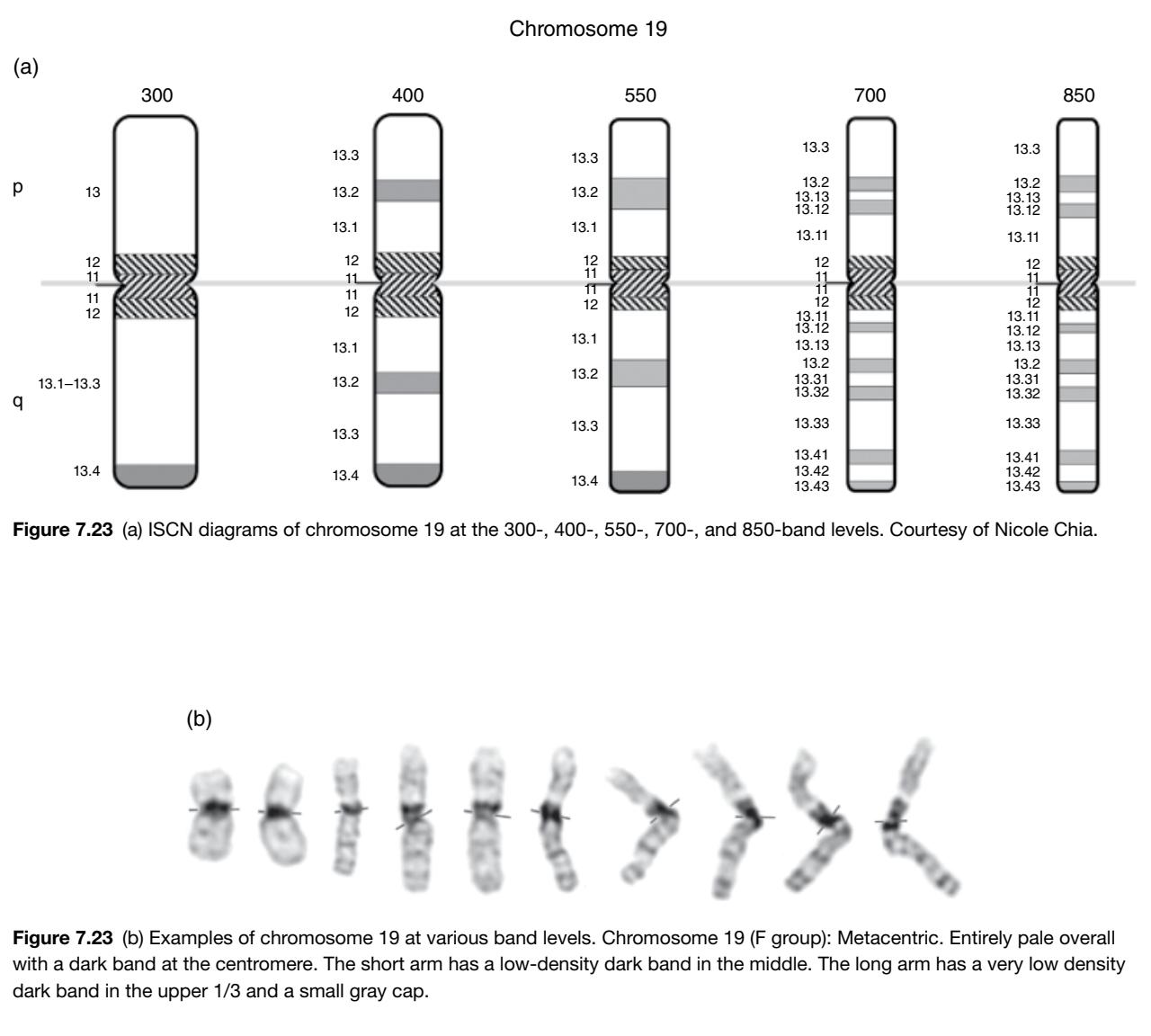


Figure 7.22 (d) FISH variation: The pericentromeric region of chromosome 18 may show variations with FISH probes for the alpha satellite region. The smaller CEP 18 signal is the more commonly seen variation and the larger signal can be seen as a larger pericentromeric region on the G-banded metaphase chromosome pair. G-band pair contributed by Cedars Sinai Medical Center Cytogenetics Laboratory. The variable size of the pericentromeric heterochromatin on chromosome 18 and the tendency of the heterochromatin to occur in large blocks can cause the alpha satellite FISH probes for the centromere of 18 to appear as extra signals in interphase cells. FISH image contributed by the Human Genetics Department, National Health Laboratory Service, Groote Schuur Hospital, Cape Town. See insert for color representation of this figure.



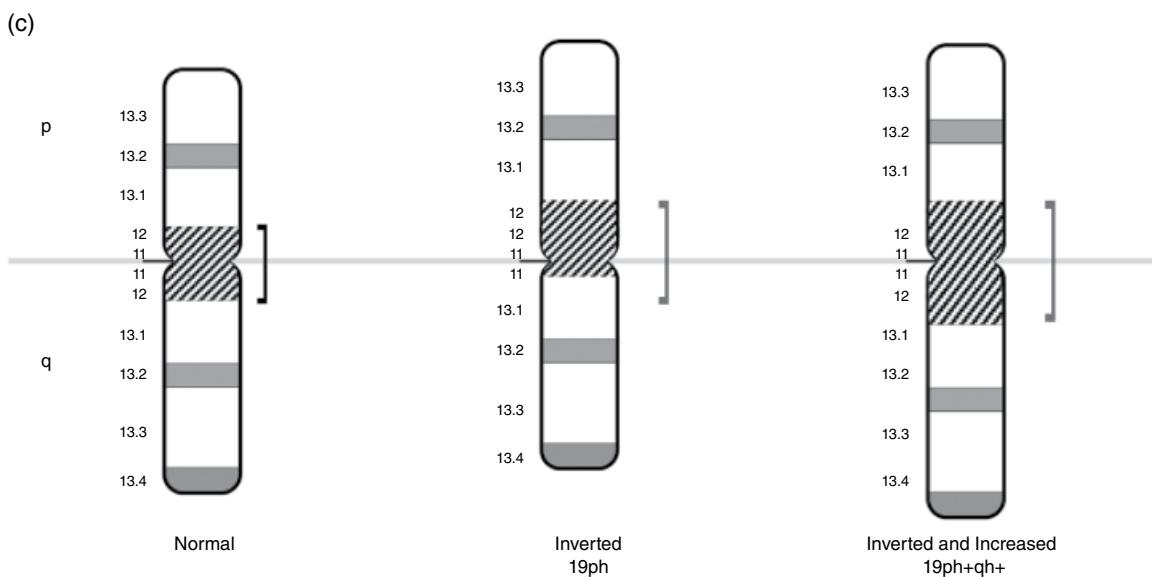
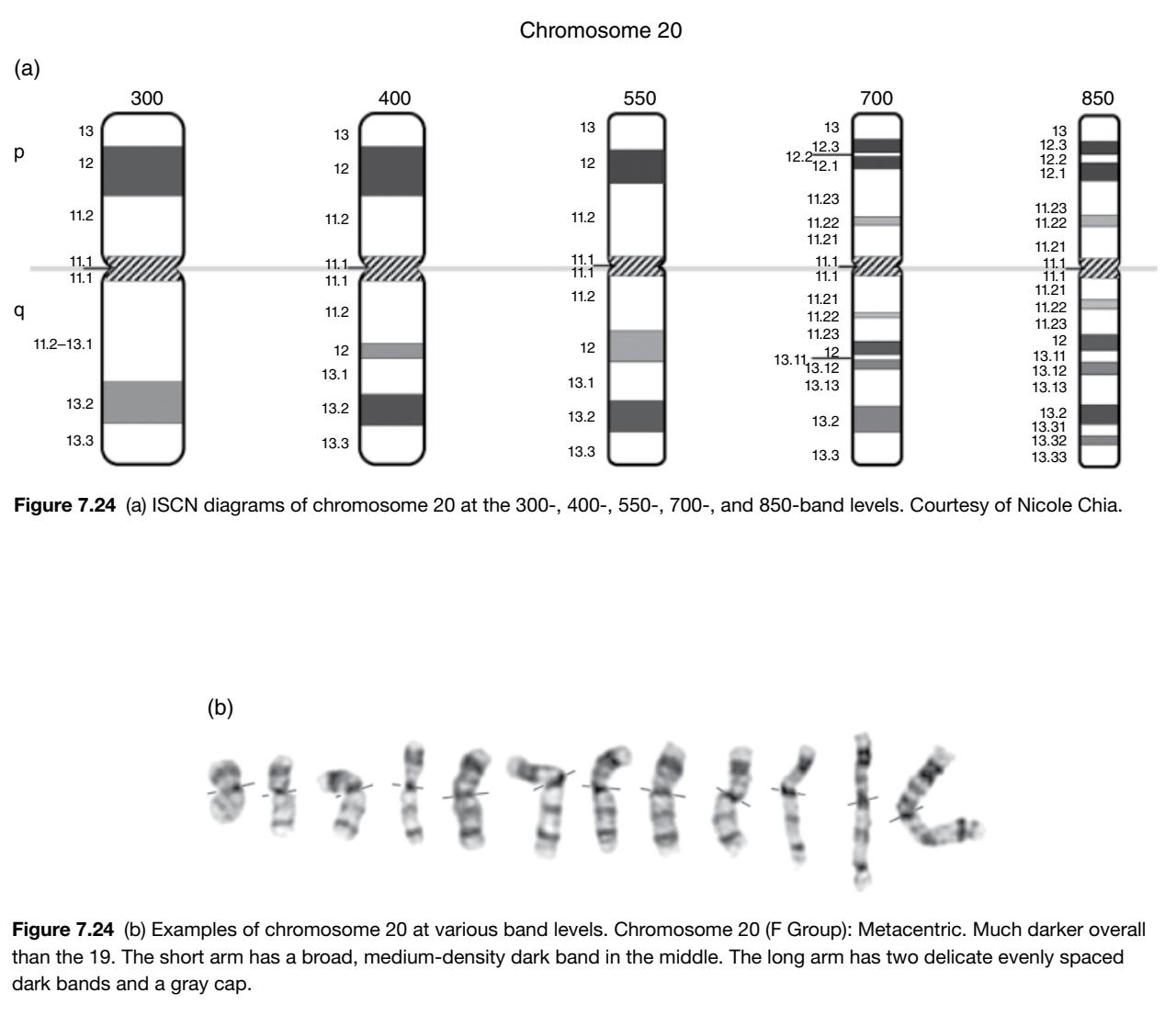


Figure 7.23 (c) Common banding variations for chromosome 19. The example on the right shows both position and size variation; therefore, its nomenclature description must reflect both normal variations by adding a plus (+) sign after the 19ph and qh positions.



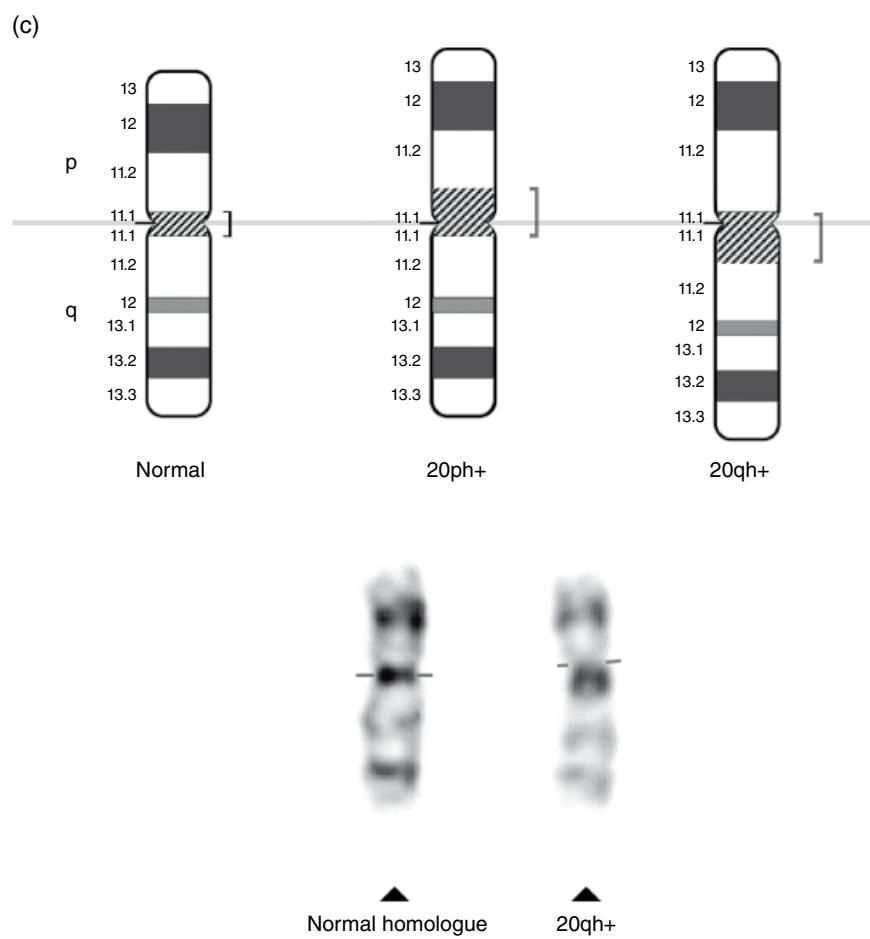
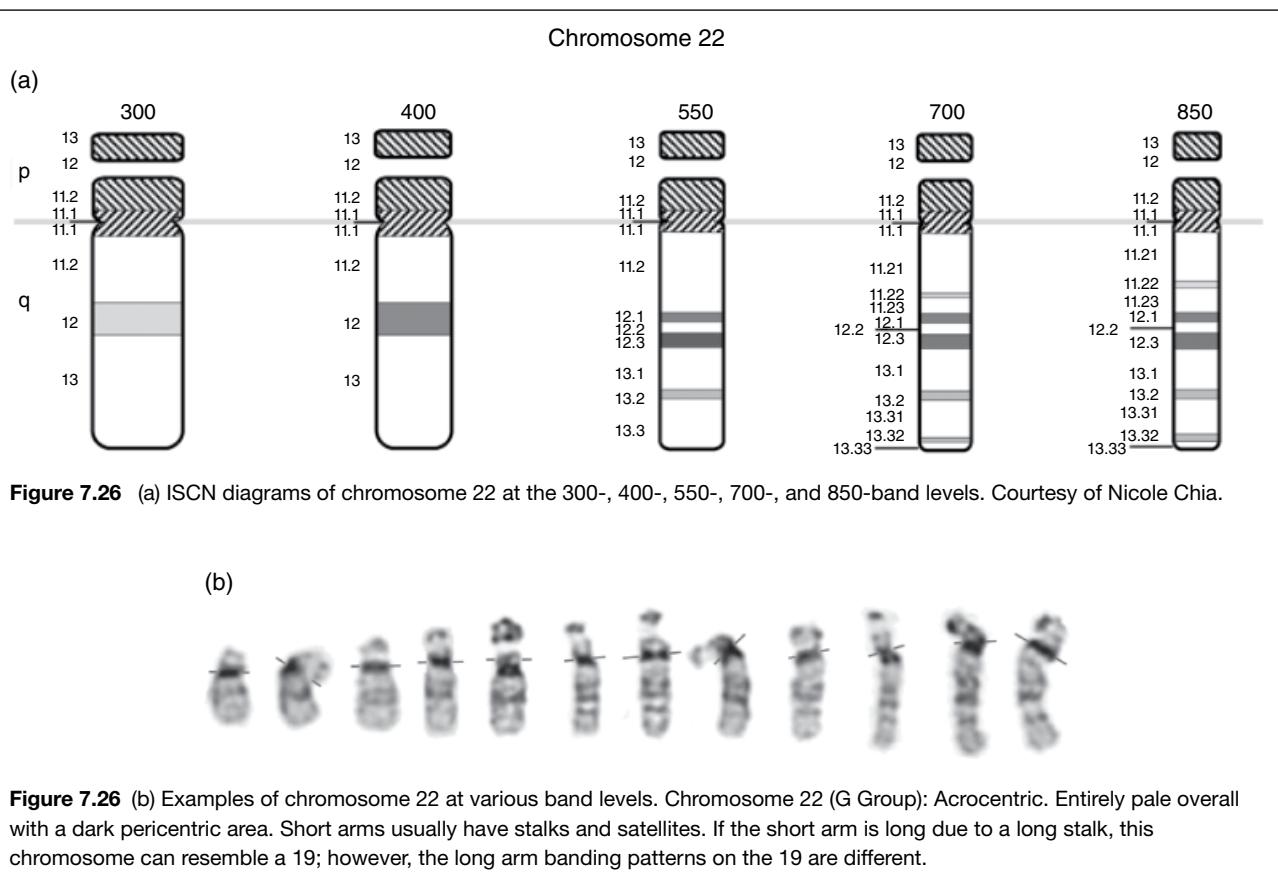
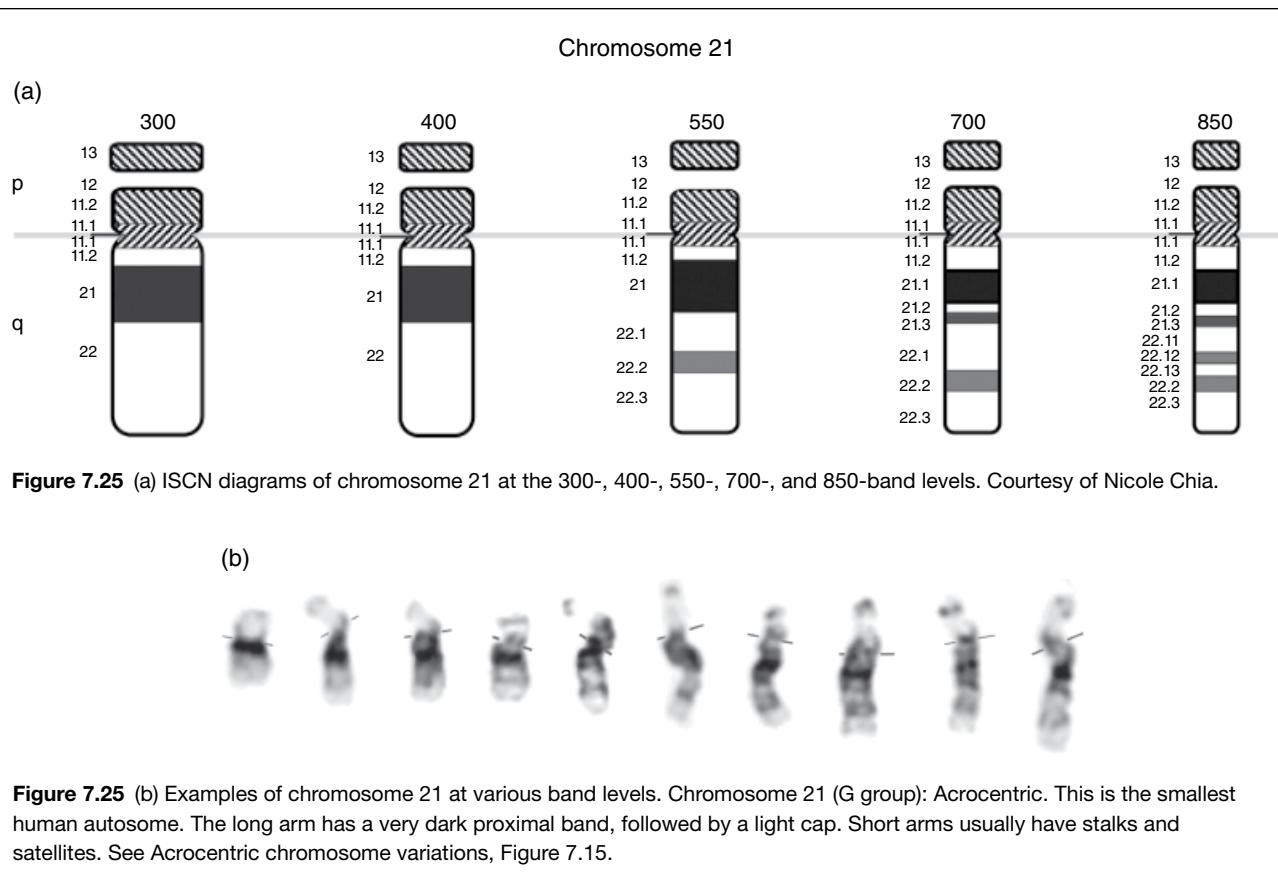


Figure 7.24 (c) Common banding variations for chromosome 20.



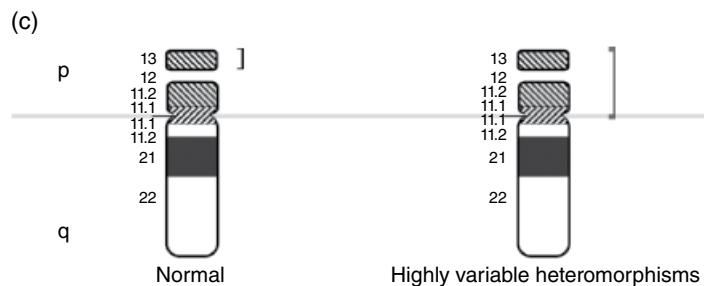


Figure 7.25 (c) Common banding variations for chromosome 21. See Acrocentric chromosome variations, Figure 7.15 and Figure 7.16.

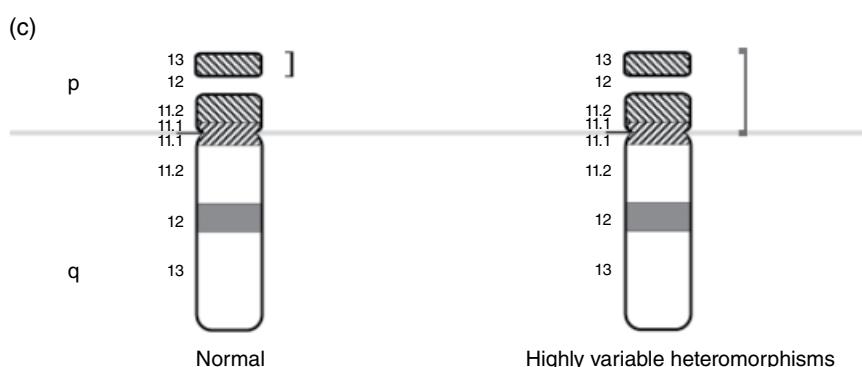
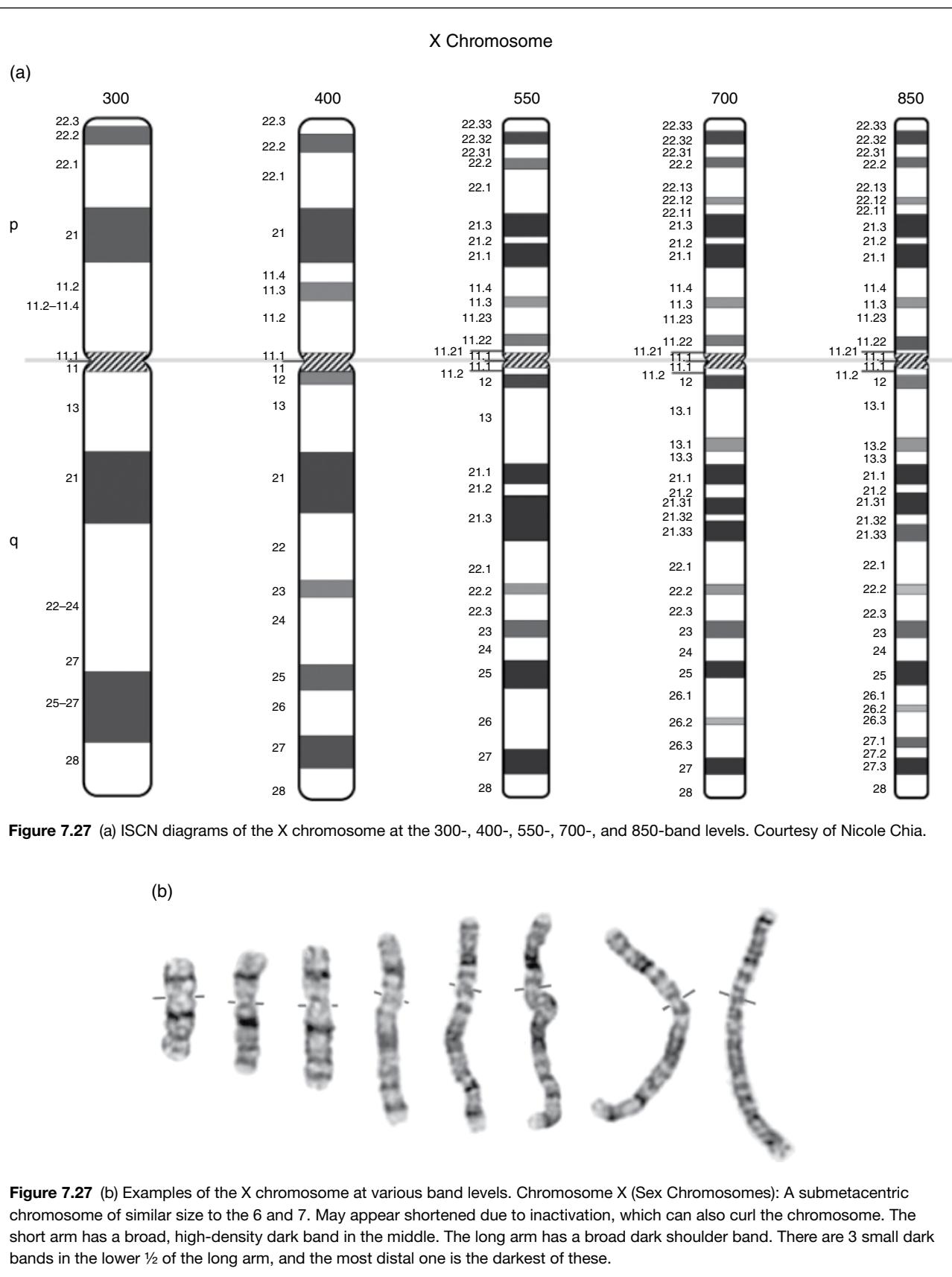


Figure 7.26 (c) Common banding variations for chromosome 22. See Acrocentric chromosome variations, Figure 7.15. Chromosome 22 may exhibit very large G-band dark material on the short arm that is Q-band very bright and may represent the Yqh (DYZ1) region. There is no phenotypic consequence for the variant, even in females (1).



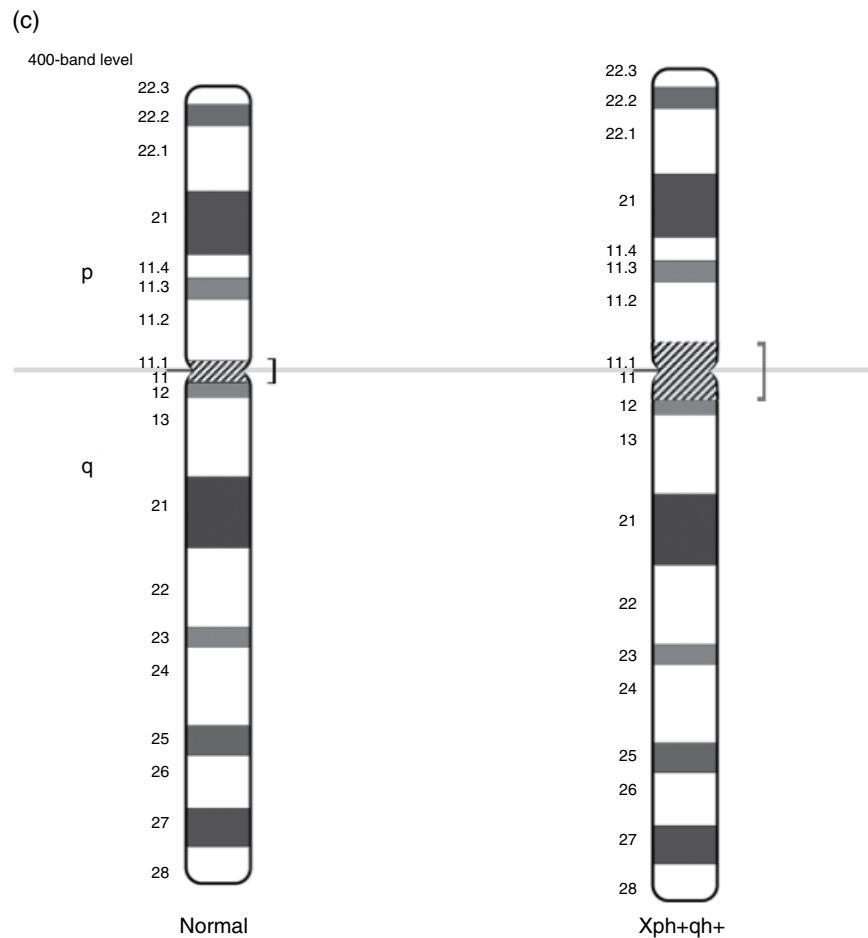
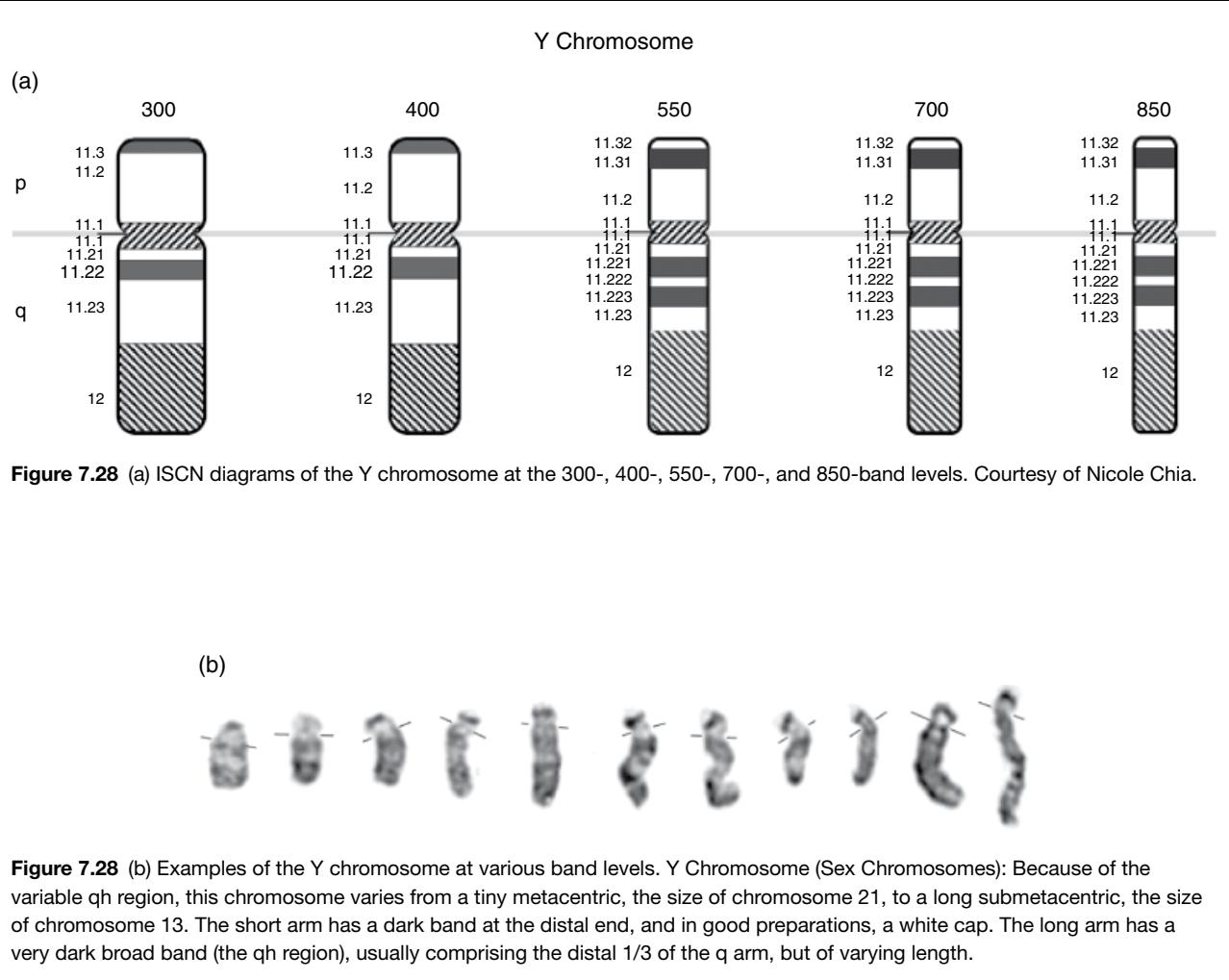


Figure 7.27 (c) A common banding variation for the X chromosome. Pericentromeric variants are rare but may include an increased or decreased size; FISH probes for the DXZ1 region may also exhibit a range of sizes from nearly invisible to very large.



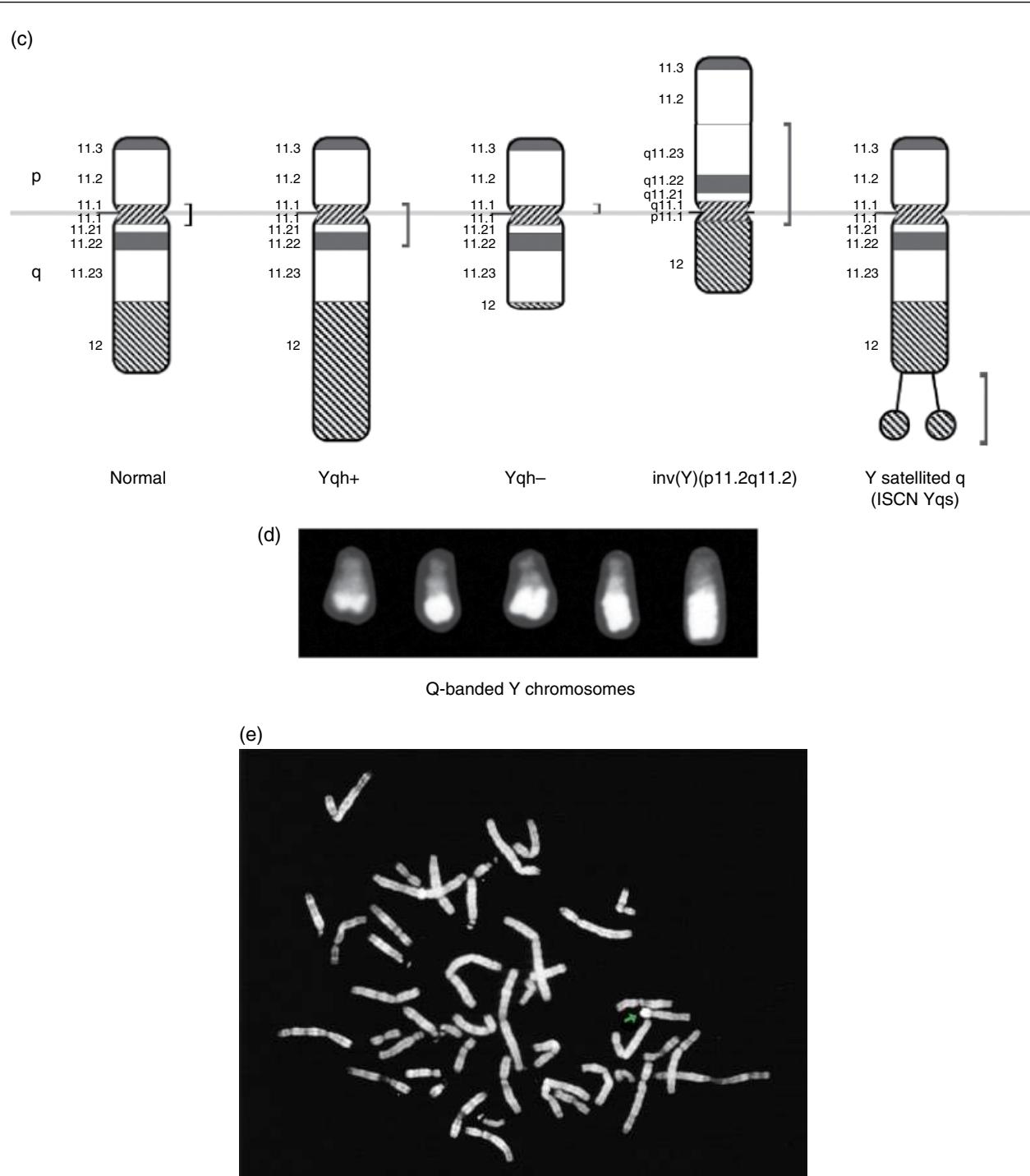
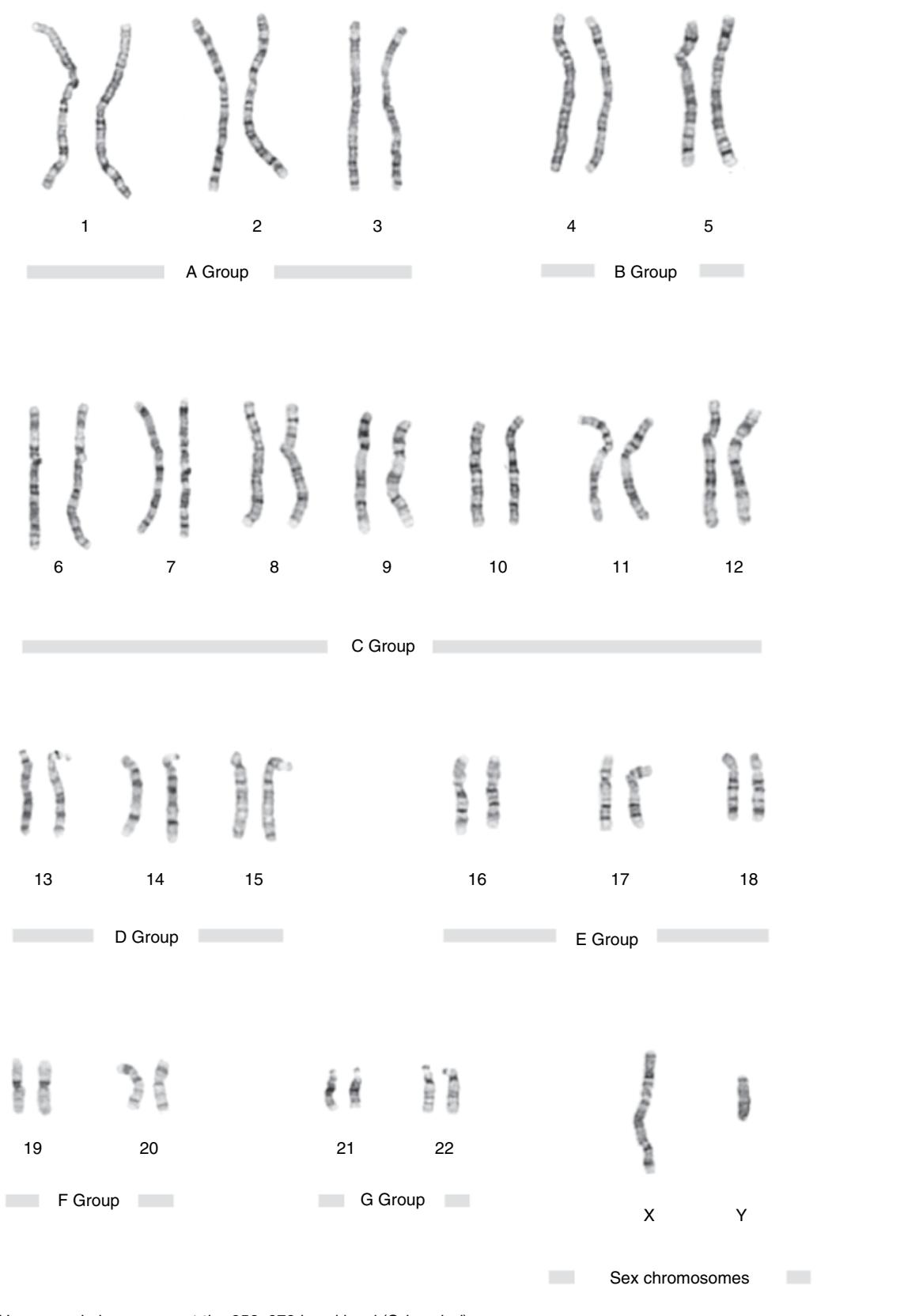


Figure 7.28 (c) Common variations in G-banded Y: The Yqh region (G-band dark, Q-band bright, DYZ1 positive region) is one of the most strikingly variable regions in the human genome. (d) Q-banded Y panel reproduced from Olson SB, Magenis RE, and Lovrien EW. Human chromosome variation: the discriminatory power of Q-band heteromorphism (variant) analysis in distinguishing between individuals, with specific application to cases of questionable paternity. *Am J Hum Genet* 1986;38:235–252s, with permission from the publisher. (e) Other variations for chromosome Y: The heterochromatic variable Yqh chromatin can be found as a variant on the short arm of acrocentric chromosomes, especially the 15 and 22. This is a Q-banded cell and the arrow points to a chromosome 15 with the bright Y heterochromatin present as an abnormal variant in this female patient. FISH using DYZ1 will confirm the finding.

**Figure 7.29** Human male karyogram at the 650–670 band level (G-banded).

7.3 Determination of G-banded chromosome resolution

As a rule, the better the banding resolution is, the better will be the chance of finding a small chromosome abnormality. This rule is more reliable for deletions and duplications than for centromere displacement (inversions, neocentromeres) because the constriction of the centromere becomes more difficult to visualize as the chromosomes become longer and thinner. However, there is a need to quantify the banding resolution of a given cell (on the analysis sheet) or chromosome study (on the final report) so that the limits of resolution can be inferred. There are several methods for determination of band levels.

7.3.1 Vancouver method

The Vancouver method [4] involves counting bands in segments for some chromosomes (e.g., chromosomes 1, 11, and 12) and all bands in others (chromosomes 10 and X). Count only G-positive bands and do not count the centromere. Total the bands then look at a chart (Table 7.1) that has increments of 50 bands at the lower range and 100 bands at the higher end to determine haploid band resolution.

7.3.2 Johnson and Stallard method

Johnson and Stallard [5] used a method that quantifies the number of light and dark G-bands present on one chromosome 10 (see Table 7.2).

Some laboratories account for a difference in resolution between the two chromosome 10 homologues in a cell by counting both homologues and dividing the total by 2 before using the haploid parameters.

7.3.3 Welborn method

The Welborn method [6] involves counting all dark and light bands on one homologue each of chromosomes 1 and 2, counting the centromere as a band in each arm. Total the bands from both chromosomes and then multiply by 6 to get the haploid band resolution, since chromosomes 1 and 2 represent 1/6 of the genome.

7.3.4 Band determination quality control

Band resolution methods should be validated both in the laboratory and for each technologist in the laboratory. The method may have a small acceptable range or standard deviation between the band estimates of any two technologists in the laboratory looking at a specific metaphase cell.

Table 7.1 Haploid band resolution determination by the Vancouver method

Chromosome	Resolution level			
	350	450	550	850
1p31-p32	1	1	3	3
10	5	5	12	19
11p	2	2	5	6
12q	4	5	8	14
X	6	8	12	18
Total	18	21	40	60

Table 7.2 Haploid band resolution determination using chromosome 10 by the Johnson and Stallard method

No. of bands present	Haploid banding level
12	375
13–14	400
15–16	425
17–18	450
19–21	475
22–23	500
24–25	525
26–28	550
29	575
30	600
31	625
32	650
33	675
34	700
35	725
36	750
37	775
38	800
39	825
40–41	850

Acknowledgments

Special thanks to Gret Boyd of BoyDog Design (gret@boydogdesign.com) for most of the illustrations in this chapter.

Glossary

Acrocentric: A structurally normal chromosome with the centromere located near the end of the chromosome, with a ratio of short arm to long arm of 1 : 7. In humans, the nucleolar organizing regions (NORs) are located on the short arms of this group.

Band: Visible region of a chromosome appearing darker or lighter in stain intensity than its neighboring region. The ISCN assigns each band a name by its chromosome number, arm (p or q), the region, and band within the region. For example, band 11q23 denotes chromosome 11 long arm, region two as defined by the ISCN, band three in that region. As chromosomes elongate, the bands subdivide into smaller sub-bands, and are denoted by a decimal point followed by the sub-band number.

Banding resolution: The number of ISCN-defined G-bands in a haploid set of chromosomes, which defines the quality of chromosome analysis and its utility in the detection of chromosome abnormalities.

Cap: A telomeric band, either G-band light or dark.

Centromere: The primary constriction; point of attachment of the spindle fibers.

Dark band: A G-band that is denser and darker than its neighboring bands. The dark bands are made up of A-T-rich DNA, are late replicating, and relatively low in gene content.

Distal: Closer to the telomere than to the centromere.

Heteromorphism: Variation in size, shape, or staining properties between homologous chromosomes.

Inversion: A two-break rearrangement with a 180-degree reversal of the segment before it is reinserted and the breaks repaired. If it involves the centromere, it is known as pericentric, and if not, paracentric.

Light band: Also a band, but lightly staining, compared with its neighboring bands. The light bands are rich in G–C DNA, early replicating, and are relatively more gene dense.

Metacentric: A chromosome with the centromere located near the middle of the chromosome, with a ratio of short arm to long arm of 1 : 1 to 1 : 1.3.

NOR: Nucleolar organizing region. Located in humans in the stalk of the short arm of acrocentric chromosomes.

p-arm: Short arm.

Pericentric: The area around and including the centromere.

Proximal: Closer to the centromere than to the telomere.

q-arm: Long arm.

qh region: The heterochromatic region in the centromeric and/or long arm regions of chromosomes 1, 9, 16, and Yq that have variable length and/or band patterns. The “q” refers to the long arm of a chromosome and the “h” refers to the heterochromatin (see Chapter 8, Table 8.4).

Secondary constriction regions: Chromosomal centromeres are called primary constrictions, and the secondary constrictions are those that may cause the chromosome to pinch in again as though there were another centromere present. These are caused by either the presence of heterochromatin (e.g., at the distal end of some 9qh regions), or by the presence of stalks and satellites at the end of the acrocentric chromosomes (chromosomes 13, 14, 15, 21, and 22).

Satellites: The knobs that are found on the distal short arm of most acrocentrics. They are located above the chromosome stalks, where the NORs are located.

Shoulder band: If a chromosome is analogous to a person with a head and neck (p-arm and centromere), then the shoulder is just below the centromere. Chromosomes with a distinct dark band in this region are said to have a dark shoulder band, such as is seen in chromosomes 4 and 10.

Stalk: The long thin chromatin to which the satellites are attached. The NOR regions are located in the stalk regions.

Submetacentric: Chromosome with the centromere located closer to one end of the chromosome than the other with a short arm to long arm ratio of 1 : 1.3 to 1 : 7. Chromosomes that structurally show an arm ratio as low as 1 : 1.3 are sometimes called metacentric.

Telocentric: A chromosome with no short arm, not usually found in human chromosomes but common in other mammals, such as mouse.

Telomere: The ends of the chromosome arms, with the DNA sequence TTAGGG in multiple repeats.

Window: A region of G-band light material surrounded by two dark bands, such as is seen on the 3p, 6p, 8p, and 9p.

Window frame: The two dark bands surrounding a lighter “window” region, if they are distinct.

References

1. Wyandt HE, Tonk VJ. *Atlas of Human Chromosome Heteromorphisms*. Norwell MA: Kluwer Academic Publishers, 2008.
2. ISCN 2013: An International System for Human Cytogenetic Nomenclature. Shaffer LG, McGowan-Jordan J, Schmid M, eds. Basel: S. Karger Publishers, 2013.
3. Olson SB, Magenis RE, Lovrien EW. Human chromosome variation: the discriminatory power of Q-band heteromorphism (variant) analysis in distinguishing between individuals, with specific application to cases of questionable paternity. *Am J Hum Genet* 1986;38:235–252.
4. Josifek K, Haessig C, Pantzar T. Evaluation of chromosome banding resolution: a simple guide for laboratory quality assurance, *Applied Cytogenetics* 1991;17(4):101–105.
5. Stallard R, Johnson W. Nonsubjective method for estimating the resolution of banded chromosomes. *Am J Hum Genet* 1983;35:155A.
6. Welborn J, Welborn R. Banding resolution of human chromosomes: A method of accuracy and simplicity. *Am J Med Genet* 2005;47(8):1140–1143.

CHAPTER 8

ISCN: the universal language of cytogenetics

Marilyn S. Arsham¹ and Lisa G. Shaffer²

¹(retired), Western Connecticut Health Network, Danbury, CT, USA

²Paw Print Genetics, Genetic Veterinary Sciences, Inc., Spokane, WA, USA

8.1 Introduction

This chapter has been based on the standards recommended by the international standing committee on human cytogenomics nomenclature, including new sequence-based cytogenomic nomenclature developed in collaboration with the Human Genome Variation Society (HGVS) sequence variant description working group, as defined in the *ISCN 2016: An International System for Human Cytogenomic Nomenclature (2016)* report published in 2016 [1]. Section locations will be referenced in order to aid the reader in further investigation. Any rule or standard that has changed in an ISCN publication subsequent to 2016 will supersede any rule or standard that has been described in this chapter. Laboratory policy differing from any topic described in this chapter will also supersede this chapter's recommendation.

Because this chapter has been developed as an introduction to nomenclature, not every topic has been covered that is included in *ISCN 2016* [1]. Among those not included are meiotic nomenclature [1, Chapter 12], chromosome breakage [1, Chapter 10], subtelomeric metaphase *in situ* hybridization [1, section 13.2.2], *in situ* hybridization on extended chromatin/DNA fibers [1, section 13.4], reverse *in situ* hybridization [1, section 13.5], chromosome comparative genomic hybridization [1, section 13.6], region-specific assays [1, Chapter 15], sequence-based assays [1, Chapter 16], and various other topics dealing with complicated rearrangements or specialized testing.

The primary emphasis of this chapter is on writing karyotypes for conventional cytogenetics and metaphase/interphase FISH (fluorescence *in situ* hybridization) reporting. When demonstrating a specific event in a karyotype example in this chapter, we may bold the symbol or event for attention; however, the reader should be aware that no symbol or character will appear bold in an actual karyotype. See Chapter 9, Constitutional chromosome abnormalities, to learn how each structural abnormality introduced in this chapter is formed.

8.2 Language

For a student of the 21st century, it is hard to imagine a world without cell phones and personal computers, but for geneticists of the 1960s and 1970s, communication among laboratories in other states and countries relied on expensive long-distance landline phone calls or time-delayed “snail” mail. It was a time when mouth-pipetting with ungloved hands was the norm, when sterility was controlled by a Bunsen burner flame on an open bench, and when the rite of passage as a technologist in a cytogenetics laboratory was the painfully unavoidable, acid-blistered thumbprint; yet the discoveries from these challenging times still influence the basic processes today. One of the most remarkable of all accomplishments from this noncomputerized era was the creation of a universal cytogenetic language that would enable geneticists across the globe to communicate about interesting cases and share in discovery and innovation.

Nomenclature for cytogenetic-based karyotypes originated at a conference in Denver in 1960 [2], where a select group of participants established a standard for human chromosome nomenclature, which is still the cornerstone of human cytogenomics today. Subsequent advances were incorporated from conferences held in 1963 (London) [3], 1966 (Chicago) [4], 1971 (Paris; introduced

banding) [5], 1978 (consolidated into one ISCN publication) [6], 1981 (high resolution banding) [7], 1985 [8], 1991 (neoplasia) [9], 1995 (FISH) [10], 2005 (300- and 700-band level idiograms) [11], 2009 (idiogram redrawn, microarray) [12], and 2013 (region-specific assays) [13]. A history of these conferences is provided at the beginning of each ISCN publication.

This international system for human cytogenomic nomenclature (ISCN), which was initially developed through the generous support of the March of Dimes Foundation, was designed to be so simple, and yet so flexible, that its basic structure is still in practice today. Recognizing the symbols that define the parts of a chromosome and the events affecting these parts, along with how they are put together, is the heart and core of ISCN and what this chapter will discuss.

8.2.1 Centromere

The critical structure for maintaining an organism's chromosome balance is the centromere (cen). Also known as the primary constriction, it can sometimes be seen as a small colorless sphere at the constricted junction of a chromosome's duplicated arms. Human chromosomes have only one centromere (monocentric), positioned near the center (metacentric), towards the end (acrocentric) or somewhere in-between those two points (submetacentric) (see Figure 8.1). Telocentric (at the end) positions may exist in other species, but not in humans.

When functioning properly, the centromere brings order to cell division by anchoring each identical chromosome arm towards opposite poles, thus ensuring a balanced migration to the two daughter cells (see Chapter 6, Chromosome stains, for an explanation of, and protocols for, specific stains that can be used to distinguish centromeric structures). This stability, however, can become disrupted when more than one centromere is present (see 8.5.2, Dicentric), unless the chromosome can compensate for the extra or missing centromere (see 8.5.2, Pseudodicentric, and 8.6.5, Neocentromere derivative), or the centromeres are so close together that they function in concert (see 8.6.7, Acrocentric derivatives).

In addition to its influence on cell survival, the centromere is also central to almost every aspect of ISCN nomenclature. Generally speaking, the number of independent centromeric structures in a cell will determine the cell's total chromosome count; therefore, a structure that appears to have two centromeres (see 8.5.2, Dicentric) will still be counted as one chromosome. Centromere origin will determine the chromosome's placement both in the pictorial arrangement of chromosomes (see 8.2.4, Karyogram) and in the symbolic description of their abnormalities (see 8.3, Karyotype). The single, random presence of a noncentromeric chromosomal fragment (i.e., acentric, abbreviated ace) is presumed to be the result of cultural artifact, and thus would not be included in the total chromosome count, nor would it be defined within the symbolic cell description (karyotype); however, it would be recorded if the specimen is being evaluated for breakage studies [1, section 10.3]. On the other hand, a fragment that demonstrates mitotic stability through its recurring presence would be included in the karyotype, but may (see 8.6.5, Neocentromere derivative) or may not (see 8.7.8, Double minute) be included in the chromosome count.

The third influence is the centromere's visual separation of a metaphase chromosome into two arm lengths – referred to as the shorter p (for *petit*) and longer q arm (some cytogeneticists recall a discussion that the short and long arms were designated p and q, because, in mathematical terms, $p + q = 1$). Geneticists at the 1960 Denver Conference [2] used these arm ratios to

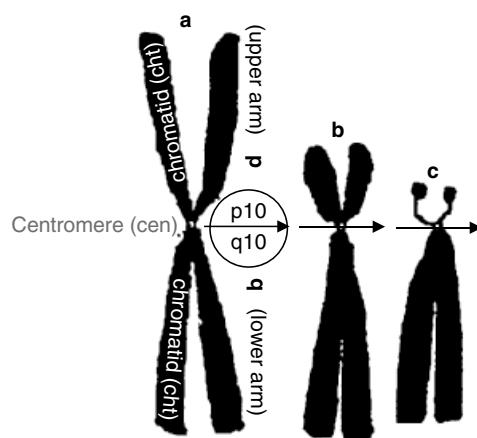


Figure 8.1 Human centromere positions. Duplicated sister chromatids are held together by a centromere. (a) **Metacentric:** centromere is centrally positioned; arms are of near equal length. (b) **Submetacentric:** centromere off-center, creating upper and lower arms of different lengths. The shorter arm (in normal chromosomes) is the upper or p arm, and the longer arm is the q arm. (c) **Acrocentric:** centromere near the end, with only a small p arm. The inherited stalk (pstk) that connects the satellite (ps) to the p arm is a nucleolus organizing region (NOR).

propose the A to G group classification, by arranging homologous pairs, called autosomes, in decreasing size and similar centromeric positioning, and then sequentially numbering each pair from 1 (largest) to 22 (smallest) for identification purposes. The remaining two were presumed to be the sex chromosomes, X and Y. Autosomes are now referred by their number, rather than the original group letter, but some laboratories may still spatially separate the seven autosomal groupings for visual orientation in the karyogram.

Finally, but of great importance to ISCN, is the centromere's pivotal role in band numbering, which begins at the centromere (p10, pronounced p-one-zero, for the short arm and q10 for the long arm) and proceeds in opposite directions towards their respective telomeres, or terminal ends (pter, pronounced "p-ter," for the short arm and qter for the long arm). When describing the relationship of band positions on a chromosome arm, a distal direction moves away from the centromere, towards the arm's terminal end, and a proximal direction moves towards the centromere (see Figure 8.2). For example, a band described as being more distal to another indicates that it is further from the centromere, which also means it is closer to the terminal end, in relation to the other band. Because band numbering begins at the centromere, a distal band will also have a higher band number than a more proximal band on the same chromosome arm. The centromere is so central to a cell's survival that it is no wonder the creators of this cytogenetic language used these essential structures as "ground zero," that is, p10 and q10, for band numbering.

8.2.2 Homologue

A normal, mitotic metaphase cell will show twenty-two *pairs* of autosomes, that is, 22 pairs of visually matching homologues (also spelled homologs), and two sex chromosomes, with the understanding that heteromorphic variation (see 8.8.2, Heteromorphic variations) can contribute some visual differences between each matching pair. Likewise, condensation discordance between homologous chromosomes, which results from asynchronous replication of the pair during synthesis, has been reported as high as 14% [14], which can also add to visual differences between normal homologues. The only truly "odd couple" are the X and Y sex chromosomes in males; yet, they too are a genetic pair, as proven by their unique head-to-head association during crossing-over in meiosis [1, Chapter 12]. In fact, an error in this meiotic association may result in the transfer of the *SRY* gene locus (sex-determining region of the *Y* chromosome) to the X sex chromosome, leading to sex reversal XX males and XY females [15].

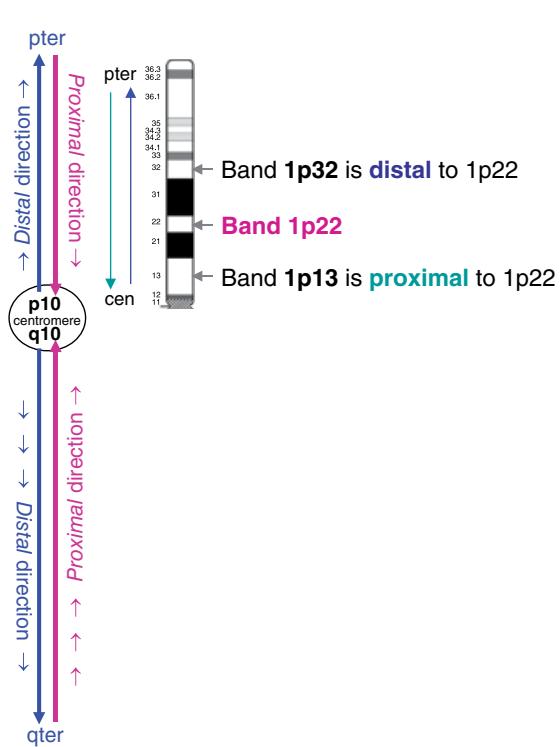


Figure 8.2 Distal vs. proximal. Bands are sequentially numbered in a **distal** direction; therefore, numbers will increase as they move farther from the centromere and closer to the terminal end of that same arm. Band 1p22 is proximal to band 1p32, because it is closer to the centromere, but it is distal to band 1p13, which is closer to the centromere.

ISCN diagram of human chromosome 1 at 400-band level (© 2009 Nicole Chia) used by permission of the artist. Reproduced from *ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013*. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

Each mitotic homologue in turn is composed of two chromatids (cht) that are held together by the centromere. Genetically speaking, these chromatids are two identical chromosomes awaiting separation to opposite daughter cells. While being held together by the centromere, however, they are referred to as sister chromatids (sc), and the unit they create is the metaphase chromosome (Figure 8.1).

Three cytogenetic aides – the idiogram, karyogram and karyotype – are essential tools for identifying each homologue, comparing their appearances, and communicating these results to other professionals in the field. *ISCN 2016* (1, section 2.2.1) recommends that the term idiogram be used to refer to the “diagrammatic representation of a karyotype.” The term karyotype refers to the “normal or abnormal, constitutional or acquired, chromosomal complement of an individual, tissue or cell line,” and the term karyogram is used to reference a “systemized array of the chromosomes prepared either by drawing, digitized imaging, or by photography, with the extension in meaning that the chromosomes of a single cell can typify the chromosomes of an individual or even a species” [1, section 2.2.1, p. 7]. Because a language cannot exist without an alphabet, we begin this trilogy with the idiogram.

8.2.3 Idiogram

The idiogram is the roadmap of our genetic language, a schematic representation of the bands one would expect to see on a normal chromosome. The Paris Conference (1971) report [5] introduced a unique identification pattern for each of the 24 distinct human somatic chromosomes by using the intensely-stained, AT-rich, late-replicating Q-bands (revealed via fluorescence using quinacrine mustard stain) and G-bands (produced by digestion with trypsin and stained with Giemsa or similar color stain), along with reverse R-bands to verify the CG-rich, early replicating, gene-rich, G-light bands [1, sections 2.3.1, 2.5]. This new level of definition provided geneticists not just with the ability to pinpoint the location of a break and compare the effects of the gene(s) in that region with the clinical picture, but also with the capability to explain that information to anyone in the world.

Original landmark regions and bands followed a specific numbering pattern for all chromosomes (see Figure 8.3). Shaded areas within the p and q arms were sequentially numbered in a distal direction, moving from centromere (p10 or q10) to their respective terminal ends (pter and qter). These original landmark regions (that is, the first digit of a band notation, e.g., q1) changed at the proximal border of an intensely staining G-band. Landmark bands, or the second digit of the band notation (e.g., q11), in turn, increased at the interface of intermediary shading within its landmark region. As resolution improved, landmark bands further differentiated. These became sub-bands, which were numbered in similar fashion after a decimal “point” (as it is pronounced), for example, 17p11.2 (see Figure 8.4). The original 1971 idiogram can be seen in the appendix of *ISCN 2016* [1, chapter 19].

ISCN 2016 [1, Figure 5] defines five band-level idiograms for each chromosome – at 300-, 400-, 550-, 700- and 850-band resolutions. These levels represent the estimated total number of visible bands that can be seen at various stages of the mitotic cycle, with those in prophase showing the highest definition (i.e., 850-band level). Some prophase chromosomes may even reveal sub-bands beyond the 850-band definition (see Figure 8.5), but for practical purposes, 550- to 850-band levels are considered sufficient for cytogenetic analysis [1, section 2.4]. Because every chromosome arm is numbered sequentially from centromere to terminal end, numbers by themselves are not unique, even between the p and q arms of the same chromosome; therefore, the arm is always included in a band notation, e.g., q11, and the chromosome number, for example, 7q11, may precede the arm, if needed for clarity.

8.2.4 Karyogram

A karyogram is the pictorial alignment of the 22 pairs of homologous autosomes from one metaphase cell, sequentially numbered from 1 to 22 (largest to smallest) by their unique band patterns, and the two sex chromosomes, XX (female) or XY (male) (1, section 2.2.1). Alignment of normal homologues is centered with the centromere so that bands can be compared equally between the two arms of the homologous pair. Some rearrangements, however, may visually benefit from an alternate alignment. Therefore, laboratory convention ultimately determines karyogram placement and orientation.

Rearrangements are generally positioned in the karyogram based on the origin of their centromere (Figure 8.6). Certain rearrangements, however, especially whole arm rearrangements (see Figure 8.7), cannot distinguish which partner retained its centromere; therefore, karyogram placement will become dependent on other factors, for example, which arm is involved (p before q), and which involved chromosome has the highest karyotype order priority, generally, X before Y before autosome in increasing numerical order. Unidentifiable structures, e.g., centric markers and rings (see 8.7, Symbols of uncertainty), are placed in a separate section of the karyogram; for example, some software analytical systems will use an extra row below the autosomes in the karyogram for these structures.

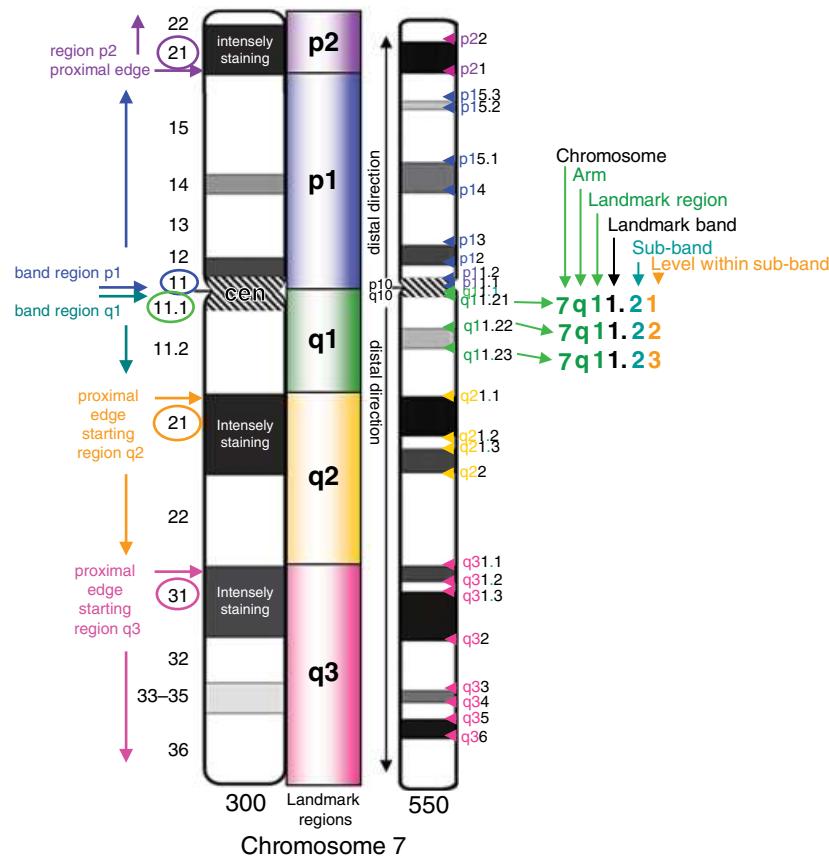


Figure 8.3 Band derivation. Early geneticists used G- and Q-band patterns to identify 86 staining regions, 66 of which were marked by intensely stained bands. These intense segments subdivided a chromosome arm into “landmark” regions, which is still today the first digit of the band number after arm designation. In this figure chromosome 7 has one intensely staining region in the short arm, which will divide the p arm into p1 and p2 regions. The long arm shows two intense bands, thus forming three regions (q1, q2, q3) [1, Table 3]. Band 7q11.2 demonstrates the breakdown of a sub-band into three further band levels: q11.21, q11.22, and q11.23 at the 550-band level for this chromosome. See insert for color representation of this figure.

ISCN diagram of human chromosome 7 at 300 and 550-band level (© 2009 Nicole Chia) used by permission of the artist. Reproduced from *ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013*. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.



Figure 8.4 Band notation. When reading a band notation, each digit after the chromosome number (or letter) is pronounced separately in order to emphasize its numerical independence, e.g., “seventeen, p, one, one, point, two”. Chromosome arm is always included in a band notation; chromosome number is used when clarification is needed.

Historically speaking, the karyogram was not always as informative as it is today. For the 1960s, banding techniques were not yet standard protocol and solid staining revealed minimal detail. Anyone who has worked with nonbanded chromosomes can appreciate the obvious limitation of these early karyograms (which were called karyotypes at that time). Nonbanded homologues were paired by size and similar centromere positioning into seven autosomal groups, A–G, and two sex chromosomes, X and Y, but their precise identity within each group was guesswork, except for the A (chromosomes 1–3) and E (chromosomes 16–18) groups, which have more exaggerated differences in centromere positions. Selecting the X sex chromosome was also a challenge without the aid of visual band patterns, because its size and centromeric position resembled other C-group chromosomes.

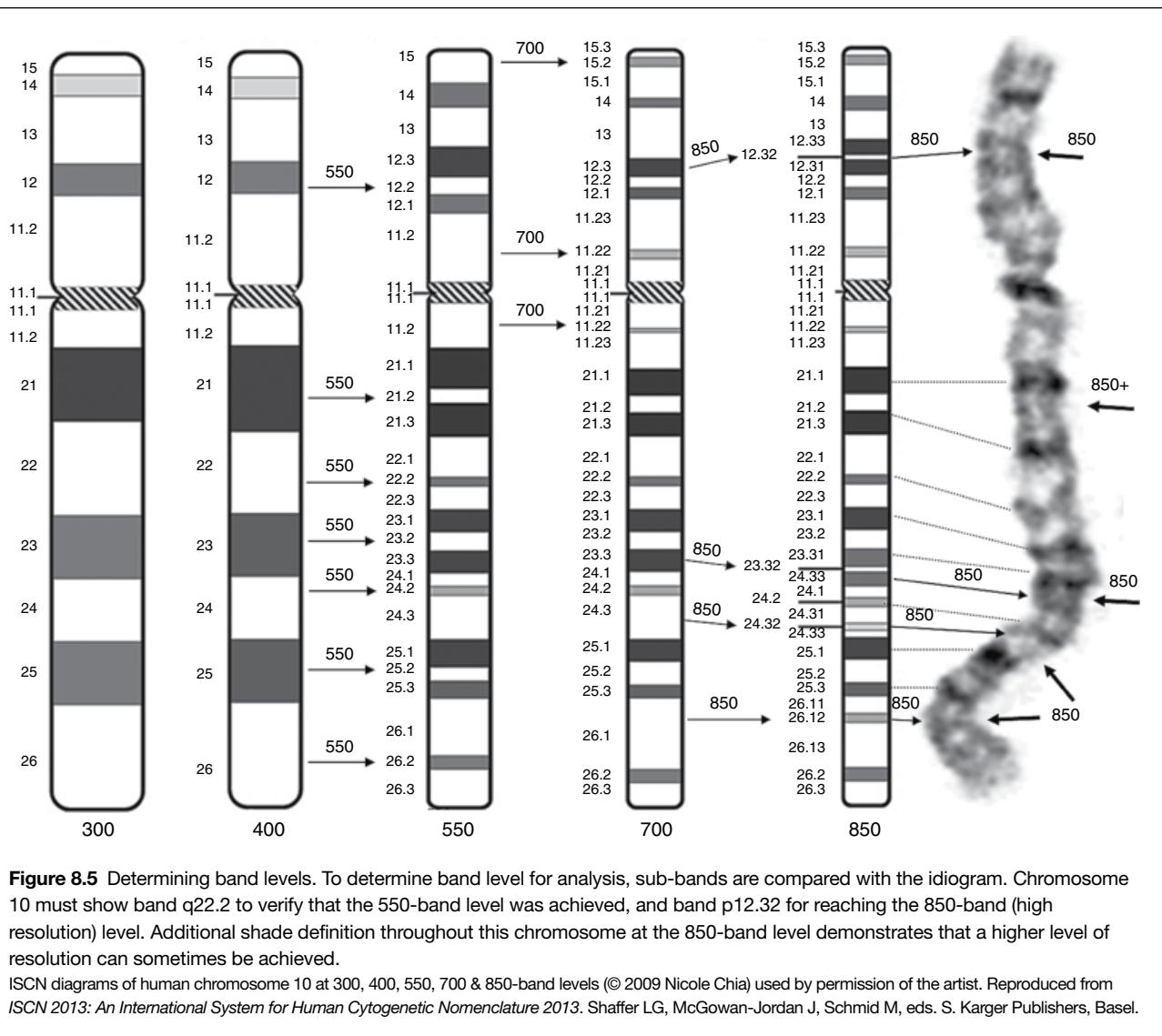


Figure 8.5 Determining band levels. To determine band level for analysis, sub-bands are compared with the idiogram. Chromosome 10 must show band q22.2 to verify that the 550-band level was achieved, and band p12.32 for reaching the 850-band (high resolution) level. Additional shade definition throughout this chromosome at the 850-band level demonstrates that a higher level of resolution can sometimes be achieved.

ISCN diagrams of human chromosome 10 at 300, 400, 550, 700 & 850-band levels (© 2009 Nicole Chia) used by permission of the artist. Reproduced from *ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013*. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

In fact, it was not uncommon in those days to distinguish sex by the number of C- and G-group chromosomes that were present, for example, 15 C-group and five G-group being male; 16 C-group and four G-group being female.

This reliance on assumption led to an interesting dilemma after the discovery of banding, when geneticists learned that the smallest G-group autosome, by definition the 22nd chromosome, was actually responsible for Down syndrome. The name for the syndrome was already established as Trisomy 21; therefore, the chromosome numbers and placement on the karyogram were switched, rather than changing the syndrome's name. Hence, chromosome 21 is still today the smallest human autosome [16], and also the only autosome (in its normal form and excluding normal polymorphisms) within the human karyogram that is smaller than its succeeding chromosome.

We now turn to the last segment of our trilogy, and the remainder of our chapter – the karyotype.

8.3 Karyotype

The unique language of cytogenetics that translates what is analytically observed from multiple cells of a specimen into a universally understood string of alpha-numeric symbols is the karyotype. In other words, the karyotype is to chromosomal constitution as blood type is to blood – it is a way for geneticists to explain the chromosomal makeup in brief, symbolic formulae. It is thus regarded as one of the most important communication tools of our field. A normal human karyotype includes the total number of centric chromosomes per cell and the sex complement, that is, 46,XX (normal female) or 46,XY (normal

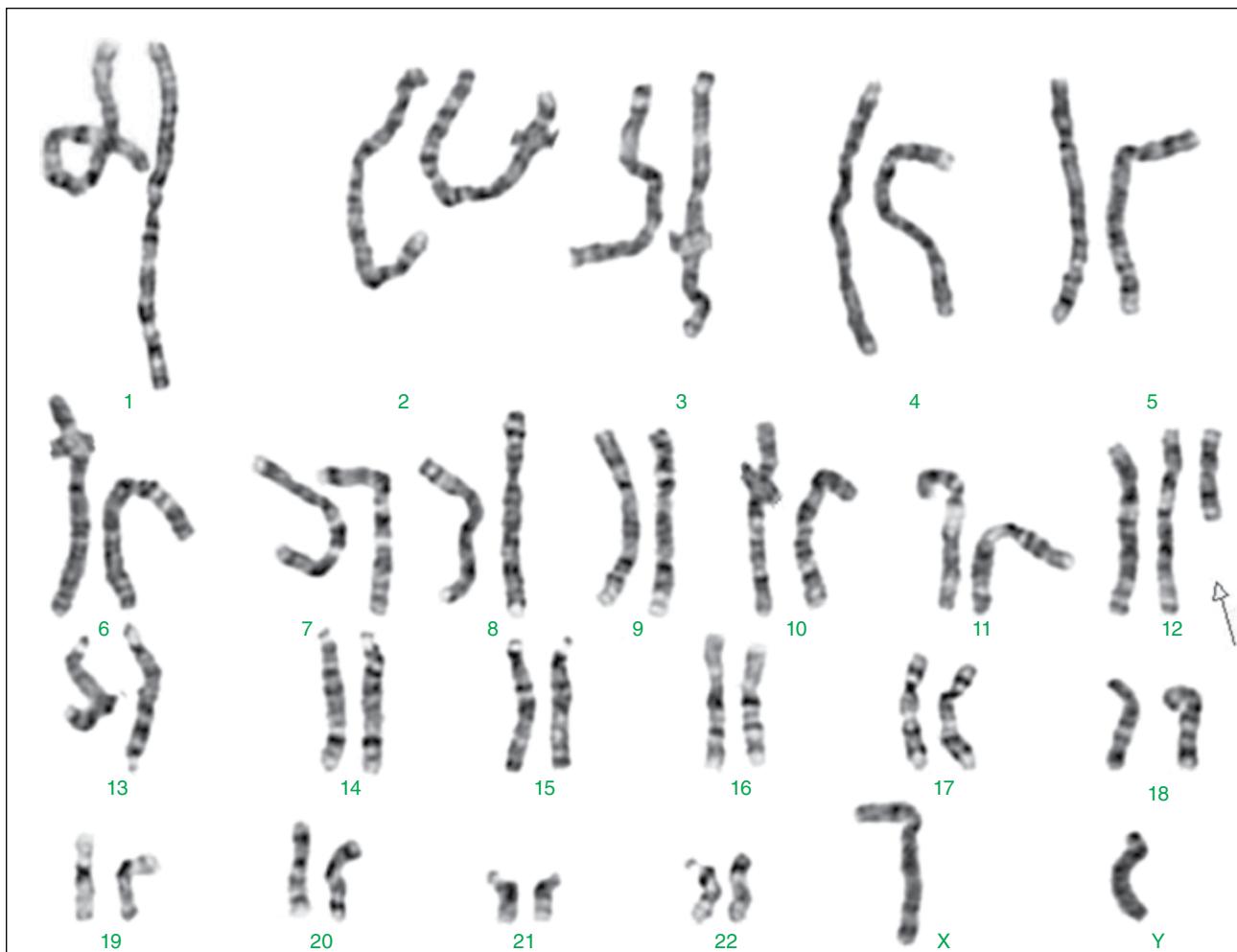


Figure 8.6 Abnormal karyogram. Chromosomes from a cell are paired in a karyogram by banding patterns into 22 autosomal groups (1–22) and two sex chromosomes (X and Y). Generally speaking, chromosomes are aligned at the centromere; however, some pericentric inversions may be more easily compared if aligned by their terminal ends. An arrow is used to point to any abnormal structure, which in this karyogram is an additional isochromosome for the short arms of chromosome 12.

male), separated by a comma, without spaces. No additional description is necessary unless a reportable change has occurred (see 8.8, Random versus reportable).

The reader should also keep in mind that the definition of “normal” in cytogenetic terms is limited by the resolution at which a change can be visualized; for routine cytogenetics, this translates to a band size of approximately 5–10 megabases (Mb) of DNA [1, section 2.5]. Therefore, not all “normal” appearing karyograms are genetically normal, but they are considered normal at the level of detection that routine chromosomal analysis can provide.

8.3.1 Chromosome count

Excluding those events that qualify a karyotype’s chromosome count (see later), the total number of clonal, independent, centric structures in a cell or cell line is generally the first item described in a karyotype, followed by a comma and the sex chromosome complement. Random, nonclonal gains or losses of centric structures, or the presence of acentric fragments, will not change the count, nor will they be described in the karyotype, unless they meet reportable standards (see 8.8, Random versus reportable). The replacement of a normal homologue with a rearranged copy of the same chromosome will also not change the count, nor will the karyotype show any loss or gain, other than what can be interpreted from the karyotype description of its abnormal replacement. “When normal chromosomes are replaced by structurally altered chromosomes, the normal ones should not be recorded as missing.” [1, section 9.1].

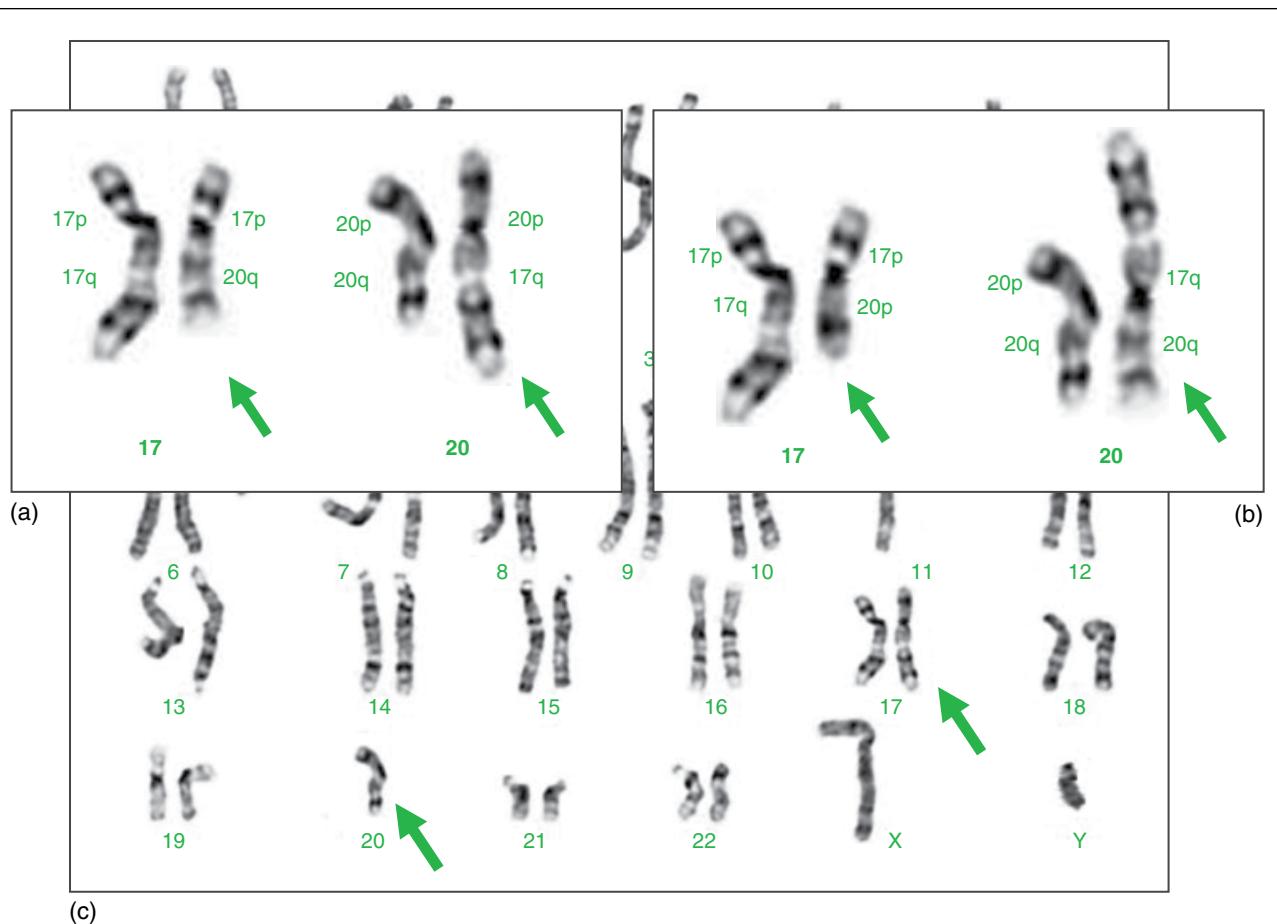


Figure 8.7 Karyogram placement with whole arm translocations. Because either partner (or both) could potentially have contributed its centromere in a whole arm translocation (see 8.6.7, Nonacrocentric whole arm derivatives), the chromosome with the short arms of highest karyotype order priority, i.e., X before Y before autosomes in ascending numerical order, will be placed first in the karyogram. (a) In this p-to-q arm rearrangement between chromosomes 17 and 20, the chromosome holding 17p (attached to 20q) is placed alongside the normal homologue 17, and its 20p/17q partner is placed alongside the normal homologue for chromosome 20. (b) If a p-to-p arm rearrangement occurs, 17p (attached to 20p) will be positioned alongside the normal homologue 17. Its 17q/20q partner will be placed alongside the normal 20 homologue, with alignment being with the two long arms of chromosome 20. (c) If one of the two translocation partners is lost, creating an unbalanced situation, the rearrangement becomes a derivative chromosome and is placed in the karyogram following highest karyotype order priority, irrespective of which arm is present. For example, if only the 17q/20p derivative chromosome is present, it will be placed next to the normal 17 homologue, parallel with the 17q arms. Arrows will point to both the derivative and the empty position. The resulting imbalance in this karyogram is a loss of one copy of 17p and 20q.

Like balancing an equation, one might then expect that all pluses and minuses described in a karyotype should add up to the chromosome count (e.g., $46 + \# \text{ gains} - \# \text{ losses} = \text{chromosome count}$), and for the majority, it does. For example, a +21 described in the karyotype would reflect a gain of one chromosome: 47,XX,+21. Not all gains or losses, however, use a plus or minus sign. Some examples are:

- Gain or loss of a sex chromosome in constitutional studies will not show a plus or minus sign to justify the 47,XXY or 45,X chromosome count (see 8.4.4, Sex chromosome aneuploidy).
- Double minutes are not counted but are reported in the final report, described in the karyotype without plus signs, and are included within the karyogram (see 8.7, Symbols of uncertainty).
- “Balanced” acrocentric rearrangements involving centromeric breakpoints will also not use a minus sign, but again, the 45-chromosome count in the karyotype would still indicate the loss (see 8.6.7, Acrocentric derivative for a more complete explanation).

- Likewise, a composite karyotype, by definition, combines clonal events that have been seen in some but not all of the cells described within the composite (see 8.7.10, Composite karyotype); it is, therefore, often out-of-balance with the chromosome count.

For these reasons, verifying the chromosome count by balancing the sum of its described events without understanding the descriptive nature of the events being counted may not always work; therefore, the karyotype writer or reader must understand the exceptions.

Although chromosome count is generally first in a karyotype, certain events that qualify this count will require its symbol to be placed prior to the count, as in mos for mosaicism, chi for chimerism, and end for endoreduplication. A space will separate the alphabetic symbol from the numerical count. Nonalphabetic symbols, such as the double slant line (//) for chimeric clones [1, section 4.1], which is used in donor–recipient bone marrow transfusion studies karyotypes, would not require a space, for example, //46,XY (see 8.9.1, Constitutional mosaicism, 8.4.1, Polyploidy and endoreduplication, and 8.10.12, Bone marrow transplant chimerism).

8.3.2 Event symbols

To simplify our discussion throughout this chapter, we are using the term “event” to represent any independent change to the 46,XX or 46,XY description, whether it be numerical or structural, balanced or unbalanced, interchromosomal or intra-chromosomal, acquired or constitutional, abnormal or normal variation, and reportable or not reportable. Event symbols can be alphabetical, mathematical, and/or grammatical (see Tables 8.1 and 8.2) and should be followed by the centric chromosome(s) involved in the event, if identifiable.

Alphabetic symbols are meaningful letter abbreviations that represent a specific event. They generally range from one to four letters, as in t for translocation (single letter code), cp for composite (two letters), del for deletion (triplet), and idic for isodicentric (four letters), although there are exceptions (see Table 8.1 for a list of alphabetical symbols being used in this chapter).

Mathematical signs, and other symbols associated with quantity, include the plus sign for a gain (+21), minus for loss (-3), multiplication (mar \times 2), using no spaces, for multiple copies (which is only used with repeated, structurally abnormal descriptions), the slant line (/) as a separator for multiple cell lines and clones, and the approximation or tilde (1~3) sign, using no spaces, to indicate a range or mean value. For example, the short-form karyotype for a numerical gain of a structurally normal chromosome 21 (referred to as trisomy, indicating three copies of the same “body” or chromosome) in a female patient would be written 47,XX,+21, using a comma, no spaces, to separate the abnormality from the sex complement. Chromosome count will also increase because an additional centric structure is present. Describing gains or losses of sex chromosomes in a karyotype are handled differently in constitutional studies and will be discussed under section 8.4, Numerical events.

The most commonly used **grammatical** symbol is the comma. As mentioned above, it separates the total number of chromosomes from its sex chromosome complement, and separates each described event (numerical or structural, balanced or unbalanced) within a karyotype string of events. No spaces are placed before or after each comma, for example, 47,XX,+21 and because the comma indicates continuation, it never ends the karyotype.

Another commonly used grammatical sign is the parenthesis, which is generally, but not always, used in two adjoining sets, for example, del(16)(p13.1), without intervening spaces or punctuation. This format keeps the event’s chromosome(s) and respective breakpoint(s) separate but connected to each other.

Other grammatical symbols include the semicolon (;) when separating different chromosomes and their respective breakpoints within adjoining parentheses; the question mark (?) when a structure is unidentifiable, or the precise breakpoint is not certain; and the period (.ish) when more than one method is being reported in the same karyotype. This period should not be confused with the decimal “point” that precedes the sub-band notation described in 8.2.3, Idiogram.

8.3.3 Structural breakpoint

Structural events involve chromosome breakage. A chromosome break (chrb), also referred as an *isolocus* or *isochromatid* break, is defined as “discontinuity at the same locus in both chromatids of a single chromosome” [1, section 10.2.1]. Defining a structural change in the short form karyotype includes the event’s symbol, the chromosome(s) involved, and the involved breakpoint(s). Chromosome breaks *may* become reportable if the same event is seen in more than one cell (see 8.8, Random versus reportable). A gap (g), on the other hand, is a “nonstaining region (achromatic lesion)” in one sister chromatid arm (chtg) [1, section 10.2.1]. Gaps (chtg and chrg) and chromatid breaks (chtb) are

Table 8.1 Alphabetic symbols. This table provides a list of selected symbols that are being used in this chapter

Symbol	Explanation
ace	acentric fragment
add	additional material of unknown origin
amp	amplified
arr	array
c	constitutional anomaly
cen	centromere
chi	chimera, containing more than one constitutional cell line from more than one zygote
con	juxtaposition of two different labeled sequences (FISH)
cp	composite
ct	chromothripsis
del	deletion
der	derivative chromosome, derived from multiple rearrangements within a single chromosome or a previous rearrangement between two or more different chromosomes; the reciprocal pair is lost, creating an unbalanced karyotype
dic	dicentric
dim	diminished signal size (FISH)
dmin	double minute
dn	de novo
end	endoreduplication
enh	enhanced signal size (FISH)
fra	fragile site
GRCh	Genome Reference Consortium human
hsr	homogeneously staining region
i	isochromosome
idem	used in neoplastic subclones to indicate the presence of aberrations previously defined in the first-mentioned, most basic clone (stemline); additional variations follow the comma after idem
idic	isodicentric
inc	incomplete
inh	inherited
ins	insertion
inv	inverted
ish	in situ hybridization
mar	marker of unidentifiable chromosomal material containing a centromere
mat	of maternal origin
mos	mosaic, containing more than one constitutional cell line (from one zygote)
n	haploid number for the species (23 for humans)
neg	no presence of the rearrangement being investigated
neo	neocentromere, a newly formed or activated centromere in a noncentromeric region
nuc ish	nuclear/interphase in situ hybridization (FISH)
p	short arm
pat	of paternal origin

Table 8.1 (Continued)

Symbol	Explanation
pos	detection of the rearrangement being investigated
psu dic	pseudodicentric, i.e., when one centromere in a dicentric chromosome is inactivated
psu idic	pseudoisodicentric, when an isochromosome is formed by a break within the opposite arm, not at p10 or q10 AND one centromere is inactivated
pter	terminal end of the short (p) arm
q	long arm
qdp	quadruplicated
qter	terminal end of the long (q) arm
r	ring chromosome
rea	recombinant
rob	Robertsonian or whole-arm translocation between two acrocentrics in constitutional karyotypes only
rsa	region-specific assay
s	satellite
sce	sister chromatid exchange
sdl	sideline
sep	separation of adjacent locus FISH signals as a result of rearrangement
sl	stemline or most basic clone
stk	satellite stalk
t	translocation
tas	telomeric association
tr	triradial
trc	tricentric
trp	triplicated
upd	uniparental disomy
wcp	whole chromosome paint

generally regarded as a by-product of cultural processing, especially if the culture had been treated with reagents to improve resolution quality, for example, 5-bromo-2'-deoxyuridine. Random occurrences are not included in a karyotype, but would be recorded in breakage studies. This chapter does not discuss the requirements for scoring and documenting fragile sites, breakage and clastogenic exposure studies; therefore, the reader should consult Chapter 10 in *ISCN 2016* [1].

Selecting the precise breakpoint of a structural event is not difficult when the break occurs within a band: it simply is that band. In Figure 8.8, for example, the leftmost chromosome demonstrates a chromatid break (chtb) clearly within band 16p13.1. The challenge begins when the break appears at the border between two bands. The general rule is to choose the more *distal* band [1, section 4.2], but when either position can be a likely candidate, the “or” symbol can be used, for example, del(16)(p13.1 or p13.2).

Suboptimal morphology in some neoplastic tissue may also hinder the capability to pinpoint the band within a landmark region; therefore, it is also acceptable to use the region number, for example, del(1)(p3), or to follow the region number with a question mark (see 8.7, Symbols of uncertainty), for example, del(1)(p3?), which indicates that the break occurred within landmark region 3, but any further localization is uncertain [1, section 4.2]. *ISCN 2016* also adds that “breakpoints within the same rearrangement or karyotype string can be at different levels of resolution reflecting the precision of the karyogram.” [1, section 4.2, p. 40]

Table 8.2 Nonalphabetic symbols. This table is a partial list of nonalphabetic symbols used in this chapter. The list does not include every symbol; therefore, the reader should refer to Chapter 3 in *ISCN 2016* [1] for a more complete list

Example	Symbol	Definition and uses
46,XY,del(7)(pter→q32:)	ARROW →	ARROW indicates the intervening region(s) between two points in the detailed karyotype system.
46~58<2n>,XX,+...	BRACKETS, angle <n>	ANGLE BRACKETS with ploidy (<i>n</i>) level are placed immediately after the total chromosome count, indicating the ploidy level at which the karyotype is being written. It is used when the count range can span two different potential bases [1, section 11.2].
46,XY[20]	BRACKETS, square [20]	SQUARE BRACKETS with cell count are placed at the end of each cell line when multiple lines exist, as in mosaic or chimeric cell lines in constitutional karyotypes; after recipient:donor cell lines in transplant studies; after individual clones; at the end of all neoplastic (cytogenetic and FISH) karyotypes; and optionally following relevant, individual events within a composite karyotype.
(pter→p11.2::p11.2→qter)	COLON, double ::	DOUBLE COLON indicates a break-reattachment event in the detailed karyotype description.
46,XY,del(7)(pter→q32:)	COLON, single :	SINGLE COLON indicates a break, but no reattachment in the detailed karyotype description.
46,XX,del(7)(q32),+21,-22	COMMA,	The simple COMMA indicates continuation and is placed between each independent event or locus designation in a karyotype string. No spaces are used before or after the comma. For example, it: a) separates the total chromosome count from the sex complement: 46,XY b) is placed after the last normal sex chromosome if the karyotype continues with additional description: 47,XY,+21 c) separates each event unit within a karyotype string of events or within and between parentheses describing the names and signal patterns of co-hybridized sets.
46,XY,+21,-22	MINUS and PLUS –, +, ++	When placed before a chromosome or event within a karyotype, PLUS or MINUS signs represent a gain or loss, respectively. These signs can also be used to describe heteromorphic size or stain intensity by placing the sign after the region being described. They will also indicate presence (+), absence (–), or duplication (++) of a locus when written after the targeted probe name in metaphase FISH karyotypes.
48,XX,+mar×2	MULTIPLICATION ×	MULTIPLICATION sign (×2) indicates the number of identical copies. It is most frequently used in hybridization karyotypes to reflect the number of observed signals per probe, but is also helpful when writing duplicate copies of a structural event. It is <i>not</i> , however, used for aneuploidy of structurally normal chromosomes.
46,XX,del(7)(q32)	PARENTHESES ()	PARENTHESES (single or adjacent) hold the elements associated with an event together as one unit or set.
46,XY,del(17)(p11.2p11.2).ish	PERIOD .	PERIOD (not to be confused with the sub-band “Point”) is used to separate different testing types, for example, 46,XY.ish with metaphase FISH, 46,XY.nuc.ish with nuclear FISH, and 46,XY.arr with microarray. Some call this a “dot.”
	PLUS +	SEE MINUS and PLUS above

Table 8.2 (Continued)

Example	Symbol	Definition and uses
22q12.1	POINT .	The POINT (not to be confused with the ‘Period’ above) delineates the sub-band level from the landmark band and region (see Figure 8.7).
46,XY,t(7;?)(q22;?)	QUESTION MARK ?	QUESTION MARK (?) indicates uncertainty. Question marks must be placed directly before the actual value in question (see 8.7, Symbols of uncertainty). Derivatives with unknown centric origin will also use the ? as its identity, and will appear at the end of the karyotype, before rings, markers, and double minutes.
t(7;14)(q22;q32)	SEMICOLON ;	SEMICOLON separates more than one chromosome in a specific rearrangement, and their respective breakpoints in an adjoining second set of parentheses.
46,XY[3]//46,XX[17]	SLANT LINE, double//	DOUBLE SOLIDUS (two forward slant lines) is used to separate chimeric cell lines and recipient/donor marrow transplant karyotypes.
mos 47,XX,+21[15]/46,XX[5]	SLANT LINE, single/	SINGLE SOLIDUS (single forward slant line) separates multiple cell lines (constitutional) or clones (neoplastic) and will thus appear in tandem with mos and chi in constitutional karyotypes (shown in example). It is also used in FISH karyotypes to indicate contiguous loci.
47~50,XY,+1~4mar[cp20]	TILDA ~	TILDA sign represents a range, as in a chromosome count range (47~50), a variable presence (+1~4mar), or equally potential breakpoint locations (p11~22).
47,XY,+der(<u>1</u>)t(<u>1</u> ;1)(p36.3;q21)	UNDERLINE 1	UNDERLINE beneath a number distinguishes one homologue from the other, as when homologues are involved in the same or identical events; this allows for sidelines to be traced without ambiguity.

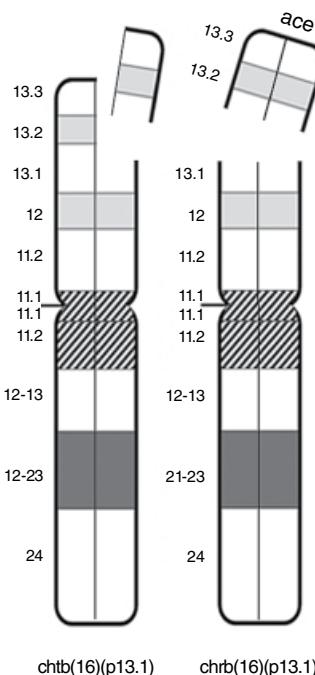


Figure 8.8 Chromatid versus chromosome break. A chromatid break (chtb) shows full discontinuity of a section of the arm, but its sister chromatid remains intact. A chromosome break (chrb) involves both sister chromatids. Because the fragment has no centromere (ace), it will become lost, leaving chromosome 16 deleted in subsequent generations.

ISCN diagram of human chromosome 16 at 300-band level (© 2009 Nicole Chia) used by permission of the artist. Reproduced from ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

8.3.4 Spaces in a karyotype

Although spaces are NOT generally used in a karyotype string of events, there are special conditions where a space is required:

- an alphabetic symbol following another alphabetic symbol (dual symbols) will be separated by a space, for example, psu dic for pseudodicentric [1, section 9.2.4] or mar mat for a marker chromosome of unknown chromosomal origin that has been inherited from the mother [1, section 4.1];
- an alphabetic symbol preceding a whole number will be followed by a space, for example, when the mosaic (mos) symbol must be used to indicate a karyotype with more than one constitutional cell line from a single zygote, e.g., mos 46 [1, section 4.1];
- a space will appear both before and after the symbol or (see 8.7, Symbols of uncertainty), for example, (p13 or q13) [1, section 5.3];
- symbols con (connected) and sep (separated) also use a space both before and after the symbol. These symbols are used when describing a positional change to the normal signal pattern in certain interphase FISH strategies, for example, (ABL1 con BCRx1) or (5'CBFB sep 3'CBFBx1) (see 8.10.8, Interphase fusion karyotype and 8.10.9, Break-apart probe strategy) [1, section 13.3.2].

A select list of symbols can be found in Tables 8.1 and 8.2. For a more complete list, the reader is referred to *ISCN 2016* [1, Chapter 3].

8.3.5 Short form karyotype

The most familiar karyotype form used in publications, clinical reports, and proficiency testing is the short system, which centers on the event and its breakpoint(s). It defines reportable numerical and structural changes by answering three questions – what event has occurred, which centric chromosome(s) is involved, and where the change occurred, when structural, via arm(s) and breakpoint(s) notation. Variations among karyotype formulae (see Figure 8.9) for the short form karyotype will depend upon how many breaks have involved how many chromosomes.

Intrachromosomal/one break

The simplest short system formula involves only one (known) break in one chromosome, for example, *event(chromosome)(armbreakpoint)*, with no spaces or punctuation. Because it involves only one chromosome, it is an intrachromosomal event.

46,XY,del(2)(q35)

Figure 8.10 demonstrates this presumed terminal deletion caused by a single break. The acentric segment (ace) from band 2q35 to the terminal end of the long (q) arm (2qter) of chromosome 2 is lost (deleted).

Events that will use this short karyotype formula include terminal deletions, isochromosomes, isodicentrics, centric fissions (not included in this chapter), fragile sites, additional material, and homogenously staining material (see 8.5, Structural events).

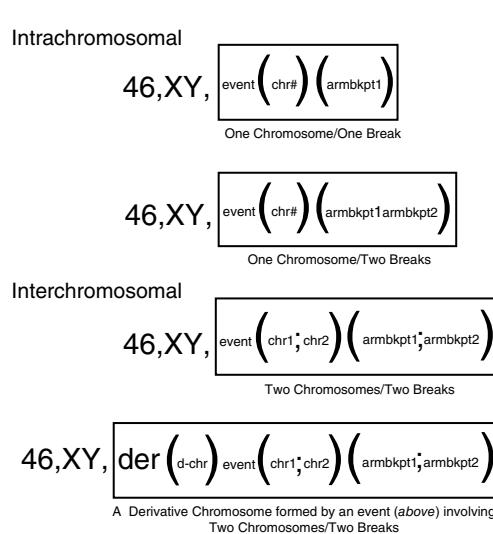


Figure 8.9 Short form karyotype formulae. Most karyotypes are written with one of these four basic formulae, based upon how many chromosomes and breakpoints are involved. There are no spaces in these formulae.

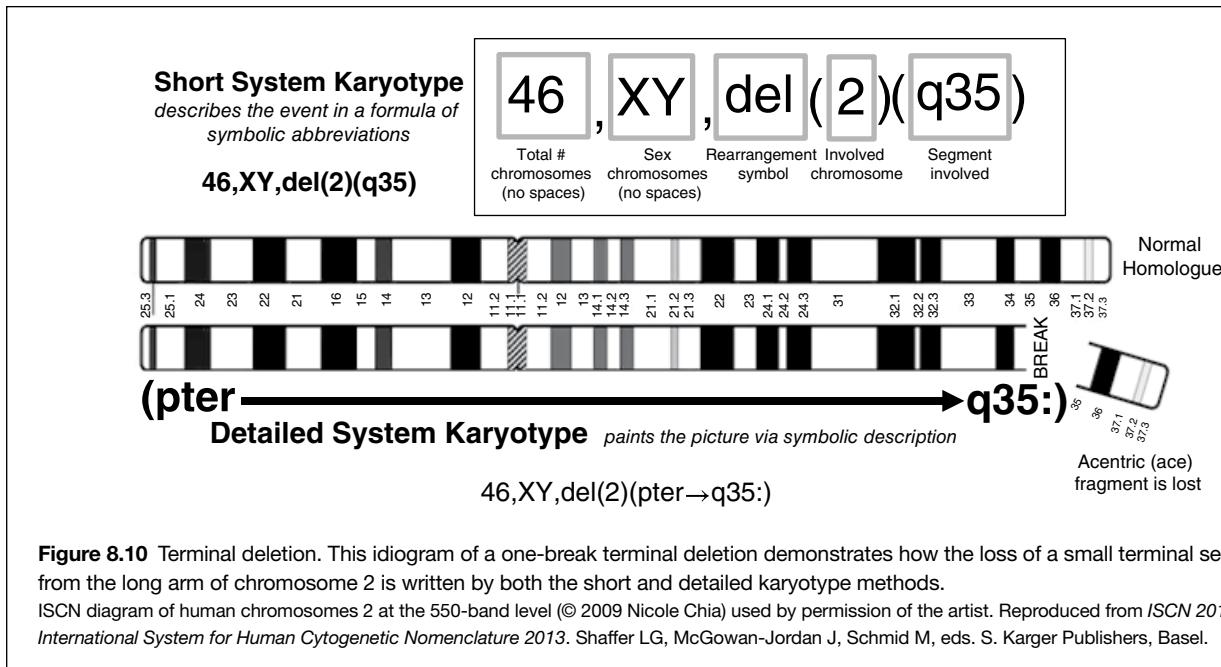


Figure 8.10 Terminal deletion. This idiogram of a one-break terminal deletion demonstrates how the loss of a small terminal segment from the long arm of chromosome 2 is written by both the short and detailed karyotype methods.

ISCN diagram of human chromosomes 2 at the 550-band level (© 2009 Nicole Chia) used by permission of the artist. Reproduced from ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

Intrachromosomal/two breaks

When two breaks occur within the same chromosome and terminal ends are not involved, an interstitial event has occurred. The karyotype for this intrachromosomal event would simply add a second breakpoint to the one-break formula, for example, *event(chromosome)(armbreakpointarmbreakpoint)*. Again no punctuation is used, because it involves the same chromosome. The order in which the breakpoints are listed depends upon which arms are affected. Breaks occurring in opposite arms, as those found in some inversions, will list breakpoints in *p-arm before q-arm* order, for example, *event(chromosome)(pbreakpointqbbreakpoint)*. Breakpoints occurring within the same arm, for example, *event(chromosome)(qbbreakpointqbbreakpoint)* will list in *proximal to distal order* relative to the centromere.

46,XY,del(16)(q22q24)

Two breaks have occurred on the long (q) arm of chromosome 16, one at q22 and the other at q24; the arms rejoined, excluding the q22 → q24 segment. This interstitial deletion uses the same symbol (del) as for terminal deletions, but adds a second breakpoint. The more *proximal* q22 breakpoint is listed before the more *distal* q24 breakpoint. Because the event still involves only one chromosome, there is no punctuation between the two breakpoints.

Events that will use the two-break, intrachromosomal formula include interstitial deletions, duplications, quadruplications, microdeletions, microduplications, inversions, and rings.

Interchromosomal/two breaks

When two centric chromosomes are involved in a rearrangement, that is, interchromosomal, a second chromosome set will be added to the basic formula, with semicolons separating the two chromosome entities, for example, *event(chromosome;chromosome)(armbreakpoint;armbreakpoint)*. The order in which chromosomes are written will generally follow standard karyotype order priority – that is, X before Y before ascending autosomal number (see 8.3.7, Karyotype order priority). The two different breakpoints, which are separated by a semicolon in the second parentheses, must be placed in the same order as their associated chromosomes in the first parentheses.

46,XY,t(2;16)(q35;q24)

A balanced exchange of chromosomal material has occurred between the long arm of chromosome 2 at q35 and the long arm of chromosome 16 at q24. Both products replace their respective homologues; therefore, chromosome count does not change and no plus or minus signs are used. The karyotype will show both centric chromosomes in the first parentheses and their respective breakpoints in the same order within the second, adjoining parentheses. Following standard karyotype priority order, chromosome 2 is listed before chromosome 16. Because this event involves two different chromosomes, a semicolon separates both the chromosomes in the first parentheses and their respective breakpoints in the second parentheses.

Events that will use the two-break interchromosomal karyotype include dicentrics, rings, telomeric associations, translocations, and acrocentric derivatives (see 8.5, Structural events, and 8.6, Derivative chromosomes).

These three basic formulae will describe the majority of constitutional events reported by cytogenetic analysis. Even the derivative chromosome that is derived from a previous event will include these formulae when pertaining to its originating event. The insertion is the only event that has its own unique formula and karyotype priority, since it requires at least three breakpoints – one to receive the inserted material, and two to release the segment from its original position (see 8.5.4, Insertion).

Some neoplastic rearrangements can become quite challenging to describe. The web address at <http://www.cydas.org/OnlineAnalysis/WebExample2.aspx> provides a tool that will create an idiogram from the karyotype's description, which may help verify that the written description matches what is visually being observed [17].

8.3.6 Detailed karyotype

When the short system cannot describe a rearrangement with clarity, the detailed karyotype format can be a valuable alternative. Complex, structurally abnormal rearrangements may even use both systems within the same karyotype in order to adequately describe all events [1, section 4.3.2]. The detailed format will start with the same *what* (*event*) and *which* (*centric chromosome*) description, for example, del(2), but instead of just listing breakpoints in the last parentheses, it will describe the chromosome's actual appearance from p to q terminal ends (pter → qter), with an arrow representing all unchanged structure between descriptions. A single colon (:) will represent a break, with its breakpoint placed proximal to the colon. Adding a second colon indicates that reattachment has occurred at a new breakpoint on the distal side of the double colon (::), thus leaving the two breakpoints rejoined and its interstitial segment missing.

Intrachromosomal/one break

A terminal deletion, as its name implies, will either begin or end its detailed description with the single-colon (:) break symbol, depending upon which arm was involved.

46,XY,del(2)(pter → q35:)

The last parentheses in this detailed karyotype describes the actual appearance of chromosome 2 (see Figure 8.10), starting with the terminal end of the p arm (pter), which is present, and proceeding unchanged (via the arrow) to band q35, which is the last visible band proximal to the break (:) on this deleted chromosome. If the break had occurred in the short arm, the single colon would begin the karyotype, followed with the breakpoint on the proximal side of the colon, for example, del(2)(:p24 → qter).

Intrachromosomal/two breaks

An interstitial deletion uses a double colon (::) in the detailed karyotype to indicate where the two breakpoints rejoined, with the interstitial segment deleted. The listed order for the detailed karyotype format follows in a p → q direction.

46,XY,del(16)(pter → q22::q24 → qter)

Breakpoints on either side of the double colon indicate that the two broken edges reattached, and the intervening (interstitial) fragment (q22 → q24) was deleted. The detailed system describes its appearance from pter to qter.

Interchromosomal/two breaks

When involving two or more different chromosomes in the same event, each chromosome is described independently, separated from the next chromosome by a semicolon, no spaces, and listed in karyotype order priority: X before Y before ascending autosome number (see Karyotype order priority next). All breaks and changes from the p terminal end to the q terminal end would be defined within each chromosome. Examples are shown later in this chapter.

8.3.7 Karyotype order priority

Standard priority for determining the first-listed chromosome in a two-chromosome event is X before Y before autosomes in ascending numerical order (Figure 8.11). For example, a translocation between chromosomes 2 and 16, e.g., t(2;16)(q35;q24), will list chromosome 2 before chromosome 16. This first-listed chromosome will also determine placement order within a string of events in a karyotype, for example, 47,XX,t(2;16)(q35;q24),+12. Commas, no spaces, separate each independent event within the string. Because sex chromosomes are handled differently (see 8.4.4, Sex chromosome aneuploidy), we will use autosomes to demonstrate building a string of events.

1. **X before Y before AUTOSOME**
46,XX,t(X;20)(q13;q13.3)
2. **AUTOSOMES in ASCENDING NUMERICAL order**
48,XY,+7,+12
47,XY,del(7)(q22q34),+12
If aneuploidy and a structural event involve homologous chromosomes:
3. **NUMERICAL before STRUCTURAL event**
47,XY,+7,inv(7)(p14q34)pat*
- If different structural events involve homologous chromosomes:
4. **ALPHABETICAL by SYMBOL**
46,XY,del(7)(q11.23q11.23)dn,inv(7)(p14q34)pat
If the same event involves homologous chromosomes:
5. **p-ARM before q-ARM**
46,XY,inv(7)(p14q34)pat,inv(7)(q21q23)
If the same event involves the same arm on homologous chromosomes but different breakpoints, the band that is closest to the centromere is listed first:
6. **PROXIMAL before DISTAL breakpoints**
46,XY,del(7)(q11.23q11.23)dn,del(7)(q22q24)
If the same two-chromosome events, e.g., translocation (t), involves the same arm and breakpoint on homologous chromosomes, and these identical, rearranged homologues are the first ones listed in their respective events, the listing order will be determined by the second-listed chromosomes:
7. **X before Y before lowest autosome number**
46,XY,t(7;12)(q36;p13),t(7;14)(q36;q32)
If the same autosome, place p-arm before q-arm
46,XY,t(7;12)(q36;p13),t(7;12)(q36;q32)
If the same arm, place in **proximal → distal** order
46,XY,t(7;12)(q36;q13),t(7;12)(q36;q24.1)

Figure 8.11 Karyotype order priority. The placement of events being described in a karyotype follows a specific priority order.

*See discussion in 8.3.8, Repeated description.

Order priority for describing a karyotype with multiple events involving homologues is as follows:

1. **Numerical before structural**, that is, the gain or loss (+ or -) of structurally normal homologues (numerical events) are listed before structurally abnormal homologues that are the sole or first-listed chromosome in their events, for example, +12,t(12;16)(q13;q24). The extra chromosome 12, however, would not precede the event if its homologue is the second-listed chromosome, for example, t(9;12)(q13;q13),+12. The key to this rule is that the additional or missing homologue(s) must be *structurally normal* and that the structurally abnormal homologue(s) must be the *sole* or *first-listed* chromosome of its event.
2. **Alphabetical by symbol**, for example, +12,del(12)(q13),inv(12)(p13q22). Because both events involve homologues, the deletion symbol alphabetically falls before the inversion (inv) symbol; therefore, karyotype order lists the numerical gain of chromosome 12 first, followed with the deletion, and then the inversion descriptions. Note: If multiple events occur within a single homologue, see 8.6, Derivative chromosome.
3. When event symbol and chromosome number are the same, priority moves to **p arm before q arm**; for example, t(9;11)(p22;q23),t(9;22)(q34;q11.2).
4. When event symbol (t), chromosome (9) and arm (q) are all the same, priority moves to the more **proximal (q13) break-point before the more distal (q34) one**, for example, t(9;11)(q13;q23),t(9;22)(q34;q11.2).

If all these parameters are the same, priority moves to the second-listed chromosome:

5. X before Y before ascending autosomal order, e.g., t(9;11)(q13;q23),t(9;22)(q13;q11.2). If the second-listed chromosomes are also homologues:
6. p arm before q arm. If the arm is also the same:
7. proximal → distal order of the breakpoints within that arm.

Exceptions to these standard karyotype rules include the following situations:

- Numerical or structural changes to constitutional sex chromosomes (see 8.4.4, Sex chromosome aneuploidy);
- Events that reserve the first-listed position for a specific purpose (see 8.5.4, Insertion);
- An event involving multiple chromosomes (see 8.5.12, Multichromosomal translocation);
- A rearranged event with more than one centromere, but only one centromere is active (see 8.5.2, Pseudodicentric, and 8.6.6, Ring derivative involving more than one chromosome);
- Rearrangements where centromere origin is unidentifiable (see 8.7, Symbols of uncertainty);
- Multi-event derivatives that use pter → qter order (see 8.6.4, Multiple events derivative) [1, Chapter 6].

Once all autosomal events have been described, symbols of uncertainty, if present, will be listed (see 8.7, Symbols of uncertainty). The detailed system may also be used either as a substitute for or in conjunction with the short system for these rearrangements, as stated in *ISCN 2016*: “It is acceptable to combine the short system (4.3.1) and the detailed system (4.3.2) for designating complex karyotypes.” [1, section 9.2.3, p. 65]

Total cell count in square brackets [20] will end the entire karyotype string of events for all neoplastic karyotypes, normal or abnormal (see 8.7.10, Composite karyotype for an exception), and after each cell line in constitutional studies with multiple cell lines.

In summary, a multi-event, neoplastic, karyotype string of events, using some of the examples in this section, could look like this:

48,XY,del(2)(q35),t(2;16)(q35;q24),t(9;11)(q13;q23),t(9;22)(q34;q11.2),+12,+del(12)(q13)[20]

48,XY: The chromosome count is now 48 because there are two extra chromosomes, +12 and +del(12)(q13). With these additional chromosomes, the total number of centric entities is 48. The remaining events are structural; without a plus sign, the reader must assume that they have replaced their normal, centric homologues. Because these events affect only autosomes, they are listed in numerical order by their *sole* or *first-listed chromosome*, for example, chromosome 2 as the sole or first-listed chromosome will be described before all events involving chromosomes 9 and 12 as the sole or first-listed chromosome.

del(2)(q35),t(2;16)(q35;q24): Without plus (+) signs, the reader must assume that these two events have involved and thus replaced both normal homologues of chromosomes 2. Because these two autosomal events involve the same sole or first-listed chromosome, their karyotype placement will follow alphabetical order by event symbols; therefore, **deletion** will be described before **translocation**. Both centric chromosomes are listed in the first parentheses of the translocation event, indicating that both listed partners (chromosome 2 and 16) are present, and are structurally abnormal. The karyogram will use arrows to indicate the three affected chromosomes – the deleted homologue 2, translocated homologue 2, and translocated homologue 16.

t(9;11)(q13;q23),t(9;22)(q34;q11.2): Following numerical autosomal order, events involving chromosome 9 as the *sole* or *first-listed chromosome* will be described next. Homologous events involving the same chromosome arm will follow proximal to distal order of their breakpoints; therefore, the event with a more proximal breakpoint (9q13) would be described before the more distal breakpoint (9q34). Omission of a plus sign on both structural events indicates that there are no normal homologues present for that chromosome. Again, the listing of both centric chromosomes within the first parentheses of the event indicates that the second-listed chromosomes (homologues 11 and 22, respectively) are also structurally abnormal. Their descriptions do not need repeating in the karyotype, because they can be assumed; however, the karyogram will indicate all four involved partners.

+12,+del(12)(q13): The gain or loss (numerical event) of a structurally normal chromosome (+12) precedes any structural event involving its homologue as the *sole* or *first-listed chromosome*; therefore, the additional, but structurally normal chromosome 12 is listed before the additional chromosome 12 with the deletion. Because both events are preceded with plus (+) signs, the reader can assume that both normal homologues are also present. The karyogram will thus show four homologues for chromosome 12 – three structurally normal copies and one deleted homologue.

[20]: A number in brackets at the end of a cell line or karyotype indicates the number of analyzed cells that shared this same karyotype. In this example, all 20 cells showed the same result.

8.3.8 Repeated description

In neoplastic specimens, complex rearrangements may evolve from rearranged structures, creating a very long and complicated karyotype. Two shortcuts are used for repeated descriptions in a karyotype, as long as they leave no ambiguity. When multiple copies of the same structurally abnormal chromosome are present, a lowercase multiplication sign and number of copies ($\times 2$), no spaces, will be placed after the initial description. The $\times 2$ shortcut, however, cannot be used for structurally normal chromosomes. We will use the bracketed cell count below to indicate a neoplastic karyotype.

46,XY,inv(12)(q13q22) × 2[20]

Two identical copies ($\times 2$) of an inverted (inv) chromosome 12, with breakpoints on the long (q) arm at q13 and q22, have replaced both normal homologues in all 20 cells analyzed; therefore, no plus sign is used and chromosome count

does not change. It should be noted that the occurrence of two identical inversions, with no other normal homologues, raises a suspicion of loss of heterozygosity (LOH) for chromosome 12 (see 8.5.13, Uniparental disomy and loss of heterozygosity).

48,XY,+15,+15[20]

Two extra chromosomes 15 are in addition to two normal homologues. Even though the extra chromosomes are identical, the karyotype must list them separately because they are structurally normal, using a plus sign for each, and chromosome count will increase to 48.

A second shortcut eliminates the repetition of breakpoints when referencing previously described events *in the same karyotype*, as long as there are no similar events to create ambiguity (e.g., same event, first-listed chromosome and breakpoint). As stated in *ISCN 2016*: “When an extra copy of a rearranged chromosome is present, the breakpoints are specified only once, at the first time it appears in the karyotype” [1, section 4.2, p. 40]. In the following example, one normal homologue of chromosome 12, along with two deleted chromosomes, are present.

- a. 47,XY,+12,del(12)(q13)×2
- b. 47,XY,del(12)(q13),+del(12)(q13)
- c. 47,XY,del(12)(q13),+del(12)

Three centromeric copies of chromosome 12 are present in this example – one normal and two deleted. The first karyotype (a) describes the normal chromosome 12 as the extra copy, and uses the suffix ×2 to indicate the presence of two identical, deleted copies of chromosome 12. The *numerical gain* of the normal chromosome 12 is described before the *structurally abnormal* copies, following karyotype order priority (as defined above). The description is simple and leaves no ambiguity, but does not provide insight into the event’s derivation, because the normal homologue for chromosome 12 is not the actual cause for the 47-chromosome count. The second (b) example is acceptable, but it repeats the breakpoint unnecessarily. The third alternative (c) shows one deleted chromosome replacing one normal homologue of chromosome 12 (thus, no plus sign), and the extra chromosome (+) as a duplicate copy of the deleted description (hence, no breakpoints). The uninvolved, normal homologue is assumed, and thus not mentioned. All three options are acceptable alternatives [1, sections 4.2, 9.3]. The last example, however, not only represents a clear representation of its formation, but it simplifies the karyotype by following the ISCN rule for omitting breakpoints in a repeated structural event.

Long, complicated karyotypes are not uncommon in neoplastic studies, as will be demonstrated later in this chapter. *ISCN 2016* introduced a new alternative for describing a string of events, and recommends using this shorter karyotype description [1, section 6] when possible.

- a. 47,XY,+7,inv(7)(p14q34)[20]
- b. 47,XY,+inv(7)(p14q34)[20]

An extra chromosome 7 is present in 20 cells from a neoplastic specimen. One of the three homologues shows an inversion and the other two are normal-appearing chromosomes 7. Previous ISCN reports wrote this karyotype with the extra, structurally normal chromosome 7 described first (a), before its structurally abnormal homologue, e.g., +7,inv(7). The new, shorter karyotype (b) combines both events and uses the inverted chromosome as the extra homologue. Both methods are acceptable by *ISCN 2016* standards, but the latter is recommended because it uses the shortest karyotype string.

47,XY,+7,inv(7)(p14q34)pat[20]

The inherited karyotype (above) from Figure 8.11 changes this paradigm. By adding unique identity to one of the homologues, origin for the duplicated copy becomes more recognizable, much like heteromorphisms and satellite appearance were used in the 1970s to suggest parental origin. For this example, therefore, the longer version is more reflective of the actual situation. Both formats are, however, acceptable by current *ISCN 2016* standards; therefore, the choice rests with the karyotype writer. See Chapter 7, Human chromosomes: identification and variations, for an incredible display of normal variations in human chromosomes.

8.3.9 Amending a cytogenetic karyotype

If cytogenetic results were reported normal, but after performing FISH, the specimen was found to have an abnormality, the cytogenetic case may be re-examined. If the abnormality is detectable after reexamination, the report can be amended. Even if only one cell showed the rearrangement by chromosomal analysis under routine studies, which at the time did not meet clonal, reportable standards, the report can now be changed because the finding has been confirmed by FISH. If, however, the abnormality is still not recognizable after reexamining the chromosomes, the rearrangement may be cryptic and not detectable by routine chromosomal analysis; therefore, the cytogenetic karyotype would not be changed [1, section 13.1], but the FISH result should be added to the karyotype string.

8.4 Numerical events

8.4.1 Polyploidy and endoreduplication

A normal gamete, which contains only one set of nonhomologous chromosomes, is said to be haploid, because it contains half the number of chromosomes required by the species. This ploidy number also sets the euploid value of n , making 2×23 (46) the expected human diploid ($2n$) chromosome count. Polyploid is a general term referring to any multiple of 23 greater than diploid. A male triploid karyotype, which contains three (tri) sets of 23 (n) chromosomes, is written 69,XXY or 69,XYY, and a tetraploid male with four (tetra) sets would be written 92,XXYY (see Table 8.3 for the use of modal numbers). When the extra sex chromosome is unknown in a cell with uneven ploidy (e.g., triploid- $3n$, pentaploid- $5n$, heptaploid- $7n$), *ISCN 2016* (1) states that “in males all sex chromosome deviations should be expressed in relation to X in haploid tumors, to XXY in triploid, to XXXYY in pentaploid tumors, and so on.” [1, section 11.2].

Endoreduplication is another specific form of polyploidy that is created when sister chromatids fail to separate during cytokinesis, resulting in a nucleus where each homologue is composed of four instead of two strands of sister chromatids (see Figure 8.12). The symbol end for endoreduplication will precede the count, separated by a space, for example, end 46,XY. This symbol clarifies that even though the metaphase may show a diploid 46-count, its DNA content is four times the amount (4×23) of a haploid (n) cell [1, section 4.1].

Table 8.3 Near-ploid guidelines. Near-ploid guidelines have been established for writing a karyotype when the ploidy range is unclear due to aneuploid complications. The difference between each ploidy level is $n \pm 11$. Using the diploid range, for example, a chromosome count that falls between 35 and 45 is considered a hypodiploid number, and count ranges that fall between 47 and 57 are hyperdiploid

Ploidy	# Range
Near-haploid (n)	≤ 34
Near-diploid ($2n$)	35–57
Near-triploid ($3n$)	58–80
Near-tetraploid ($4n$)	81–103
Near-pentaploid ($5n$)	104–126
Near-hexaploid ($6n$)	127–149
Near-heptaploid ($7n$)	150–172
Near-octaploid ($8n$)	173–195

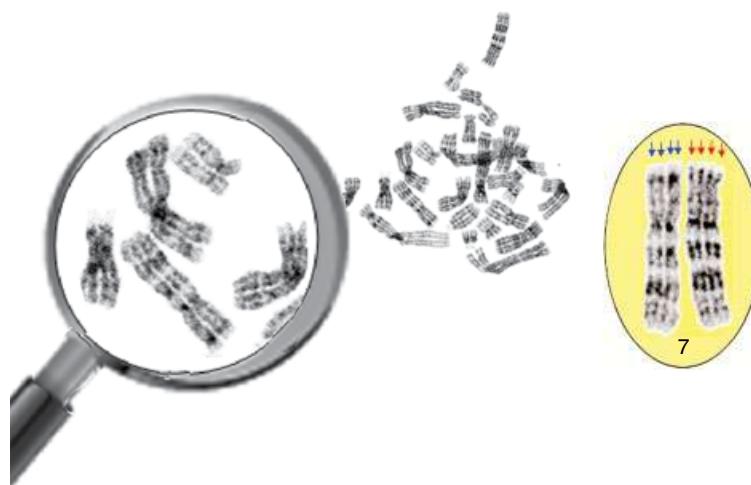


Figure 8.12 Endoreduplication. Endoreduplication (end) is a polyploid derivative that results after DNA replication, when sister chromatids fail to separate and the cell fails to divide (cytokinesis), giving rise to four-stranded chromosomes at subsequent prophase and metaphase stages.

8.4.2 Near ploidy

Modal number refers to the most common chromosome count in a tumor cell population. When the chromosome count does not equal an exact ploidy value – for example, *haploid* (n), *diploid* ($2n$), *triploid* ($3n$), *tetraploid* ($4n$), *pentaploid* ($5n$), *hexaploid* ($6n$), *heptaploid* ($7n$), and *octaploid* ($8n$) (see Table 8.3), the cell can be referred by its near-ploidy value. These values define a “*hypo*” range as a *loss* no greater than 11 from that ploidy value (n minus 1–11), and a “*hyper*” range as a *gain* no greater than 11 from that ploidy value (n plus 1–11). For example, a near-triploid count that is missing one chromosome 12, for example, 68,XXY,-12, is hypotriploid. Likewise, a near-tetraploid male karyotype with an extra chromosome 12, written 93,XXYY,+12, falls within the hypertetraploid range [1, section 11.2].

If the chromosome count does not fall within the category that reflects its more meaningful biological origin [1, section 8.1], the writer can place the corrected level at which the karyotype is being described in angle brackets, for example, 34< $2n$ >, immediately following the chromosome count, without spaces, and before the comma preceding the sex complement. Likewise, if the chromosome count is a range crossing two ploidy levels, for example, 57–62, which is not uncommon in very complicated neoplastic situations (see 8.7, Symbols of uncertainty), the writer must define the ploidy level at which the karyotype has been written, for example, 57–62< $2n$ >.

These guidelines ensure that the reader of a karyotype is in synch with the intentions of the karyotype’s author, especially when chromosome counts border a neighboring ploidy range. The bottom line for all these rules centers on ISCN as a language; therefore, karyotype structure must be properly applied to ensure that its message will be correctly interpreted by its reader.

8.4.3 Autosome aneuploidy

If euploidy represents a multiple of the 23 haploid set, then aneuploid literally means not euploid, that is, not a multiple of the 23 set; therefore, chromosome counts of 45 ($2n-1$), 47 ($2n+1$), 70 ($3n+1$), and 88 ($4n-4$) all represent examples of aneuploidy. If the only change to a karyotype is a gain (trisomy) or loss (monosomy) of one whole, structurally normal autosome, the karyotype would simply add a comma after the last sex chromosome, followed by a plus (+) or minus (-) sign to indicate a gain or loss, and the autosome number, all without spaces or parentheses, for example, 47,XX,+21. More than one gain or loss of structurally normal autosomes would be listed in ascending numerical order in the karyotype, with a comma separating each event: 48,XY,+18,+21. A specific type of monosomy, nullisomy (i.e., missing both homologues), is not a viable constitutional finding, but when found in neoplastic conditions, it would be written 44,XX,-21,-21.

Listed below are three aneuploid (trisomic) constitutional syndromes (see Chapter 9, Figure 9.2):

47,XY,+13 Patau syndrome, Trisomy 13

47,XX,+18 Edward syndrome, Trisomy 18

47,XY,+21 Down syndrome, Trisomy 21

8.4.4 Sex chromosome aneuploidy

Constitutional karyotype

Sex chromosomes are generally listed before autosomes (see 8.5.4, Insertion, for an exception), but how they will be written will depend upon whether the specimen is a constitutional or neoplastic study. For constitutional studies, multiple copies of structurally normal sex chromosomes are listed together in X before Y order, irrespective of their quantity, without spaces and without plus (+) or minus (-) signs. Karyotype examples of sex chromosome aneuploidy include the following:

45,X Turner syndrome

47,XXY Klinefelter syndrome

70,XXX Male triploid that has an extra copy of a structurally normal X sex chromosome

Neoplastic karyotype

One major difference of neoplastic karyotypes from constitutional ones is how sex chromosome aneuploidy is described. Each gain or loss of a sex chromosome as a result of neoplasia, whether structurally normal or abnormal, is written after the constitutional sex complement, using a plus or minus sign. Another distinction is including the cell count in square brackets, without spaces, at the end of all neoplastic karyotypes, normal or abnormal, and following each clone within a multiclonal karyotype.

45,Y,-X[20]

The missing X sex chromosome as a result of the neoplasia, seen in all 20 cells analyzed from a neoplastic specimen, follows the normal Y, and uses the minus sign, no spaces.

48,XY,+X,+X[20]

Two extra X sex chromosomes in all 20 cells, as a result of neoplasia, are individually listed after the constitutional XY male description. Multiple copies of a *structurally normal chromosome* cannot be described with the lowercase \times (multiplication sign); therefore, it would be *incorrect* to write the karyotype as 48,XY, \times X \times 2 (see 8.3.8, Repeated description).

Constitutional notation in a neoplastic karyotype (c)

If a known constitutional sex chromosome abnormality is seen in a neoplastic specimen, the karyotype will use constitutional karyotype standards to describe this one event, and will append a lowercase **c**, or its parental origin, **mat** or **pat**, if known, or **inh** if not known, after its description (see 8.5.8, Constitutional origin).

45,Xc[20]

Neoplastic results on a bone marrow specimen from a patient with known Turner syndrome shows no clonal abnormalities as a result of her neoplasia in twenty cells analyzed. The description follows constitutional rules, even though the karyotype is from a neoplastic tissue. The lowercase **c** verifies that the abnormal event (monosomy for the X sex chromosome) is constitutional.

A constitutional abnormality, for example missing the X sex chromosome, *cannot* be written 45,X,-X[20], which would be the description if the loss was a result of her neoplasia, nor would it be written 45,X,-Xc, because the event must follow constitutional karyotype convention. Spaces are generally not used with the **c** symbol unless it follows another symbol, for example, 47,XX,+mar **c**. Even if constitutional aneuploidy is the only abnormal finding, cell count in square brackets will still be included at the end of a neoplastic karyotype.

48,XXYc,+X[20]

This neoplastic karyotype from a patient with Klinefelter syndrome has two extra X sex chromosomes, one constitutional, and one as a result of his neoplastic condition. The lowercase “**c**” with no spaces will follow the entire XXY constitutional complement, *not* the second X, for example, 48,XXcY,+X[20], even though that chromosome is the cause for his constitutional condition. The extra X that is a result of his neoplasia, however, will follow neoplastic karyotype standards; therefore, a plus sign is used, no spaces, and a comma will separate it from the constitutional XXYc sex complement.

8.4.5 Pseudodiploid

If euploidy literally means a multiple of 23, then the karyotype 46,X,+21 poses an interesting dilemma. The count appears as if it is euploid (46), but the specimen actually shows a gain and loss as a result of two independent aneuploid occurrences, each associated with genetic consequences – that is, Down syndrome (+21) added to Turner syndrome (45,X). Even though the total number is a euploid multiple ($2n$), the chromosome makeup does not contain a full set of chromosomes, and is therefore not genetically equivalent to 2×23 . Because the numerical ploidy value of n reflects the genetic makeup contained within the 23 chromosome pairs, not merely a count, our example is called pseudodiploid [1, section 11.2]. In the same manner, the karyotype 69,XXY,-12,+21 would be pseudotriploid.

8.5 Structural events

This section will introduce the short and detailed karyotype format for most structural events. Because of their potential complexity, derivatives and symbols of uncertainty will be discussed in their own sections.

8.5.1 Deletion (del)

Deletion signifies a partial loss. When only one breakpoint can be identified, without subsequent reattachment, it is assumed that the terminal end is included in the loss and the event is called a terminal deletion.

Terminal deletion

When the distal side of a break is lost, one arm will appear truncated. Unless the broken, acentric fragment (**ace**) can regenerate a centromere, it will become lost.

46,XY,del(9)(p24)**46,XY,del(9)(:p24 → qter)**

This one-break deletion occurred within the short arm of chromosome 9. The last parentheses in the detailed karyotype, which is written in a p to q direction, must begin with the single colon to show that all material distal to the breakpoint on the p arm is missing. See Chapter 9, Table 9.1, for the phenotypic traits associated with this deletion.

In reality, telomeres are so critical to chromosome stability that when missing, chromosomes often regenerate or acquire these repetitive nucleotide sequences from a sister chromatid, homologue or nonhomologous chromosome [18].

Interstitial deletion

An interstitial deletion is created by two breaks, where the two broken edges rejoin and the intervening, acentric segment becomes lost.

46,XY,del(7)(q22q34)**46,XY,del(7)(pter → q22::q34 → qter)**

The chromosomal segment between breakpoints q22 and q34 has been deleted. Breakpoints in the short-form karyotype are listed in proximal to distal order. Because this example involves a single chromosome, no spaces or punctuation are used. The last parentheses in the detailed karyotype describes the abnormal chromosome from p-terminal to q-terminal end. The two colons represent the break and reattachment of the two breakpoints, with the intervening segment lost. To view a sample of this deletion, see Chapter 11, Figure 11.2.

Microdeletion

Routine cytogenetic analysis is limited by the size at which an abnormality can be detected, approximately 5 to 10 Mb [1, section 2.5], with higher resolution extending this range to 3–5 Mb [19]. Deletions or duplications that fall within or below the lower limits of detection [20–22] would thus benefit from more locus-specific targeting, for example, FISH or molecular-based arrays. In the short form karyotype, these cryptic events are called microdeletions or microduplications (see 8.5.3, Duplication) and are described in the karyotype by a repetition of the same breakpoint as both upper and lower limits of the break region, without spaces or punctuation.

46,XY,del(17)(p11.2p11.2)

Use of identical breakpoints indicates that an interstitial microdeletion has occurred. A deletion within band 17p11.2 on the short arm of chromosome 17 is associated with Smith-Magenis syndrome. Because the size of this deletion can span from 1.5 to 9 Mb in length, with 80–90% having a 4-Mb deletion due to recombination in low copy repeats [23], larger deletions may be detected by higher resolution chromosome analysis; however, alternate testing should be performed for confirmation. Some microdeletion syndromes, such as Williams syndrome, are generally too small to detect without FISH or molecular karyotype confirmation (see Figure 8.13).

Not all chromosomes analyzed in a metaphase cell will be at the exactly same resolution level at the same time; therefore, special attention must be given to each individual chromosome when comparing band composition and determining the band level achieved, especially for high resolution evaluation. In each of these microdeletion cases, suspected deletions should be confirmed by FISH, microarray, or similar molecular-based detection.

8.5.2 Dicentric (dic)

When a translocation involves two different chromosomes, including homologues, where each translocated segment holds its own centromere, a dicentric is formed, with subsequent loss of its acentric counterparts. Dicentrics are counted as one chromosome, even though they contain two centromeres, and the chromosome count becomes 45. Because both centromeres are now located on one derivative chromosome, the subsequent loss can be inferred and is thus not included in the karyotype. The derivative (der) symbol may be used instead of dic, but the combination of der dic is not acceptable [1, section 9.2.4]. When a dicentric chromosome involves more than one event, the karyotype should use the derivative formula [1, section 9.2.3] (see 8.6, Derivative chromosomes).

45,XY,dic(17;20)(p11.2;q11.2)

A dicentric chromosome (see Figure 8.14) was formed when a break (p11.2) within the short arm of chromosome 17 joined with a break (q11.2) within the long arm of chromosome 20. Both chromosomes retained an active centromere;

hence, the new dicentric chromosome shows two primary constrictions. Because both centromeres are present on this new structure, both chromosomes are listed in the first parentheses; and because both centromeres are active, their order will follow standard karyotype order: X before Y before autosomes in increasing numerical order. If only one centromere is active (e.g., there is only one constriction present), see Pseudodicentric, next. A semicolon, without spaces, is used to separate the two different chromosomes and their respective breakpoints.

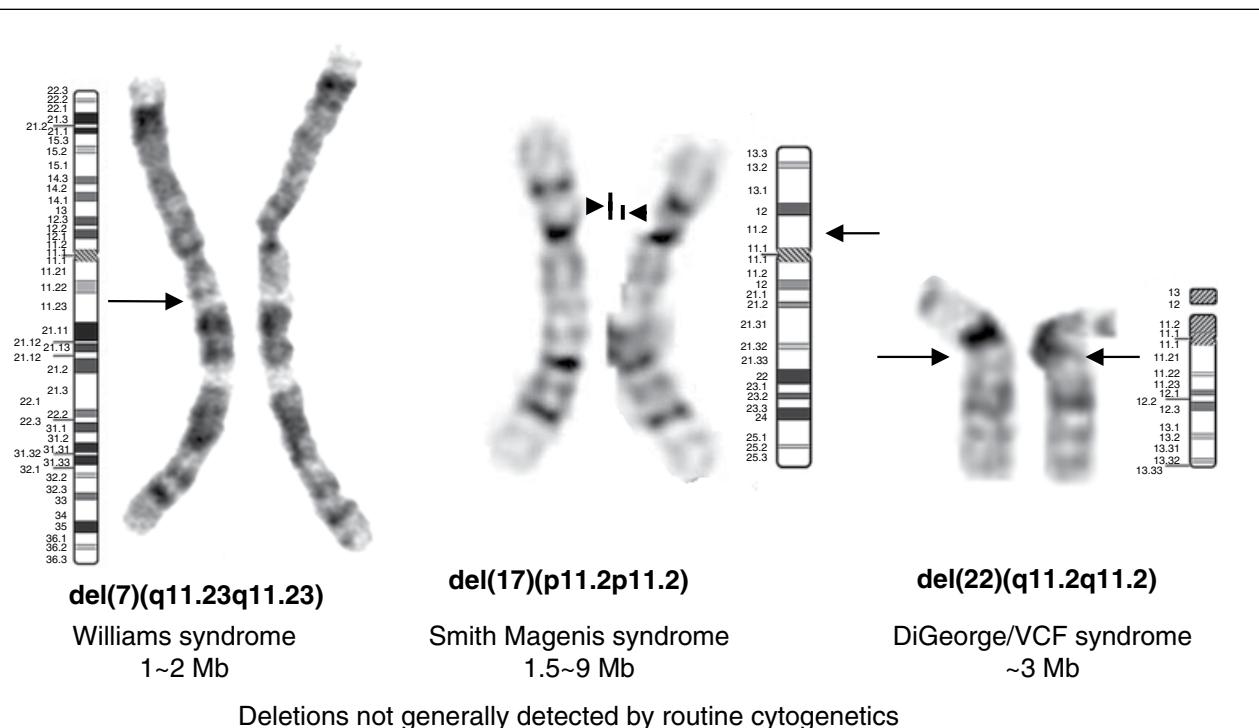


Figure 8.13 Microdeletions. Because routine cytogenetics cannot detect abnormalities less than 3~5 Mb, microdeletions are not always detected by standard cytogenetic analysis.

46,XY,del(7)(q11.23q11.23)

The microdeletion in patients with Williams syndrome affects a locus the size of 1~2 Mb¹ within the long arm segment of chromosome 7 at q11.23. Because of its small size, it is very difficult to detect by classical cytogenetics, even at the 850-band levels of high-resolution chromosomal analysis, without FISH or molecular testing for confirmation. This homologous pair was confirmed by molecular testing as positive for Williams syndrome.

46,XY,del(17)(p11.2p11.2)

The microdeletion at 17p11.2 for Smith Magenis syndrome involves a segment that measures 1.5~9 Mb in length, with a mean of 5 Mb², indicating that the larger sizes can potentially be detected by high resolution analysis.

46,XY,del(22)(q11.2q11.2)

The third microdeletion involving 22q11.2 is found in patients with DiGeorge–velocardiofacial syndrome (DGS-VCF). Its length of ~3 Mb³ makes it potentially detectable by high resolution banding. The homologues in the above figure have been aligned by the q telomere in order to accentuate the deletion.

ISCN diagram of human chromosomes 7, 17 at the 700-band level and 22 at the 850-band level (© 2009 Nicole Chia) used by permission of the artist. Reproduced from *ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013*. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

1. <http://www.medicine.nevada.edu/dept/genetics/williams.html>: University of Nevada School of Medicine Genetics Program, Director: Colleen A. Morris M.D, Genetics Division of the Department of Pediatrics. UNR.EDU/GENETICSLK.HTML. Retrieved 2/14/2013.
2. <http://moon.ouhsc.edu/kfung/jty1/neurohelp/ZNP2IE05.htm>. The Neuropathology Learning Program for Residents & Medical Students, Department of Pathology, University of Oklahoma Health Sciences Center. Retrieved 2/14/2013.
3. <http://ghr.nlm.nih.gov/condition/22q112-deletion-syndrome>. 22q11.2 deletion syndrome. Genetics Home Reference, U.S. National Library of Medicine. Retrieved 2/14/2013.

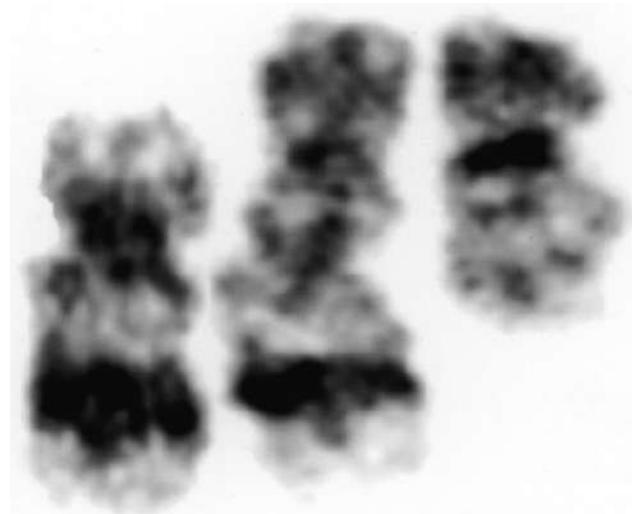


Figure 8.14 Dicentric chromosome. A true dicentric will show two centromeric constrictions. This neoplastic rearrangement involving chromosomes 17 and 20 is seen in patients with AML and MDS.

45,XY,dic(17;20)(20pter → 20q11.2::17p11.2 → 17qter)

Even though chromosome 17 is listed before chromosome 20 in the first parentheses, the detailed karyotype description in the second parentheses must “paint” a picture of the new chromosome from p to q arm. Because the p arms of chromosome 17 are attached to breakpoints on the q arms of chromosome 20, and are thus buried within the new formation, the detailed description must begin with the p arms of chromosome 20. Figure 8.14 may help visualize this description. For clarity, the detailed karyotype will include the chromosome number with each band defined. When centromere presence or position is not clear, the *cen* symbol can be added in the detailed description, for example, (20pter → cen → 20q11.2::17p11.2 → cen → 17qter).

Pseudodicentric (psu dic)

The difference between a dicentric and a pseudodicentric is the inactivation of one of the centromeres. The karyotype will list the active centromere first, irrespective of karyotype order rules, and will define the event as a psu dic [1, section 9.2.4].

45,XY,psu dic(20;17)(q11.2;q11.2)

The centromere of chromosome 20 is the only active one; therefore, it will be described first in the short karyotype, *irrespective of karyotype order priority*.

8.5.3 Duplication (dup)

Duplicated segments that have originated from the same chromosome and appear, in tandem, either in a direct or inverted position, will use the dup symbol to describe its event. Positional orientation is apparent by the order in which the breakpoints are listed in relation to the centromere [1, section 9.2.5], with direct being a proximal to distal order for their breakpoints (hence, increasing numerical order), and inverted being distal to proximal (hence, decreasing numerical order). Because the band order infers a direct or inverted duplication, the abbreviations dir and inv are no longer needed [13, section 9.2.5] and their symbols have been removed from the list of abbreviated symbols in *ISCN 2016* [1, chapter 3]. (see Addendum for *ISCN 2016* updates).

46,XY,dup(1)(q21q32)

46,XY,dup(1)(pter → q32::q21 → qter)

Chromosome 1 shows a duplication of segment q21 → q32. The proximal to distal order of the breakpoints indicates that the duplication is direct. For an example of this duplication, see Chapter 11, Figure 11.2.

46,XY,dup(7)(q32q22)

A duplicated segment of the long arm of chromosome 7 is inverted in this example, as represented by the distal to proximal order of its breakpoints.

46,XY,dup(7)(q22q22)

46,XY,dup(7)(pter → q22::q22 → qter)

Similar to the cryptic microdeletion above, a microduplication of small or cryptic genetic material will repeat the same breakpoint (q22q22) in the second parentheses.

TriPLICATION (trp) and quadrUPlication (qdp)

Tripllications and quadruplications are occasionally seen in neoplastic specimens and will use the trp or qdp symbols.

46,XY,trp(1)(q21q32)

46,XY,trp(1)(pter → q32::q21 → q32::q21 → qter)

A triplicated copy of the segment from q21 to q32 is present on the q arm of chromosome 1 (see Figure 8.15).

46,XY,qdp(1)(q21q32)

46,XY,qdp(1)(pter → q32::q21 → q32::q21 → q32::q21 → qter)

Four copies of the same region (q21 to q32) are present on one homologue of chromosome 1.

Because the presence of an inversion is not apparent when using only the short karyotype for describing tripllications and quadruplications, the detailed system may be required if orientation needs to be clarified [1, sections 9.2.14, 9.2.19].

46,XY,qdp(1)(pter → q32::q21 → q32::**q32 → q21**::q21 → qter)

Four copies of the same region (q21 to q32) are present on one homologue of chromosome 1, but the third sequence (shown in bold) is inverted. The short system cannot define positional orientation; therefore, this type of rearrangement can only be defined by the detailed karyotype.

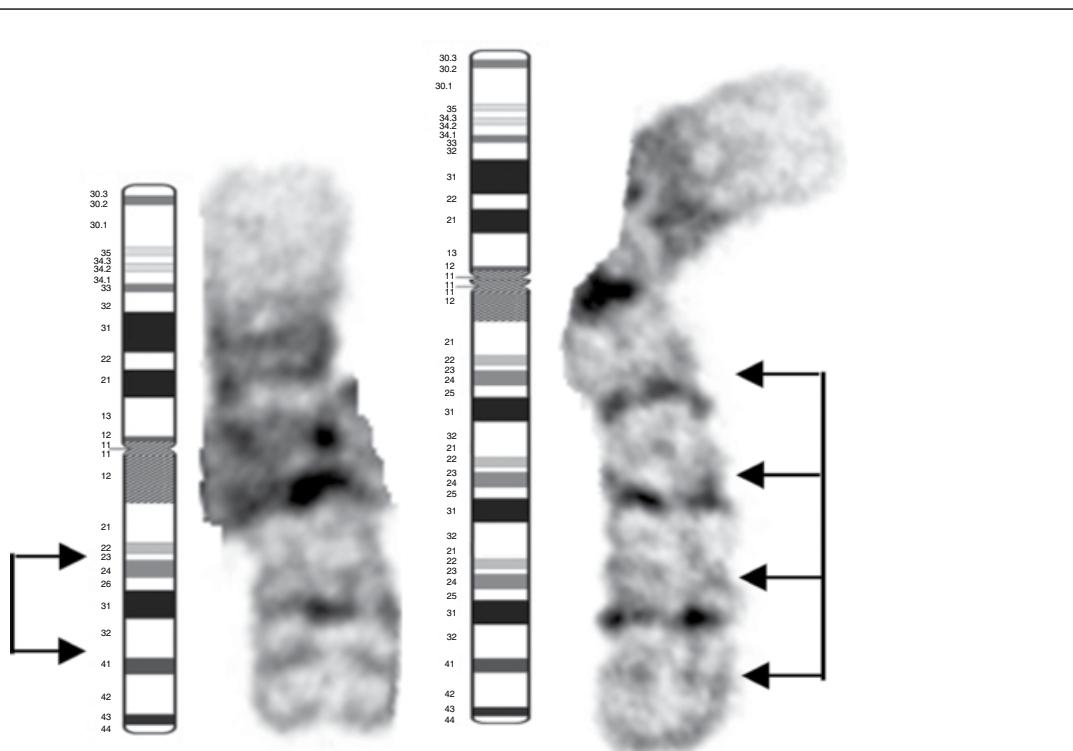


Figure 8.15 Triplication.

46,XY,trp(1)(q21q32)

46,XY,trp(1)(pter → q32::q21 → q32::q21 → qter)

Three copies of the long arm segment between q21 and q32 are present on chromosome 1.

ISCN diagram of human chromosome 1 at the 400-band level (© 2009 Nicole Chia) used by permission of the artist. Reproduced from *ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013*. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

8.5.4 Insertion (ins)

Insertions can involve a segment from a different location on the same chromosome, or a segment from a totally different chromosome [1, section 9.2.9]. Both events are formed by three breaks – two breakpoints to release the segment to be inserted, and one break within a receiving area, where the released segment can be inserted.

Intrachromosomal insertion

An intrachromosomal insertion involves only one chromosome. Three breakpoints will be described in the second parentheses, with the receiving breakpoint first, and the last two being placed in the order they are inserted. No punctuation or spaces are placed between these breakpoints, because they involve the same chromosome. The position in which the segment is inserted (direct or inverted) is understood by its breakpoint orientation (proximal to distal) within the karyotype.

46,XY,ins(7)(q31p13p15)

46,XY,ins(7)(pter → p15::p13 → q31::**p13 → p15**::q31 → qter)

In the short-form karyotype, the first breakpoint within the second parentheses (q31) is the receiving position for the last two breakpoints (p13p15), which are inserted in a distal order, p13 before p15, making this a direct insertion.

46,XY,ins(7)(q31p15**p13)**

46,XY,ins(7)(pter → p15::p13 → q31::**p15 → p13**::q31 → qter)

The breakpoints in the long arm of chromosome 7 are listed in a proximal direction, e.g., in decreasing numerical order (::p15 → p13::), indicating that the inserted segment is inverted.

Please note: We are using a bold font in karyotypes throughout this chapter in order to isolate specific sections within the karyotype that are being emphasized in the discussion. The actual karyotype does not include a bold font.

Interchromosomal insertion

An interchromosomal insertion occurs when a two-break segment from one chromosome (donor) is inserted into a one-break position of a second chromosome (receiver). The receiving chromosome is listed first, irrespective of normal karyotype placement rules. This random selection will thus override the rule for placing X before Y before lowest autosome [1, sections 4.3.1.2, 9.2.9]. As in any two-chromosome event, semicolons will separate chromosome identities and their breakpoints, but will not separate breakpoints within the same chromosome (donating chromosome).

46,XY,ins(12;3)(q15;p13**p21)**

Chromosome 12 (and its breakpoint q15) is listed first in both parentheses, irrespective of standard karyotype order, because it is the receiver of the inserted segment (p13 → p21) from chromosome 3. Breakpoints 3p13 and 3p21 are listed in proximal to distal order in the short karyotype, indicating that the inserted position is in a direct orientation to the centromere. The deleted partner is not described, because it is implicitly understood.

46,XY,ins(12;3)(12pter → 12q15::**3p13 → 3p21**::12q15 → 12qter;3pter → **3p21::3p13** → 3qter)

The detailed karyotype, on the other hand, describes *both* rearranged chromosomes in pter to qter order, with the two chromosome descriptions separated by a semicolon, no spaces. Chromosome 12 is normal from its p-terminal end until band 12q15, where the double-colon break::rejoin symbol (12q15::) shows segment 3p13 → 3p21 inserted between the two sides of the breakpoint. The proximal to distal order of the inserted breakpoints *within the long arm of chromosome 12* indicates that the insertion is direct. Unlike the short system, the detailed karyotype will also describe the new derivative (deleted) chromosome 3, using a semicolon, no spaces, to separate the two chromosome descriptions.

8.5.5 Inversion (inv)

Inversions can be de novo or inherited. Inversions that are known population variants are not included in the karyotype (see 8.8.2, Heteromorphic variations) [1, Table 1]. De novo or inherited inversions that are not known normal variants are considered structural aberrations and would be included in the karyotype. It is worth noting that individuals who carry a large pericentric inversion have an increased risk for producing viable zygotes with duplication-deletion consequences (see 8.5.9, Recombinant, later) [24].

Intrachromosomal inversions can be paracentric (involving the same arm, thus not involving the centromere) or pericentric (involving both arms, thus including the centromere). Pericentric inversions are generally easier to detect, because arm ratio is usually affected. Both forms use the intrachromosomal/two-breaks karyotype formula.

46,XY,inv(11)(q21q23)

46,XY,inv(11)(pter→q21::q23→q21::q23→qter)

Breakpoints within the same chromosome arm indicate that a paracentric inversion has occurred in the long arm (q) of chromosome 11, between bands q21 and q23. The inverted segment does not involve the centromere.

46,XY,inv(8)(p23.1q22.1)

46,XY,inv(8)(pter→p23.1::q22.1→p23.1::q22.1→qter)

A pericentric inversion was initiated by two breaks in chromosome 8, one on the short (p) arm at band p23.1, and the second on the long (q) arm at band q22.1. The inverted segment involves the centromere (see Figure 8.16).

8.5.6 Isochromosome (i)

An isochromosome (i) [1, section 9.2.11] is a mirror image of either the short (p) or long arms (q) of a chromosome, resulting in a gain (trisomy) for one arm and loss (monosomy) for the other arm. Its formation may be more complicated than a one-break deletion, but in writing the karyotype, it follows the same one-break formula. The arm described, e.g., q10, represents the duplicated arm *that is present*, leaving the opposite arm missing.

46,XY,i(21)(q10)

46,XY,i(21)(qter→q10::q10→qter)

An isochromosome for the long arms of chromosome 21 has replaced one normal chromosome 21; hence, the karyotype shows a total chromosome count of 46, but the patient has Down syndrome (Figure 8.17). No plus sign is used, because the centromere of the chromosome to which the extra material has been attached is already counted.

47,XY,+i(12)(p10)

47,XY,+i(12)(pter→p10::p10→pter)

The additional isochromosome (see Figure 8.17) indicates that the patient is tetrasomy (has four copies) for the short arms of chromosome 12, a karyotype that is found in mosaic form in patients with Pallister–Killian syndrome. Tetrasomy 21q is extremely rare, making tetrasomy 12p a more likely candidate; however, because of their remarkably similar appearance, FISH or molecular studies may be required to confirm its identity, especially in prenatal situations.

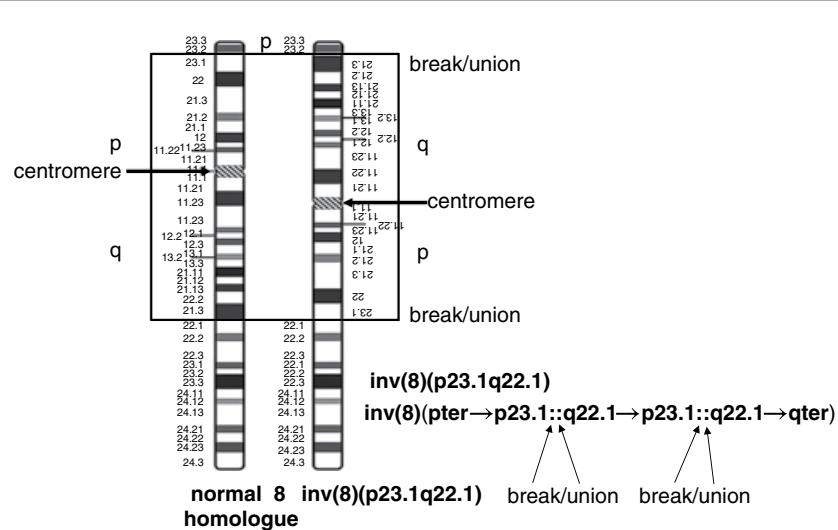


Figure 8.16 Pericentric inversion. When breaks occur on both arms (p and q) of one chromosome, and the broken edges reattach to opposite arms, a pericentric inversion has occurred. The breakpoints for pericentric inversions surround and thus involve the centromere, which will also make it easier to detect if the chromosome's arm ratio becomes distorted from this displacement. The short form karyotype will describe the event as an inversion (inv), and the reader can infer by the involved arms whether it is pericentric (different arms) or paracentric (same arm). The detailed karyotype describes the chromosome as it appears, from the end of the short arm (pter) to the end of the long arm (qter), using double colons where each break and union occurred.

ISCN diagram of human chromosome 8 at the 700 and 850-band level (© 2009 Nicole Chia) used by permission of the artist. Reproduced from ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

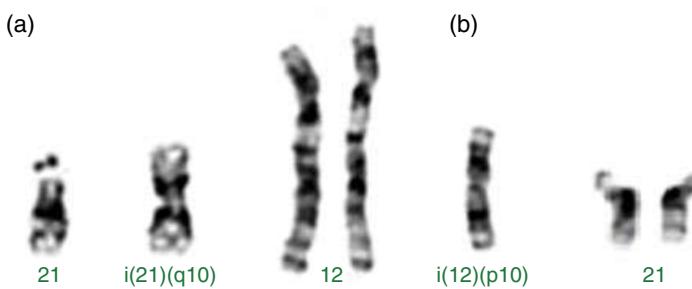


Figure 8.17 Isochromosome comparison, 21q and 12p. (a) **i(21)(q10)** Two duplicate q arms in this 21q isochromosome gives three copies of the Down syndrome critical region, making the patient positive for Down syndrome. (b) **i(12)(p10)** This extra isochromosome may look like (a), but it is actually an isochromosome for the short arms of chromosome 12, resulting in tetrasomy for that arm. The full karyogram for this cell can be seen in Figure 8.6. Mosaic tetrasomy 12p is found in patients with Pallister–Killian syndrome.

46,X,i(X)(q10)

46,X,i(X)(pter → q10::q10 → pter)

An isochromosome for the long arm of the X sex chromosome replaces a normal X, resulting in one extra copy (trisomy) for the long arm and one missing copy (monosomy) for the short arm of the second X sex chromosome in this female karyotype. Because there are no short arms in this abnormal structure, the detailed karyotype will use the long arm (q) to both begin and end its description.

47,XY,i(X)(q10)

The isochromosome is now in addition to two structurally normal X and Y sex chromosomes. The abnormal sex chromosome is placed *after all structurally normal sex chromosomes*; therefore, it will follow the Y chromosome. Chromosome count increases to 47; however, a plus sign is *not* used, because the abnormality involves *a sex chromosome in a constitutional study*. This exception would not apply to autosomes, nor would it apply to sex chromosome abnormalities as a result of neoplasia in a neoplastic karyotype. The net result of this karyotype is one copy of the short arm of the X sex chromosome, three copies of the long arm, and one Y sex chromosome [1, section 9.2.11].

8.5.7 Isodicentric (idic)

An isodicentric chromosome (see Figure 8.18) is an isochromosome where the break has occurred either above or below the centromere, leaving a partially duplicated segment of the opposite arm between the two active centromeres. Similar to the isochromosome, this chromosome has both duplication and deletion, but because it now hosts two active centromeres, it also visually shows two separate constrictions (if only one constriction is present, see Pseudoisodicentric, next). The event symbol changes to idic, and the breakpoint changes, but the karyotype formula is the same as for an isochromosome – one chromosome, one breakpoint.

46,X,idic(X)(q13)

46,X,idic(X)(pter → q13::q13 → pter)

This isodicentric has two copies of the short arm of the X chromosome and a duplicated copy of a small segment of the long arm from Xq13 breakpoint to the Xq10 centromere (see Figure 8.18). The detailed description starts as usual at the p-terminal end, passes through the centromere, breaks at Xq13 and reunites (:) into a dicentric mirror image.

Pseudoisodicentric (psu idic)

When an isodicentric shows only one active constriction, the arms will appear asymmetrical. The symbol psu idic would be used to indicate a pseudoisodicentric chromosome. Figure 8.18 demonstrates the unequal arm ratio of the pseudoisodicentric chromosome [1, section 9.2.4].

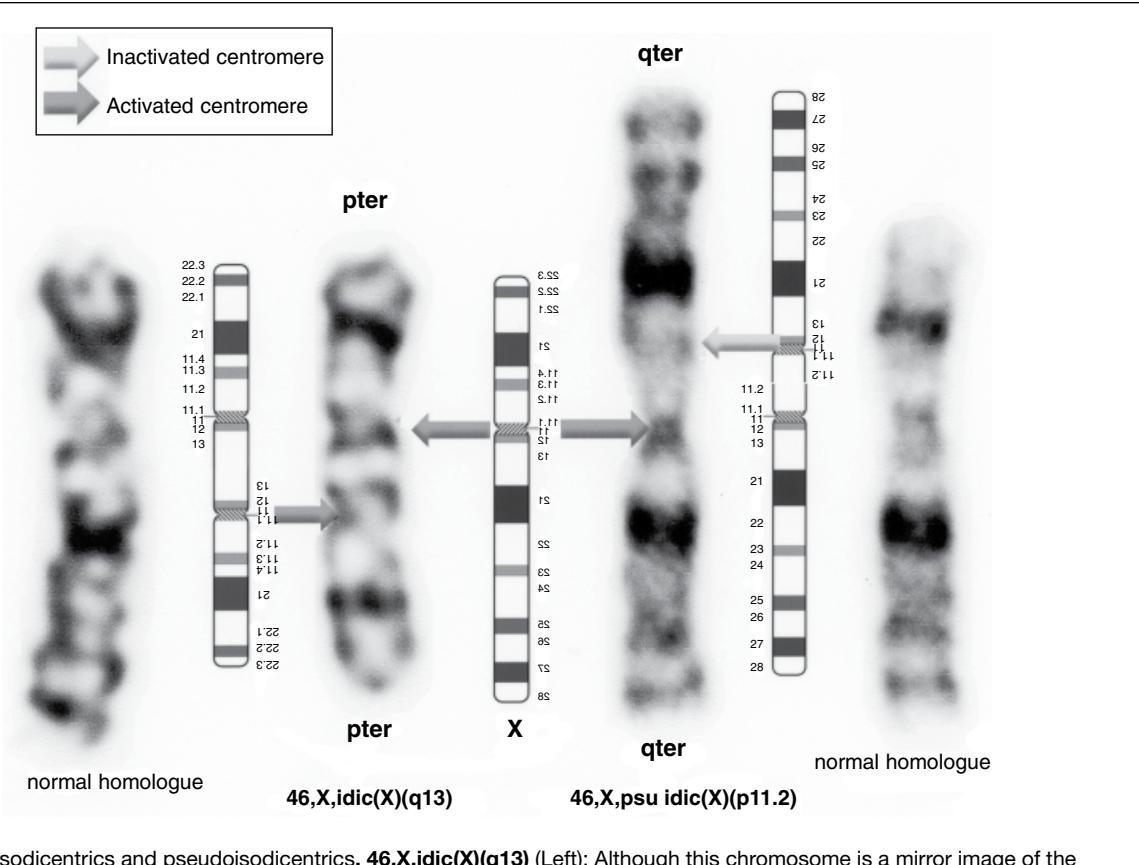


Figure 8.18 Isodicentrics and pseudoisodicentrics. **46,X,idic(X)(q13)** (Left): Although this chromosome is a mirror image of the p arms, the break occurred at band q13, not at the centromere, leaving the proximal segment of the q arm also duplicated. Both centromeres remain active, making this structure an isodicentric, with the duplicated p arm segment embedded between the two centromeres. **46,X,psu idic(X)(p11.2)** (Right): This mirror image of the q arms of the X sex chromosome includes duplication, not just of the long arm, but also of the proximal p11.2 region of the short arm. Because there is only one visual primary constriction, the chromosome is called a pseudoisodicentric. If one looks carefully, the two arms of this pseudodicentric are not equal in length. ISCN diagram of human chromosome X at the 400 band level (© 2009 Nicole Chia) used by permission of the artist. Reproduced from *ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013*. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

46,X,psu idic(X)(p11.2)

46,X,psu idic(X)(qter → p11.2::p11.2 → qter)

The break has occurred in the short arm at Xp11.2, with the duplicated short arm (Xp10 to Xp11.2) buried between the duplicated long arms. This forces the detailed karyotype to describe this chromosome from qter to qter order. A space separates the two symbols, psu and idic.

8.5.8 Constitutional origin (mat, pat, dn, inh, and c)

Symbols that explain the inherited origin of chromosomal material are mat for maternal, pat for paternal, and inh when an abnormality is inherited but origin is not confirmed. Specifying mat or pat is preferred, if origin is known [1, section 4.1]. If neither birth parent is found to carry the abnormality, the symbol dn can be used to indicate a de novo abnormality. These symbols of parental origin are generally placed without a space directly after the event's description, for example, 46,XY,del(22)(q11.2q11.2)mat; however, a space will be used when it follows another symbol, for example, +r mat. Neoplastic karyotypes will use a "c" to distinguish a constitutional event, for example, +21c, from an event that is the result of neoplastic disease (see 8.9.2, Neoplastic clonal evolvement).

8.5.9 Recombinant (rec)

A recombinant chromosome is an inherited, “structurally rearranged chromosome with a new segmental composition resulting from *meiotic crossing-over*” [1, section 4.5, p. 46]. The recombinant **rec** symbol can only be used in constitutional studies when it meets both of the following conditions:

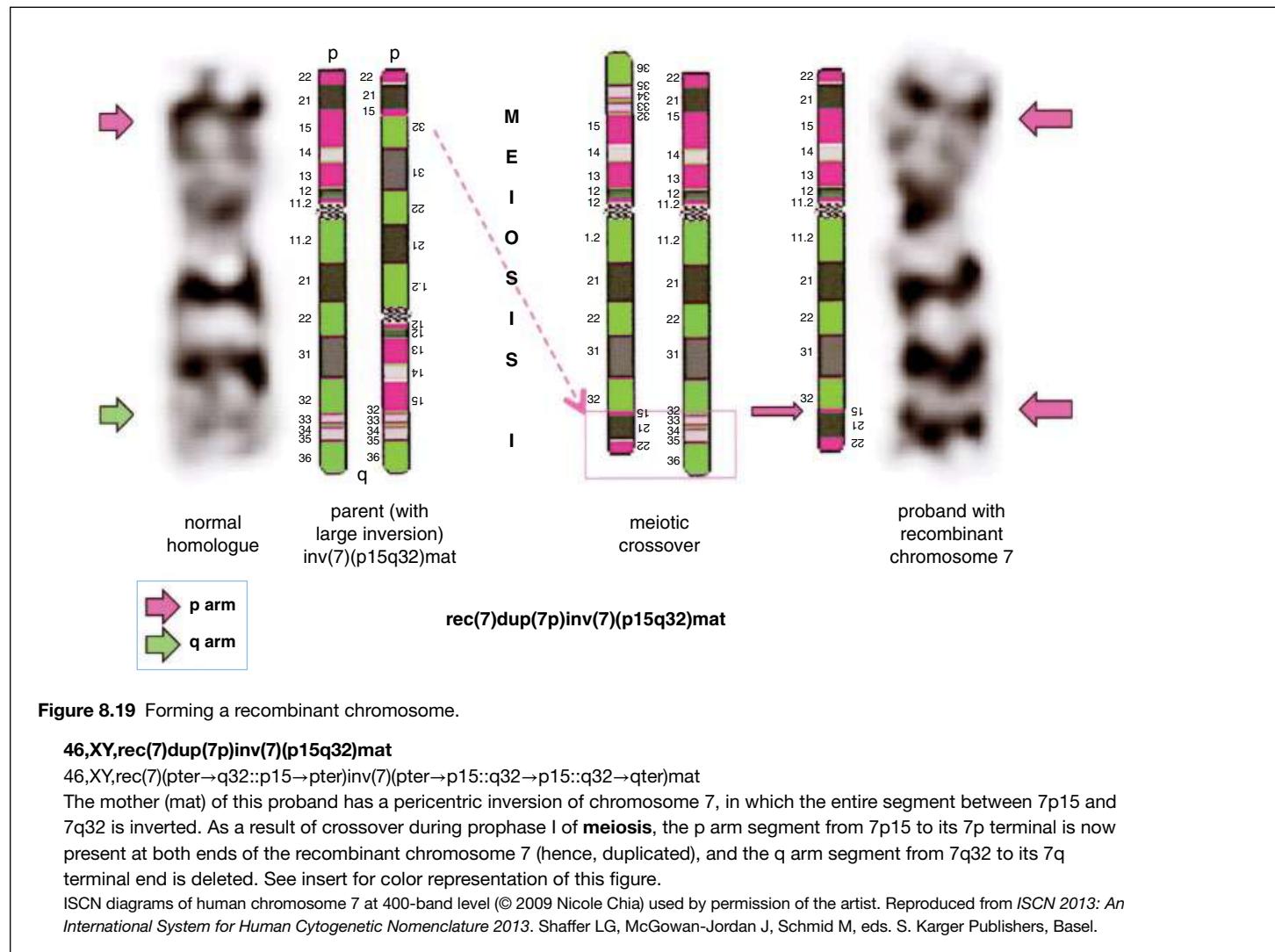
1. one parent is a known carrier of an inversion or insertion (e.g., a large inversion within chromosome 7 was present in the father of the example later);
2. *and* an unbalanced meiotic exchange between the rearranged chromosome and its homologous counterpart occurred during the pachytene stage of meiosis [1, Figure 9].

If the inversion has not been verified in a parent, or the inherited inversion or insertion did not undergo de novo meiotic rearrangement, the event would be described as a derivative. Likewise, if the rearrangement is a result of an acquired condition, or of any event other than meiotic crossover, the rearranged chromosome would also be described as a derivative.

46,XY,rec(7)dup(7p)inv(7)(p15q32)pat

46,XY,rec(7)(pter→q32::p15→pter)pat

The proband (i.e., person initially referred for testing)’s recombinant chromosome 7 was formed by unbalanced meiotic exchange as a result of a large pericentric inversion inherited from his father. The father’s karyotype, inv(7)(p15q32), is included in the short karyotype. The detailed karyotype paints a virtual picture of the unbalanced, recombinant chromosome, showing that the duplicated short (p) arm segment from 7p15 to 7pter (see Figure 8.19) has replaced the distal end of the long arm from 7q32 to 7qter [1, sections 4.5, 9.2.3].



8.5.10 Ring of known centric origin (r)

A ring (r) chromosome (see Figure 8.20) is formed when breaks occur in both arms surrounding a centromere, and the broken edges join together. It can involve one chromosome or multiple chromosomes, and can have one or more centromeres, as in dicentric (dic r) and tricentric (trc r) rings. When the centromere source within a ring chromosome is known, the ring description is placed in karyotype order, i.e., X before Y before autosomes in increasing numerical order (see 8.3.7, Karyotype order priority) by centromere identity. The first parentheses following the symbol will indicate which centromere is present in that event. If two different centromeres are present within one structure, both centric chromosomes will be listed in the first parentheses, with the first listed chromosome being selected following standard karyotype order. Rings of unknown origin are discussed under 8.7, Symbols of uncertainty.

47,XY,+r(18)

This patient has an extra centric ring in addition to two normal chromosomes 18, leaving a trisomic condition for the regions present. Because rings are centric formations, the total chromosome count will also increase. Breakpoints need not be included if they are not clearly identifiable.

46,XY,r(18)(p11.3q23)

46,XY,r(18)(::p11.3 → q23::)

A ring chromosome has replaced one chromosome 18 homologue (see Figure 8.20). A break has occurred in the short arm at p11.3 and the long arm at q23, leaving a loss (monosomy) of the segment from 18p11.3 to the terminal end of the short arm (e.g., p11.3 → pter) and from 18q23 to the terminal end of the long arm (e.g., q23 → qter).

47,XY,+dic r(7;18)(p11.2q32;p11.2q22)

A dicentric ring involving chromosomes 7 and 18 was formed by the union of breakpoints 7q32 with 18p11.2 and breakpoints 7p11.2 with 18q22, each chromosome retaining its centromere. A space separates the two alphabetic symbols, dic and r. The two contributing chromosomes are written in karyotype priority order. Likewise, a tricentric ring will use the symbol trc with the three chromosome descriptions following the same basic order as described in 8.5.12, Multichromosomal translocation.

Because of mitotic instability, ring chromosomes originating from a single cell may form different structural appearances, for example, larger or smaller sizes, figure eights, nonring formations, loss or extra copies. Placing the mos symbol before the event's description will inform the reader that the structure's appearance was inconsistent, but clonal.

47,XX,+mos r(18)(p11.2q21.1)

47,XX,+mos r(18)(::p11.2 → q21.1::)

The additional (+) presence of a ring chromosome, derived from a centric segment of chromosome 18, has been seen in some but not all cells analyzed [1, sections 9.2.3 and 9.2.15].

A ring involving two chromosomes, but with only one centromere present, will be written as a derivative (see 8.6.6, Ring derivative involving more than one chromosome) [1, section 9.2.15]. Additional ring formations are described under 8.7, Symbols of uncertainty.



Figure 8.20 Ring chromosome.

46,XY,r(18)(p11.3q23)

46,XY,r(18)(::p11.3 → q23::)

This monacentric ring was formed as a result of breaks occurring on opposite arms. The new structure retains the centromere, but has lost genetic material distal to the two breakpoints. Ring structures may show structural instability during mitosis, which can lead to a variety of altered appearances and numerical variations.

8.5.11 Telomeric association (tas)

When describing telomeres in multichromosomal telomeric association (tas), the karyotype order for listing the involved chromosomes will follow the order of their association, starting with the end chromosome with the highest priority – that is, X before Y before lowest autosome value [1, section 9.2.16]. The most distal band number is selected for the short form karyotype, while the telomeric symbol (pter or qter) is used in the detailed karyotype. The detailed karyotype will also describe the unassociated terminal ends of the first and last chromosomes in the linear string. Although they may appear as one entity, individual chromosomes are counted independently.

46,XY,tas(5;12)(q35;p13)

46,XY,tas(5;12)(5pter → 5qter → 12pter → 12qter)

Chromosome 5 is numerically lower than chromosome 12, and thus will begin the string of telomeric associations. The telomeric region of the long arm of chromosome 5 is in association with the telomeric region of the short arm of chromosome 12, leaving their opposite arms as unassociated ends, as shown in the detailed karyotype.

46,XY,tas(5;12;8;16)(5q35;12p13;8p23;16q24)

46,XY,tas(5;12;8;16)(5pter → 5qter → 12pter → 12qter → 8pter → 8qter → 16qter → 16pter)

This example expands the basic concept above with additional partners, leaving the terminal ends being the short arm of chromosome 5 and the short arm of chromosome 16.

8.5.12 Translocation (t)

Reciprocal translocation

When a chromosome exchanges a segment of its arm with another chromosome, a translocation has occurred (Figure 8.21). Speaking constitutionally, if the rearrangement is balanced and the patient has no clinical symptoms, he or she may not even be referred for genetic testing until or unless experiencing reproductive problems. In neoplasia, however, a seemingly “balanced” rearrangement may not be as innocent as it appears. Certain genetic juxtapositions have become critical diagnostic indicators for specific hematological abnormalities (see Chapter 11, Cytogenetic analysis of hematologic malignant diseases). These rearrangements disrupt critical coding or create new codes that will alter a gene’s end product, ultimately leading to the clinical features associated with the particular neoplastic disease.

46,XY,t(9;22)(q34;q11.2)

A “balanced” translocation (t) between chromosomes 9 and 22 has exchanged material distal to breakpoints at 9q34 and 22q11.2. Also referred as the Philadelphia chromosome (Ph) or *BCR/ABL1* (pronounced “B-C-R-able”) rearrangement, this “balanced” exchange juxtaposes the *ABL1* gene on chromosome 9 at q34 with the *BCR* gene at q11.2 on chromosome 22, and has become the diagnostic indicator for chronic myelogenous leukemia (CML).

46,XY,t(9;22)(9pter → 9q34::22q11.2 → 22qter;22pter → 22q11.2::9q34 → 9qter)

The detailed karyotype starts the same as the short form, for example, 46,XY,t(9;22), but will replace the second parentheses with a description of the translocated chromosome (9) and its partner, chromosome 22, separated by a semicolon, no spaces. The regions distal to the two breakpoints, that is, 9q34 → 9qter and 22q11.2 → 22qter, are exchanged via two break:rejoin event.

Whole arm translocation

To be eligible as a whole arm translocation, a rearrangement must: (i) exchange entire arms of the involved chromosomes, (ii) be balanced, and (iii) have a centromere of unknown origin. Chromosome count remains at 46, and no plus sign precedes the event symbol. Karyotype format is similar to a reciprocal translocation, but using only centromeric breakpoints (p10 and q10) to reflect the arm that is present. In other words, if the short arm is present, p10 is used as the breakpoint.

The first-listed chromosome will be selected by the short arm of highest priority order - that is, X before Y before lowest numerical autosome. Karyogram position will also be determined by the short arm of the chromosome of highest priority. An unbalanced rearrangement, however, should be described as a derivative chromosome (see 8.6.7, Nonacrocentric whole arm derivative).

46,XY,t(17;20)(p10;q10)

46,XY,t(17;20)(17pter → 17p10::20q10 → 20qter;20pter → 20p10::17q10 → 17qter)

This whole arm translocation involves the short arm of chromosome 17 being attached to the long arm of chromosome 20 (see Figure 8.7). Its counterpart, the short arm of chromosome 20 that is attached to the long arm of chromosome 17, need not be described in the short karyotype, because as a balanced karyotype, its presence is inferred. The detailed karyotype, however, will describe both rearranged chromosomes, using the semicolon as a separator.

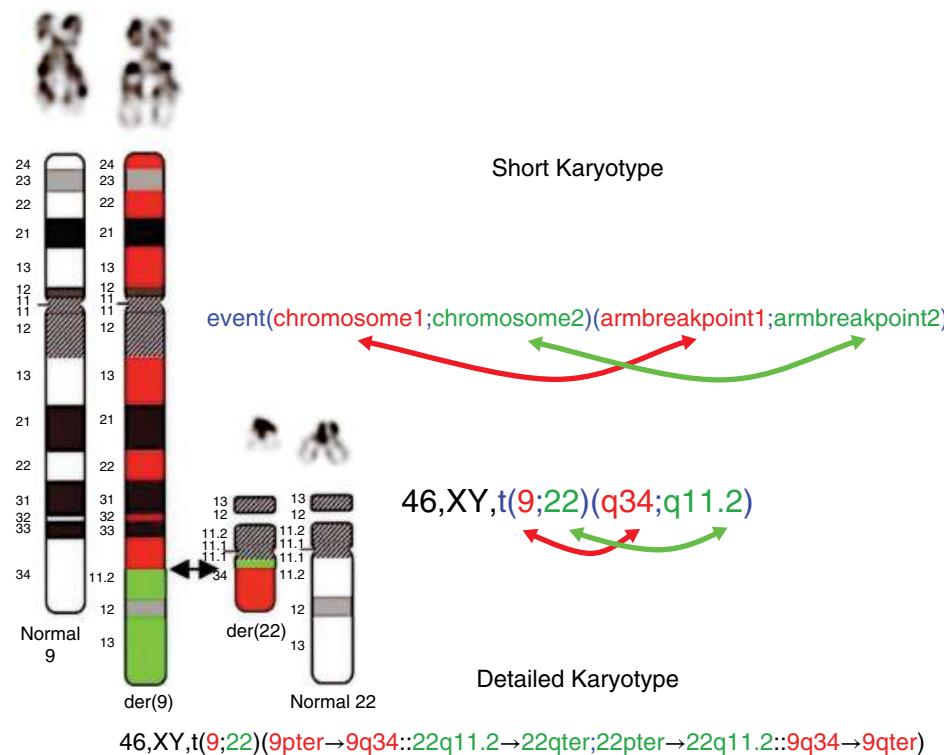


Figure 8.21 Reciprocal translocation.

The short form karyotype uses the semicolon to separate the two different translocated chromosomes and their respective breakpoints. The detailed karyotype begins the same way, but the last paragraph will describe both partners from p terminal to the break and reunion and then proceed to the terminal end, with a semicolon separating the two chromosome descriptions. Centromere origin will determine a derivative's placement in a karyogram or karyotype. See insert for color representation of this figure.

ISCN diagram of human chromosomes 9 and 22 at 400-band level (© 2009 Nicole Chia) used by permission of the artist. Reproduced from *ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013*. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

Although acrocentric or Robertsonian translocations are treated in a similar manner, they incur a loss of the short arms and are thus described under 8.6.7, Acrocentric derivative.

Multichromosomal translocation

When multiple chromosomes are involved in a translocation, the first-listed chromosome will be of highest priority – that is, X before Y before lowest numerical autosome. Once that first chromosome is selected, the order will depend upon which chromosome received its broken segment. This chain reaction continues, until all involved chromosomes have been listed. If done correctly, the last chromosome should be the one donating its segment to the first chromosome, thus completing a virtual circle.

When rearrangements involve multiple chromosomes in complex patterns, chromosome numbers may be included with their respective breakpoints in the second parentheses. For more complex multichromosomal rearrangements, see *ISCN 2016* [1, section 9.2.17.1].

46,XY,t(5;12;8;21)(q31;p13;q22;q22)

46,XY,t(5;12;8;21)(5pter→5q31::21q22→21qter;5qter→5q31::12p13→12qter;8pter→8q22::12p13→12pter;21pter→21q22::8q22→8qter)

This example (see Figure 8.22) does not involve sex chromosomes; therefore, chromosome 5, being the lowest autosomal number, is listed first. Chromosome 12 receives the segment from chromosome 5 and donates its segment to chromosome 8, who in turn passes its segment to chromosome 21. The last chromosome (21) donates its segment back to chromosome 5, and the circular exchange is complete.

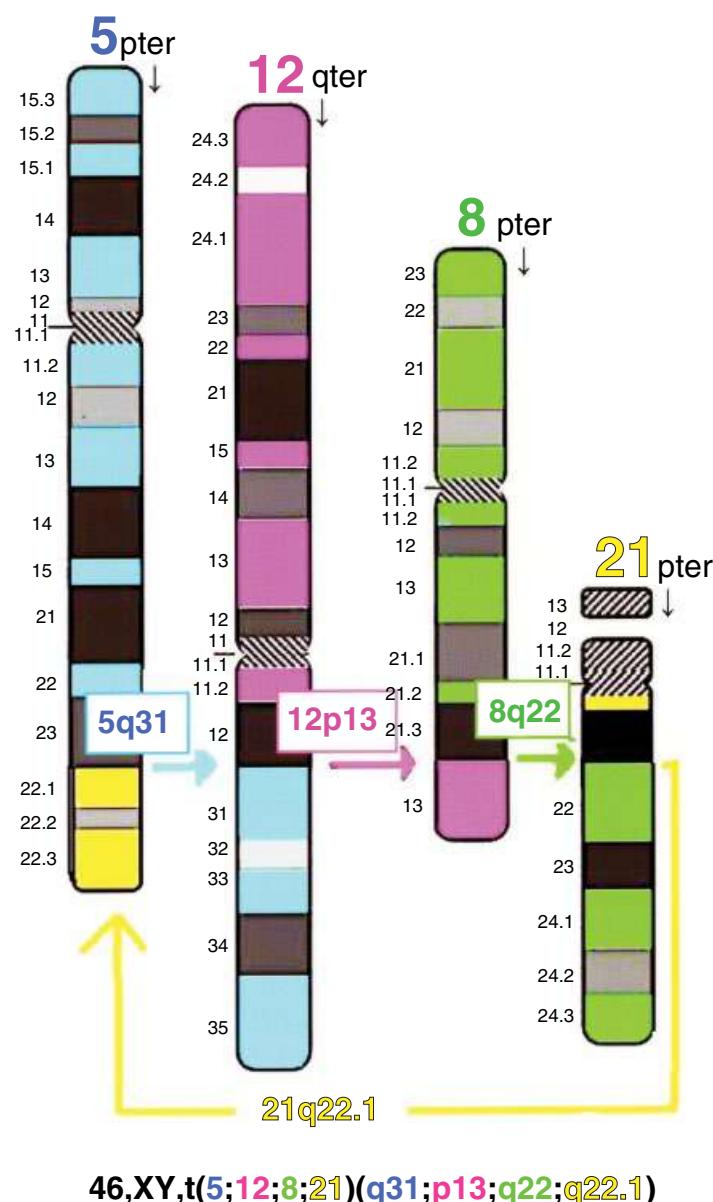


Figure 8.22 Four-break translocation.

46,XY,t(5;12;8;21)(q31;p13;q22;q22.1)

When writing a translocation involving more than two chromosomes, the first-listed chromosome will follow standard karyotype order, e.g., X before Y before lowest autosome number. The remaining order will depend upon which chromosome is the receiver of the previous chromosome's segment. The last chromosome will be the one donating the segment to the first chromosome. See insert for color representation of this figure.

ISCN diagram of human chromosomes 5,8,12, and 21 at 400-band level (© 2009 Nicole Chia) used by permission of the artist. Reproduced from *ISCN 2013: An International System for Human Cytogenetic Nomenclature 2013*. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

8.5.13 Uniparental disomy (upd) and loss of heterozygosity (LOH)

As explained in Chapter 10, Genomic imprinting, the discovery of uniparental disomy (upd) revealed the cell's innate ability to "autocorrect," that is, to rescue either a potentially lethal monosomy outcome by retaining two copies of the same chromosome, or rescue a lethal trisomic outcome by eliminating one, unfortunately the wrong homologue. In both cases, what is left behind after the rescue is a homologous pair from the same parent. Because each homologue still contains the correct amount of genetic material, on the surface it would seem that the proband is genetically balanced, but this is not the case when involving imprinting genes (those that are specifically affected by parental origin). The formula for writing a "upd" karyotype is upd(chromosome)origin, for example, 46,XY,upd(15)pat. See 8.6.7, Acrocentric derivative, for an interesting example of upd as a result of the inheritance of a homologous acrocentric translocation.

Segmental uniparental disomy as an acquired condition is also being discovered with alarmingly high incidences in association with certain neoplastic diseases, such as MDS (myelodysplastic syndrome) [25,26]. Because the problem is technically the loss of somatic heterozygosity, rather than meiotic reproductive error, the molecular abbreviation **LOH** for loss of heterozygosity is used to refer to these situations. Routine cytogenetics or FISH cannot detect segmental homozygosity with absolute confidence, even if the homologues exhibit heterogeneity; therefore, microarray studies, such as SNPs (single nucleotide polymorphisms), would be needed to detect LOH if suspected.

8.6 Derivative chromosomes (der)

A derivative chromosome by definition is "a structurally rearranged chromosome generated either by a rearrangement involving two or more chromosomes or by multiple aberrations within a single chromosome" (1, section 9.2.3, p. 62). The first parentheses that follows the der symbol will explain which participant(s) of that prior event contains a centromere, followed with a description of the event that originated the derivative product. No spaces or punctuation separate the symbols and their parentheses, leaving the entire description a single unit. Semicolons, however, will still be used to separate interchromosomal events within their respective parentheses.

8.6.1 One centric derivative

When only one centric partner is present from a previous event, making the karyotype unbalanced, the first parentheses will list the centric chromosome that is present, followed by a description of the previous event.

46,XY,der(22)t(9;22)(q34;q11.2)

This unbalanced karyotype describes a derivative chromosome replacing one chromosome 22 that previously had been in rearrangement with chromosome 9 (see 8.5.12, Reciprocal translocation); however, both homologues of chromosome 9 appear normal. The end result is trisomy for 9q34 → qter, monosomy for 22q11.2 → qter, and most importantly, a juxtaposition of the *BCR/ABL1* loci on chromosome 22. It should be noted, however, that this result raises several complex scenarios that may require further investigation, including loss of heterozygosity (homozygosity) for chromosome 9 (see 8.5.13, Uniparental disomy and loss of heterozygosity).

8.6.2 Homologue derivative

When identical rearrangements involve homologous chromosomes, the resulting derivative(s) may require one of the homologues to be underlined for clarity. This enables the karyotype to separately track clonal evolution for each homologue [1, sections 4.1, 9.2.3, and 9.2.17.1]. Either homologue can be underlined (1, section 9.2.3); however, once designated in the karyotype, that same homologue would continue as the one underlined [1, section 9.2.3].

46,XY,der(1)t(1;1)(p36.2;q21)

A prior unbalanced translocation between chromosome 1 homologues resulted in a partial trisomy of its long (q) arms (Figure 8.23). The derivative replaces one homologue, giving a net result of monosomy for the region from 1p36.2 → 1pter and trisomy for 1q21 → 1qter [1, section 9.2.3].



Figure 8.23 Rearrangement between homologues.

46,XY,der(1)t(1;1)(p36.2;q21)

Homologous exchange in this example resulted in partial trisomy for the long arm of chromosome 1 from q21 to the q terminal end. The extra segment is attached to the p arm of chromosome 1 at p36.2.

8.6.3 Isoderivative

An isochromosome that is formed from a derivative chromosome is called an isoderivative.

46,XY,ider(20)(q10)del(20)(q11.2q13.3)

This rearrangement created a mirror image duplication of the long (q) arm of chromosome 20 after a prior deletion of most of the q arm [1, section 9.2.3]. Figure 11.2 in Chapter 11, Cytogenetic analysis of hematologic malignant diseases, includes an image of this isoderivative.

8.6.4 Multiple events derivative

When more than one independent, structural event has occurred, the karyotype event must be written as a derivative [1, section 9.2.3].

46,XY,der(16)del(16)(p13.1)del(16)(q24)

46,XY,der(16)(:p13.1 → q24:)

Both arms show terminal deletions (p13.1 and q24). The short form must describe each event involved in this derivative separately. The detailed karyotype simplifies the karyotype by describing only the end product, beginning and ending with single colons, that is, (:p13.1 → q24:).

When a derivative chromosome involves multiple events, they are described in pter → qter order, irrespective of the alphabetical order of their associated symbols.

46,XY,der(16)del(16)(p13.1)add(16)(q24)

46,XY,der(16)(:p13.1 → q24::?)

Two events have occurred within chromosome 16 – a deletion to one end and an addition of unidentifiable material to the other end. The karyotype will list breakpoints in pter to qter order, irrespective of the alphabetical order

of their event symbols; therefore, the deletion (del) of the short arm will be described before the addition (add) of the long arm. See 8.7, Symbols of uncertainty, for rules governing the addition of unidentifiable chromosomal material.

8.6.5 Neocentromere derivative

If an acentric segment forms a new (neo) centromere (see Chapter 9, section 9.2.5, Neocentromeric or analphoid chromosomes) at a position that generally does not contain a centromere, the der or neo symbol can be used. The short system may not always be sufficient for describing this event.

47,XY,+der(1)(qter → q23:)

47,XY,+neo(1)(qter → q23:)

An extra chromosomal structure results in a trisomic presence of the distal portion of the long arm of chromosome 1. Because this region does not normally include a centromere, a new (neo) centromere has been generated at breakpoint 1q23. In the absence of a short arm, the detailed karyotype will begin its description with the terminal end of the long arm. Either symbol (der or neo) is acceptable.

When needed for clarity, the position of the neocentromere can be indicated by the neo symbol [1, section 9.2.13], for example, **47,XY,+der(1)(qter → q23 → neo → q23:)**.

8.6.6 Ring derivative involving more than one chromosome

Rings that are formed from more than one chromosome will adapt to the formula that best explains its formation (see 8.7.6, Rings of unknown origin). A monocentric ring derived from two chromosomes would be written as a derivative, with the chromosome providing the centromere listed first.

47,XY,+der(18)r(18;7)(p11.2q22;q11.2q32)

Chromosome 18 holds the centromere of the ring; therefore, it is described before the numerically lower chromosome 7. It will also be positioned in the karyogram with its homologue. The karyotype is unbalanced, with trisomy for the segments on chromosome 18 from p11.2 → q22 and chromosome 7 from 7q11.2 → 7q32 [1, section 9.2.15].

8.6.7 Whole arm derivative

Acrocentric derivative

Most acrocentric whole-arm translocations originate from breaks in the short arms, making them dicentric [1, section 9.2.17.3]; however, without special tests to verify centromeric origin, both partners are listed in the first parentheses. Generally speaking, the use of centromeric breakpoints in the karyotype, e.g., p10 or q10, reflects the arm that is *present* in the cell (see 8.5.12, Whole arm translocation); therefore, acrocentric derivatives will use q10 for both partners in the second parentheses. Loss of their respective p-arm counterparts, which by itself supports acentric behavior, has little known clinical effect on the carrier, because they involve nucleolus organizing regions (NORs). In some situations, however, a bisatellited marker chromosome is present in some or all cells analyzed, which could have clinical significance, depending on where the breaks have occurred and what genetic material is involved. For example, if the break involved the Prader–Willi/Angelman syndrome gene locus on 15q, the subsequent imbalance of its imprinting genes could potentially affect the proband. The symbol **rob** for the Robertsonian translocation is another option for describing whole arm translocations, but only if they involve acrocentric chromosomes [1, section 9.2.17.3], and only in constitutional, *not* neoplastic (acquired), karyotypes [1, section 9.2.17.3]. Its applicability, therefore, is limited, but of historical importance.

45,XY,der(13;21)(q10;q10)

The q arms of two acrocentric chromosomes, 13 and 21, have merged at the centromere, making the chromosome count total at 45 independent, centric chromosomal bodies. Breakpoints in the second parentheses (q10;q10) indicate that the long arms are present. Although the 45-chromosome count represents an unbalanced state, the loss involves NORs, for which there is no known negative effects on the carrier.

46,XY,der(13;21)(q10;q10),+21

This 46-chromosome karyotype indicates that the derivative has replaced one chromosome 13; therefore, the result is one normal chromosome 13, two normal chromosomes 21, and a whole-arm derivative made of the q arms from chromosomes 13 and 21. This gives a net total of three copies of the long (q) arms for chromosome 21, and the proband would have Down syndrome.

45,XX,der(21;21)(q10;q10)

45,XX,der(21;21)(21qter→21q10::21q10→21qter)

When a translocation involves both homologues for a chromosome, leaving no normal chromosome for that set, as shown in this derivative for chromosome 21, the patient's risk of having an abnormal child would become 100%, because there is no normal chromosome 21 to pass on. Hypothetically speaking, if a child inherits this structure from her father, and no normal homologue from her mother, the net result would reflect uniparental disomy (see 8.5.13, Uniparental disomy and loss of heterozygosity), and the karyotype would be written: 45,XX,upd der(21;21) (q10;q10)pat [1, section 8.4].

Nonacrocentric whole arm derivative

Similar to the acrocentric derivative above, whole arm translocations of nonacrocentric chromosomes become derivatives when their partners are lost, leaving an unbalanced karyotype. The interchromosomal/two-breaks karyotype formula is used, listing both chromosomes in the first parentheses following the derivative (der) event symbol. The breakpoints will reflect which arms are present.

46,XX,+9,der(9;18)(p10;q10)

46,XX,+9,der(9;18)(9pter→9p10::18q10→18qter)

This particular whole-arm derivative (see Figure 8.24), seen in *BCR/ABL1* negative chronic myeloproliferative disorders [27], reflects a net gain of the short arm of chromosome 9 and a net loss of the short arm of chromosome 18. Both presumed centromere-carrying chromosomes are placed in the first parentheses following the der symbol. The lost arms (9q and 18p) need not be defined because their absence can be inferred.

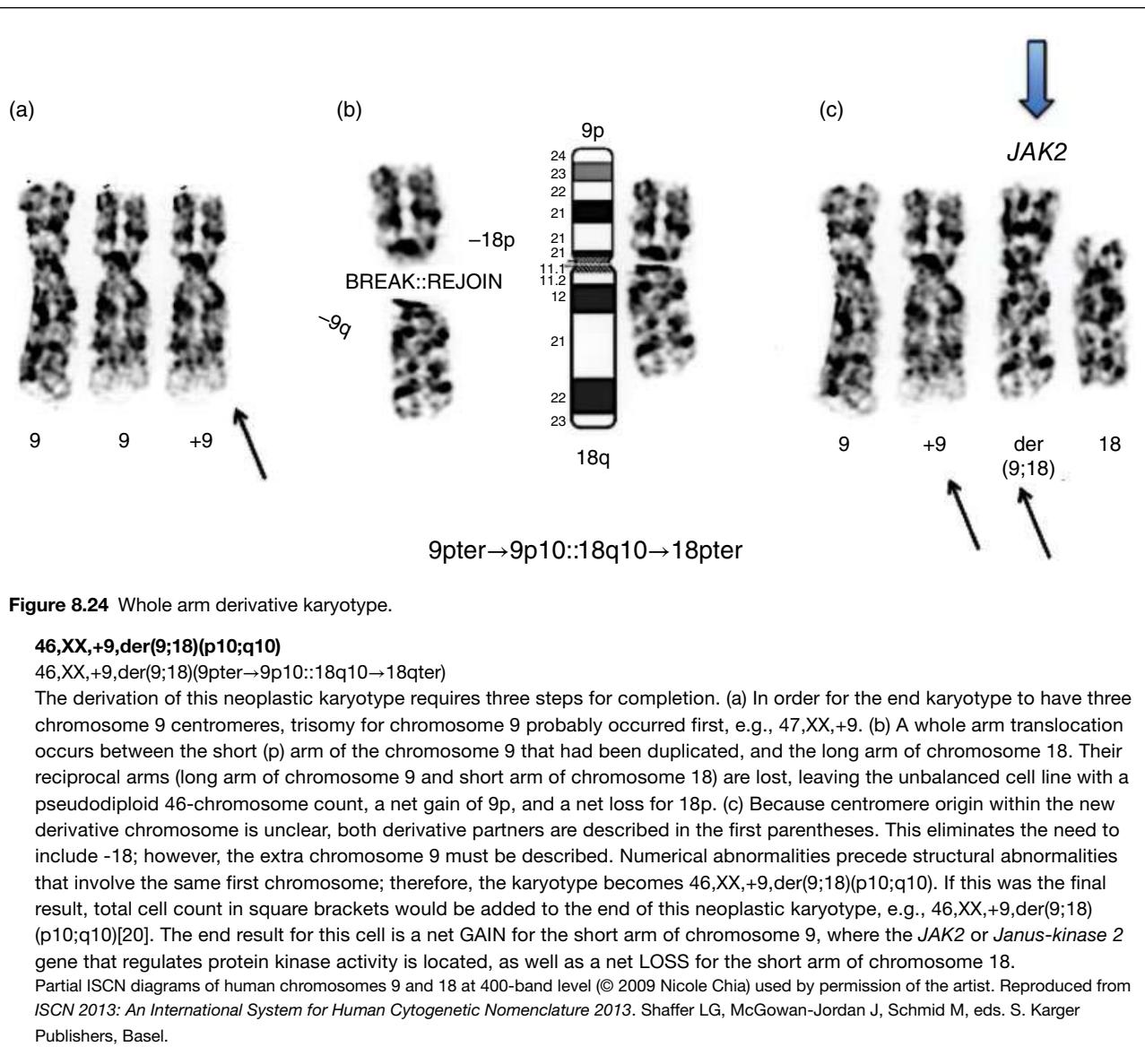
8.7 Symbols of uncertainty

One of the great strengths of ISCN is its ability to describe the indescribable or questionable [1, Chapter 5]. This is accomplished with the help of the following symbols:

?	uncertainty
add	additional material of unknown origin
hsr	homogeneously staining region
or	alternatives (spaces are used before and after)
~	approximate; uncertain band location; range of numbers
r	ring
mar	marker
dmin	double minute (pronounced mi-noot, as in very small)
inc	incomplete
cp	composite

8.7.1 Uncertainty (?)

When identity is unclear, a question mark is placed directly before the item in question. Positioning the question mark is important, as the wrong placement will lead to a wrong conclusion by the reader. For example, if the event is questioned, the mark would precede the symbol, for example, ?del. If the chromosome identity is uncertain, the question mark would precede the suspected chromosome number, for example, del(?5). If the breakpoint is unclear, the question mark would be placed before the specific band at which its identity is no longer absolute – for example, 46,XY,del(5)(p?15.1) establishes that a



terminal deletion has occurred on the short (p) arm of chromosome 5, but it questions which region is involved. On the other hand, 46,XY,del(5)(p15.?1) is able to pinpoint the break to band p15, but is unclear which sub-band is affected. See 8.7.5, Approximation range for additional options.

The question mark is also used when an unidentifiable, clonal chromosomal structure containing a centromere must be described within the karyotype, or a segment of a known chromosome is in rearrangement with this unidentifiable, centric chromosome.

47,XY,+3,-7,-20,+der(?)(?;1)(?;p11.2),+mar,inc[20]

47,XY,+3,-7,-20,+der(?)(1pter→1p11.2::?→cen→?),+mar,inc[20]

Even though chromosome 1 is a known participant in this derivative structure, the question mark indicates that the centromere's origin cannot be identified. The event is placed at the end of the karyotype before the other symbols of uncertainty — that is, rings, markers and double minutes (see 8.3.7, Karyotype order priority). The incomplete (inc) symbol at the end indicates that additional inconsistencies were also being questioned, but could not be definitively or clonally identified at this time. Because the bracketed number does not specify that the karyotype is a composite (see 8.7.10, Composite karyotype), all twenty analyzed cells showed these same clonal abnormalities.

8.7.2 Additional material (add)

As its name implies, the triplet add is used when unknown, additional material attaches to one, *but only one*, terminal end of a chromosome (or at the breakpoint of a deletion that includes the terminal end) [1, section 9.2.1]. The short form does not use the question mark, because it is understood by the use of the *add* symbol; the detailed format, however, describes the virtual picture, and therefore requires the mark.

add(9)(p11.2)

add(9)(?:p11.2→qter)

Unidentifiable material is attached to the short (p) arm of chromosome 9 at band p11.2. The detailed system will describe the affected chromosome from pter to qter order, where the region from the p-terminal (pter) to band p11.2 has been replaced with unknown (?) material as a result of a breakage-reunion (::). This example results in a loss of most of the p arm of chromosome 9, and a gain of unknown material.

When additional, unidentifiable material “is attached to both arms of a chromosome and/or replaces more than one segment in a chromosome” [1, section 9.2.1, pg 61], the derivative symbol is used, e.g., 46,XY,der(16)add(16)(p13.3)add(16)(q24) (1, section 9.2.3).

8.7.3 Homogeneously staining region (hsr)

Homogeneously staining regions are amplification within the genome that appear as unbanded, inserted material within a chromosome arm. When hsr is present, irrespective of size or origin, the karyotype states only the breakpoint where the material has been inserted.

46,XY,hsr(9)(p22)

46,XY,hsr(9)(pter→p22::hsr::p22→qter)

Unidentifiable, homogeneously staining material has been inserted into the short arm of chromosome 9 at band 9p22 (Figure 8.25). Using the hsr symbol does not assume that the repetitive material is from chromosome 9; it only indicates that the unidentifiable material is homogeneously stained, that is, without significant band identifiers.

When a chromosome contains either more than one hsr region or a structural change in addition to the hsr, the event is described as a derivative [1, section 9.2.8].

8.7.4 Or

Symbol **or** is used for alternative interpretations of an aberration, as when both interpretations form identical-appearing structures, both are cytogenetically observed, or both are confirmed by additional testing. It also uses a space before *and* after the symbol.

46,XY,del(7)(q11.2q22 or q22q32)[20]

Band widths and appearance in this neoplastic karyotype make it difficult to determine with confidence which breakpoint is involved without further testing.

Another potential for using the **or** symbol is when different breakpoints have affected the same chromosome, but *not at the same time*.

46,XY,add(16)(p13.3 or q24)

This karyotype describes additional, unidentifiable material on either arm, but not appearing at the same time; it is either one *or* the other, not both together. If these different, unidentifiable additions were to appear simultaneously on a chromosome, the derivative formula would be used [1, 9.2.1, 9.2.3] (see 8.7.2, Additional material), for example, 46,XY,der(16)add(16)(p13.3)add(16)(q24).

8.7.5 Approximation (~) range

When a precise breakpoint can only be localized to a range within a segment, rather than one specific position with certainty, or when the copy number of rings, markers or double minutes varies within the cells examined, the approximation or tilde (~) sign can be used to indicate upper (most distal) and lower (most proximal) boundaries [1, Chapter 3].

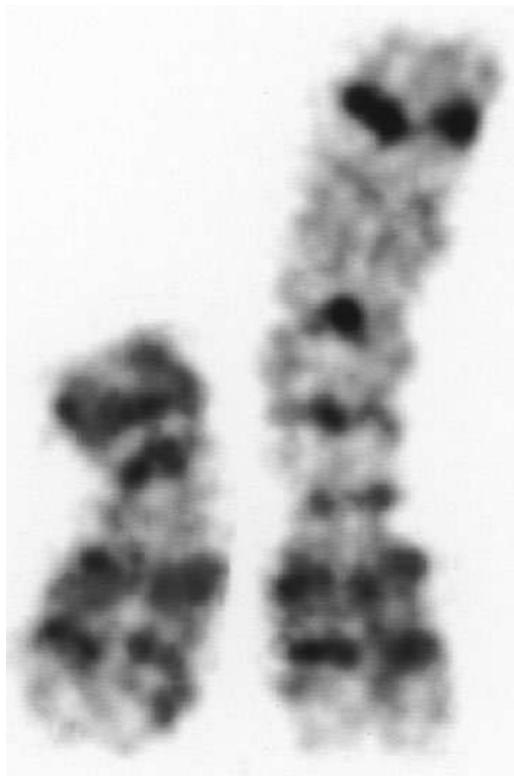


Figure 8.25 Homogeneously staining region (hsr). Chromosome 9 shows an insertion of an unidentifiable, homogeneously staining region.

46,XY,del(16)(p13.1~13.3)

A deletion was observed in the short (p) arm of chromosome 16, either at p13.1, p13.2 or p13.3 [1, section 5.2]. All band positions between and including the two range values (p13.1 and p13.3) are potential breakpoints. Although one might think the *or* symbol could also be used to write this karyotype (see 8.7.4, Or), by definition it cannot, because it does not imply that the range between the two positions are equally valid breakpoints.

8.7.6 Rings of unknown origin (r)

A ring can be monocentric or multicentric; it may involve only one chromosome or it could involve segments from multiple chromosomes [1, section 9.2.15]. Its original chromosomal source is not always identifiable; therefore, several different methods are used to describe a ring chromosome. Ring chromosomes with a known centromere source have already been discussed under 8.5.10, Ring of known centric origin. When a ring's identity is not known, it is placed at the end of the karyotype with other unidentifiable structures, in the following order [1, Chapter 6]:

- derivative chromosome with an unknown centromere, for example, +der(?);
- ring of unknown identity (+r);
- marker of unknown identity (+mar);
- double minute (dmin).

47,XY,-18,+21,+r

Even though chromosome 18 is missing, karyotypes are not based on assumption [1, section 9.2.3]; therefore, one cannot assume that the ring contains material from the missing chromosome 18, nor that the centromere is from chromosome 18, without confirmation by tests like FISH or chromosome microarray analysis. Without certainty, the writer must describe what is seen, not what is assumed to have happened; therefore, without knowing the origin of the centromere, the ring

symbol is placed after all known autosomal description, before marker descriptions, if also present. Trisomy 21 was added in this example to emphasize the ascending numerical placement order of autosomes; therefore, the karyotype would list -18 before +21, and both before the ring of unknown identity.

As a result of its potential instability, ring chromosomes can appear in an assortment of shapes and sizes, e.g., double rings, figure eights, broken segments, and so forth, but the karyotype need only describe the original ring formation. This instability can also lead to multiple copies of the same or different appearing ring structures. When more than one clonal but unidentifiable ring structures are present, the number or range of copies will precede the ring symbol, without a space, as shown in the next examples.

48,XY,-18,+3r[20]

This neoplastic karyotype indicates the presence of 48 centric bodies. Chromosome 18 is missing, and three, unidentifiable ring structures are present. This description does not, however, explain whether the three rings are identical [1, section 9.2.15].

If ring structures are unidentifiable, but clonally distinguishable, ascending *Arabic* numbers can be appended as a suffix.

48,XY,+r1,+r2[20]

Two clonally different, but still unknown ring structures are present in the 20 cells analyzed.

The numerical notation that indicates different morphological ring entities, e.g., r1 and r2, should not be confused with the karyotype description for a ring involving chromosome 2, for example, r(2), which encloses the involved chromosome number in parentheses [1, section 9.2.15].

50,XY,+r(2),+8,+r1,+r2[20]

A ring involving chromosome 2 is described in ascending numerical order by its chromosome identity, thus before the extra chromosome 8. The two clonally different rings of unknown origin, r1 and r2, are placed at the end of the karyotype.

When more than one different, clonal ring formation is present, and one (r1) has multiple copies, the times (x) sign with its copy number will follow its ring notation, without spaces [1, section 9.2.12].

49,XY,+r1x2,+r2[20]

Two different, but unidentifiable clonal ring structures (r1 and r2) are present. One ring structure (r1) has two copies (**x2**) per cell and the other ring has one copy (+r2) per cell. Together they total three additional centric rings, bringing the chromosome count to 49.

One last variation is the ring chromosome that has been inherited from a parent, e.g., of maternal or paternal origin.

47,XY,+r mat

An extra ring chromosome has been shown to be inherited from the mother (**mat**). Because it follows another alphabetic symbol (r), a space must separate the two symbols (see 8.3.2, Event symbols).

Additional ring examples can be found under 8.5.10 Ring of known centric origin, 8.6.6 Ring derivative involving more than one chromosome, and 8.7.10 Composite karyotype.

8.7.7 Marker (mar)

49,XY,+r1,+r2,+mar[20]

The marker, like the ring above, is a chromosome of unknown origin. It follows the unknown ring chromosome at the end of the karyotype. Because it is considered a centric structure, it will also be preceded with a plus sign, and will increase chromosome count. Most of the karyotype rules previously described for rings will also apply for markers (see 8.7.10, Composite karyotype for handling varying copies of markers). The bracketed number at the end [20] indicates the number of cells analyzed in a neoplastic study.

1. Two markers (not necessarily the same) per cell would be written **48,XY,+2mar[20]**.
2. Two different, distinguishable, clonal marker forms would be written **48,XY,+mar1,+mar2[20]**.
3. Additional copies of the same clonal marker will use a times (x) symbol: **49,XY,+mar1x2,+mar2[20]**.
4. A de novo constitutional marker (c) in a neoplastic karyotype, or one that is known to be inherited from a parent (mat or pat), will use a space to separate the two alphabetic symbols: **47,XY,+mar c[20]** or **47,XY,+mar mat[20]**.

8.7.8 Double minute (dmin)

Unlike rings and markers, double minutes are not considered “true” centric bodies, and thus do not affect chromosome count and are not written with a plus sign. If seen in two cells, however, they are considered clonal and would be recorded in the karyotype after any unknown centric marker description [1, section 9.2.12]. If multiple copies occur, a number range could also be placed before the symbol; however, unlike rings and markers, the range would *not* be preceded with a plus (+) sign.

46,XY,5dmin[20] using an absolute numerical value indicates that all cells had 5 dmins;

46,XY,-22dmin[20] indicates that the cells showed approximately 22 dmins (as a mean number) per cell;

46,XY,5~22dmin[20] defines a definitive range that was not less than 5 and not more than 22 dmins per cell.

8.7.9 Incomplete (inc)

When suspicious but inconclusive structural or numerical changes are observed, the incomplete symbol can be placed at the end of the karyotype string, before the cell count in square brackets, for example, 48,XY,+1,+19,inc[20], to indicate that the karyotype is incomplete. Because it is strongly recommended that karyotypes be as complete as possible, the use of this symbol should be used with caution [1, section 5.4].

8.7.10 Composite karyotype [cp20]

Every effort should be made to identify the levels of clonal evolution in neoplastic karyotypes [1, section 11.1.5] (see 8.9.2, Neoplastic clonal evolvement), but there are times when rearrangements are too complex and clonal evolvement too convoluted to be able to describe the events meaningfully. In these situations, a composite karyotype can be used that will describe each clonal event, even though they may not have been seen in every cell (see 8.8, Random versus reportable, for exceptions) [1, section 11.1.5]. A range for chromosome count would be used when applicable, for example, 47–49, and the lowercase cp (for composite) would be placed before the total cell count within square brackets at the end of the description, for example, [cp20] [1, section 11.1.5]. The requirement for observing three cells for an identical loss is overridden if that same chromosomal loss is associated with another, reportable clonal abnormality [1, section 11.1.1].

47~50,XX,+1,+3,+17,+22,+mar,-22dmin[cp20]

Each cell in this composite karyotype had from one to four extra chromosomes, although five different, ‘countable’ chromosomes are described. The discrepancy indicates that not all cells showed all chromosomal gains, but all gains were clonal, that is, present in at least two cells, and that their absence was not due to random occurrences [1, section 11.1.5]. The presence of approximately 22 double minutes is neither included in the total chromosome count range, nor written with a plus sign.

47~50,XX,+1[5],+3[4],+17[14],+22[7],+mar[2],-22dmin[18][cp20]

A more informative option for writing the same composite karyotype above would be to include individual cell counts in square brackets following each event [1, section 11.1.5]. This allows the reader to appreciate the frequency of each event, but does not provide how events relate to each other. For example, in this karyotype we learn that only four out of 20 cells showed a gain of chromosome 3, but we do not know if those same cells also included a gain of chromosome 22.

46~48,XY,-18,+1~3r[cp20]

In a simpler example, a range of one to three clonally occurring rings, without further distinction, are present. The varying number of rings (+1~3) is reflected in the chromosome count (46~48), and the karyotype becomes a composite (cp20).

47~48,XY,+1~2mar[cp20]

Similar to the above, one to two extra copies of a marker chromosome are present in the 20 cells analyzed, making the karyotype a composite.

48~50,XY,+r1x1~3,+r2[cp20]

If there are two or more clonally distinguishable, but unidentifiable centric rings present, and one of the rings (e.g., r1) shows a range of extra copies (e.g., from 1 to 3 copies) in the 20 cells analyzed, the karyotype will place a times (x) sign, no spaces, after the ring identity (r1), and will use the tilde (~) symbol between the lower (minimum) and upper (maximum) range values. Because they are centric, these additional copies will also affect chromosome count (48~50).

Because the karyotype author should make every attempt to describe subclone evolvement, the karyotype reader will assume from a composite description that there was no definitive pattern that would make subclone description possible.

8.8 Random versus reportable

8.8.1 Guidelines for reporting an abnormality

One of the challenges in analyzing a specimen is distinguishing true low-level mosaicism from random loss, gain, or rearrangement as a result of cultural artifact. The former is included in the karyotype; the latter is not. Guidelines have thus been established that set a reasonable cutoff value for detecting mosaicism with a 95% confidence level when encountering a structural or numerical variance [28–30]; however, any clinically suspicious finding should be discussed with the laboratory's supervisor or director. Clonal determination in neoplastic tissue may also require special considerations, e.g., number of cells analyzed, type of culture used, time spent in culture, or type of abnormality found [1, section 11.1.1].

Unless the abnormality has been previously reported, guidelines require the presence of at least two cells with exactly the same gain (e.g., trisomy) or structural rearrangement to be considered true mosaicism (see 8.9, Multiple cell lines). Because of a slightly increased potential for chromosome loss as an artifact of the rigorous harvest and slide-making process, loss of the same chromosome in constitutional studies must be observed in at least three cells in order to become reportable.

In situ cultures of tumor tissue generally require the observation of the same numerical gain or structural abnormality in at least two metaphase cells from two different primary cultures (i.e., first-generation cultures initiated from the original tissue source) or from two distinctly separate regions on the same slide. Loss will require three such observations in order to be considered clonal; however, if two cells showing the same chromosome loss also show an identical chromosome gain(s) or structural abnormality(ies), the loss becomes reportable [1, section 11.1.1]. Prenatal in situ or flask guidelines have slightly different restrictions and are discussed in Chapter 5, Prenatal chromosome diagnosis.

Certain external factors are also known to be associated with specific chromosomal findings, e.g., advancing age with sex chromosome aneuploidy, or T-cell mitogen PHA with the t(7;14); therefore, low occurrences of suspicious numerical or structural abnormalities should be discussed with the supervisor or director. Because these guidelines were established to help distinguish cultural artifact from true low-level mosaicism, questionable gains or losses should be verified by checking the surrounding area under the microscope in order to determine whether a free-floating morphological match of the missing chromosome can be found, or if a gain can be a floater from a neighboring, incomplete metaphase cell. If these gains or losses are true and meet guideline standards, they become reportable.

On the opposite spectrum, even though most single cell findings are random occurrences, experienced technologists have encountered rare cases in which a single abnormal cell in an otherwise normal specimen showed an abnormality that was a potentially relevant finding for the patient's symptoms, especially in neoplastic studies. When further investigated via additional counts or complementary testing such as FISH, this single cell was confirmed as a true, reportable cell line for that specimen.

A single-cell occurrence becomes reportable when it has been

1. previously reported in a prior specimen of this patient [1, section 11.1.1]. This clone would be listed first, irrespective of size, if it represented a clonal predecessor to another related clone in the analysis [1, section 11.1.6]. If, however, it is a karyotypically evolved clone, it would be described after a less complicated clone, following standard karyotype order for neoplastic clonal evolution;
2. confirmed by another test – for example, FISH, or another tissue source (e.g., skin, buccal mucosa). The confirming FISH karyotype would be written following a period at the end of the cytogenetic description [1, section 11.1.1].

No general rule can foresee every possible factor that could affect a specimen's outcome; therefore, any suspicious finding should be brought to the attention of the laboratory director.

8.8.2 Heteromorphic variations

Heterochromatic regions (see Chapter 7) that surround the centromere normally show variations in both size and length. *ISCN 2016* [1] uses opposing directional cross-hatches in the idiogram to represent the various families of α -repetitive satellite DNA [1, section 2.5] that form these pericentromeric heterochromatic (h) regions, and those variably present on all acrocentric short arms and on chromosomes 1, 3, 9, 16, 19, and Y [1, section 2.4]. Generally speaking, these changes have no known effect on the carrier, but can change the morphological appearance of a chromosome. Heteromorphic variations can be written as follows:

- Size or length variations of heterochromatic (h) regions, acrocentric stalks (stk) and satellites (s) with plus (+) and minus (−) signs, for example, increase (h+, s+, stk+, Yqh+) and decrease (h−, s−, stk−, Yqh−).
- Inter- or intra-chromosomal repositioning, e.g., partial pericentric inversion (phqh), full inversion (ph), or repositioning of a heterochromatic region. Examples of interchromosomal repositioning include 3q24h, where heterochromatin has been identified at band q24 on chromosome 3, and 17qs, which indicates that satellites are attached to the long arm of chromosome 17.

Table 8.4 Polymorphisms

13ps+	increased satellite length
13pss	double satellites
13pstk+	increased stalk length
13pstkstk	duplicate stalks on the short arm (p) of chromosome 13 (stksstk)
13cenh+mat	increase in centromeric heterochromatin, which is inherited from the mother
13ps+pstk+cenh+mat	a combination of above examples on the same chromosome; therefore, no commas
13ps+,14pstk+,21cenh+mat	a combination of above examples on 3 different chromosomes; commas separate each
9ph	heterochromatin inverted into the short arm of chromosome 9
9phqh	heterochromatin on both arms of chromosome 9
Yqh-	missing (-) heterochromatic segment (h) on long arm (q) of Y sex chromosome
1qh+	increased length (+) of heterochromatin (h) on the long arm (q) of chromosome 1

- Duplicate presence (pss, pstkstk) or not at all (ps-, pstk-) [1, Chapter 7] (see Table 8.4). Additional bands have even been occasionally noted within the q12 heterochromatic areas of chromosomes 1, 9, and the Y, but because they are considered part of the variable cross-hatched region, they are not depicted in the idiogram [1, section 2.4].

When writing a heteromorphic variation in karyotype format, breakpoints are defined by the euchromatic band that is adjacent to the cross-hatched heterochromatic region in the idiogram [1, section 7.1.2]. Examples of common heteromorphic inversions are inv(1)(p13q21), inv(2)(p11.2q13), inv(3)(p11.2q12), inv(9)(p12q13), inv(10)(p11.2q21.1), inv(16)(p11.2q12.1), and inv(Y)(p11.2q11.2) [1, Chapter 2, Table 1].

8.8.3 Common fragile sites

Another form of normal variant is the fragile site, which in karyotype terms is a specific chromosome locus with a propensity for repeated chromatid or isolocus chromosomal constriction, gaps, or breaks, especially after *in vitro* exposure to chemicals that stress DNA nucleotide replication. Two classes of fragile sites have been defined – common (CFSs) and rare (RFSs), the latter occurring at a low frequency (5%) within the general population. These RFS regions of expanded trinucleotide or minisatellite DNA repeats have been associated with certain genetic diseases, such as fragile X syndrome, some cancers, and even germ-line rearrangements that could have genetic consequences [31].

Fragile sites generally occur at AT-rich, late-replicating, dark G-band (or light R-band) regions, which are known to be involved in nuclear matrix attachment, especially at the border interface with its light G-band neighbor [31]. Because it involves only one break, it will be written in the one-break, intrachromosomal karyotype format (see 8.8.4, Rare fragile sites next). Many CFSs have been molecularly identified with no known phenotypic consequence [32]; therefore, they are treated as normal variants and are not included in the karyotype unless laboratory protocol dictates otherwise [1, section 7.2].

8.8.4 Rare fragile sites

RFSs, inherited or acquired, may have phenotypic consequences, and would be reported in the karyotype if identified by cytogenetic testing. The event uses the fra symbol, and involves only one chromosome and one breakpoint.

46,Y,fra(X)(q27.3)

In this example, the male patient has a fragile site near the terminal end of the long arm (q) of the X sex chromosome at band q27.3, which is characteristic of fragile X syndrome. The structurally abnormal X sex chromosome is written after the normal Y, separated by a comma but no spaces [1, section 9.2.7].

8.9 Multiple cell lines and clones

The rules for writing a multiple cell line karyotype will depend on whether the events are inherited or acquired; therefore, these two scenarios will be defined separately below. Common to both, however, will be the use of the forward slash or solidus (/) to separate different cell lines, and the placement of total cell count per cell line within square brackets at the end of each line.

8.9.1 Constitutional mosaicism

Because of the importance of the genome to fetal development and survival, large portions of extra or missing genetic sequences are rarely found in constitutional studies, unless the involved genes hold minimal survival consequence to fetal development. This is not to imply that the individual has no biological or phenotypic consequence for this genomic excess or loss; it only means that the developing embryo has enough critical genes to develop and survive.

mos 47,XXX[35]/45,X[15]

When multiple cell lines are present in a *constitutional* study, the *abnormal* cell line of highest frequency (mainline) will be listed first, preceded by the symbol mos for mosaic (from the same zygote) or chi for chimera (from different zygotes). The symbol is separated from the chromosome count by a space.

mos 45,X[25]/47,XX,+21[25]

When abnormal cell lines show equivalent cell counts, the order will follow standard karyotype order (see 8.3.7, Karyotype order priority); therefore, X would be listed before chromosome 21.

mos 45,X[25]/46,X,i(X)(q10)[25]

When equivalent cell lines have different types of events, for example, numerical and structural, numerical will be listed first.

mos 45,X[5]/47,XXX[5]/46,XX[40]

The normal cell line, irrespective of its total cell count, is placed last, even if it has the highest cell count. In this example, both abnormal cell lines are numerical; therefore, they are arranged in increasing complexity order. (1, section 4.1)

8.9.2 Neoplastic clonal evolvement

While constitutional cytogenetics focuses on gamete and zygote development, cancer cytogenetics centers on the clone. *ISCN 2016* [1] defines a clone as “a cell population derived from a single progenitor”, which may not be “completely homogeneous because subclones may have evolved during the development of the tumor” [1, section 11.1.1, p. 84]. The association of specific chromosomal loss, gain or rearrangement with certain neoplasia has had an enormous effect on both early detection and effective, targeted therapies.

Neoplastic karyotypes generally use the same event symbols and formulae as constitutional studies, for example, forward slash for separating different cell lines, total cell count in square brackets at the end of each line, and the normal diploid line placed last [1, section 11.1.6]. Event order within each clone will also follow the same decision chain for standard karyotype order (see 8.3.7, Karyotype order priority). Constitutional abnormalities will follow standard placement order within an abnormal clone description and will append the suffix letter c (or its origin; see 8.5.8, Constitutional origin) to distinguish it from acquired neoplastic changes (e.g., +7,+21c,+mar). A clone that only has the constitutional anomaly will also append the c suffix to the event, but the clone will be placed at the end of the karyotype string in the same way that a normal clone is handled, irrespective of its frequency [1, sections 4.1, 8.3, 11.3].

Because neoplasia knows no boundaries, the karyotype must be capable of expressing this evolvement through stemline-sideline progression, starting with the most basic clone or stemline, followed by its subsequent sidelines in increasing complexity order. It is worth noting, however, that a normal-appearing neoplastic karyotype does not necessarily indicate that the cells are normal, only that there were no clonal abnormalities detected, using routine cytogenetic analysis.

Reporting cytogenetic results (donor//recipient) in bone marrow transplant situations will be discussed in section 8.10.12, Bone marrow transplant chimerism.

8.9.3 Mainline

When more than one independent tumor clone is identified in a neoplastic specimen, they are placed in frequency order (largest cell count), starting with the mainline or most frequent clone. A karyotype may also have more than one mainline if the same highest absolute cell count is shared by more than one clone.

46,XY,t(14;18)(q32;q21.3)[14]/47,XY,+12[6]

The most frequent clone, or mainline, shows a t(14;18) in 14 of the 20 cells analyzed, and is thus listed before the six unrelated cells with trisomy 12. If the cell count had been the same, the events would follow standard karyotype order and the numerical +12, which is the autosome of lower numerical value, would precede the structural t(14;18).

Certain conditions will override unrelated clones being written in decreasing frequency. For example, when a previously reported abnormality is found, it is listed first [1, section 11.1.6].

46,XY,t(14;18)(q32;q21.3)[3]/47,XY,+8[14]/46,XY[3]

The t(14;18) had been previously reported, and is thus written first.

If related clones are present with unrelated clones, the evolving clone is listed first in increasing complexity order, followed by the unrelated clones in decreasing frequency. The normal cell line if present would still be last [1, section 11.1.6].

8.9.4 Stemline (sl), sideline (sdl) and idem

The most basic clone in a series of related clonal evolvement is called the stemline (sl), and is written first in the karyotype (see Figure 8.26). Subclones, called sidelines (sdl or sdl1, sdl2, etc.), follow their associated stemline in increasing complexity, irrespective of their frequency [1, section 11.1.4]. Because a clone need not be homogeneous, this subclonal population is still considered part of the original stemline clone [1, section 11.1.1].

Idem (Latin for same) represents only those abnormalities described in the stemline. It is useful when describing limited sideline evolvement, and is recommended when two or more stemlines (sl1, sl2, etc.) each evolve into its own set of sidelines [1, section 11.1.4]. These two symbols, sl and idem, cannot be intermixed when describing a single tumor sample [1, section 11.1.2]. We will demonstrate how to use these tools to follow clonal evolution by introducing a succession of scenarios.

Scenario 1: In 20 cells using 400-band level analysis, 3 cells were normal male. The remaining cells all showed a t(14;18) translocation at q32 on chromosome 14 and q21.3 on chromosome 18.

Two clones are present – one abnormal and one normal. The abnormal clone is listed before the normal one, and the karyotype would be written: **46,XY,t(14;18)(q32;q21.3)[17]/46,XY[3]**, with the solidus separating the clones, and the cell counts included at the end of each clone. The translocation clone is both stemline (most basic clone) and mainline (most frequent clone) for this karyotype.

Scenario 2: Within the 17 cells having the above translocation, 13 cells also show a trisomy 12.

The karyotype now has three clones – one stemline, one sideline and one normal. The most basic clone, or stemline, is still the clone with only the t(14;18) translocation, and will thus remain first in the karyotype, but its total cell count has reduced to 4, e.g., **t(14;18)(q32;q21.3)[4]**. The remaining 13 cells, which show trisomy 12 in addition to the already present t(14;18), becomes its first sideline; therefore, using the sl (stemline) symbol to represent the t(14;18), its description becomes **47,sl,+12[13]**. Chromosome count also increased to 47 as a result of the extra chromosome 12. This sideline now becomes the most frequent clone, or mainline, of the karyotype, but the original, most basic clone still remains the stemline and will be listed first. The karyotype can be written in one of two ways:

46,XY,t(14;18)(q32;q21.3)[4]/47,sl,+12[13]/46,XY[3]

where the symbol sl (for stemline) indicates that every abnormality described in the stemline is present in this sideline clone (in this case, the translocation). The new sideline adds an extra chromosome 12.

46,XY,t(14;18)(q32;q21.3)[4]/47,idem,+12[13]/46,XY[3]

where idem is used instead of sl to reduce repetitive description (i.e., the stemline's translocation).

At this level of detail, both formulae are equally effective.

count,sex,event[#] / count, sl ,event[#] / count, sdl1 ,event[#] / count, sdl2 ,event[#] / count,sex[#]				
Stemline (most basic)	Sideline 1	Sideline 2	Sideline 3	Normal cell line

Figure 8.26 Clonal evolvement. In a neoplastic karyotype, each evolving clone is described independently, starting with the most basic (stemline) event(s) and continuing in increasing complexity. The number of cells counted in each clone is enclosed in square brackets at the end of each cell line, and forward slashes separate cell lines. The most basic stemline is described first, followed by its sidelines in increasing complexity.

Scenario 3: Within the above 13-cell sideline that has the translocation between chromosomes t(14;18) and the extra chromosome 12, eight (8) of the 13 cells now also show an interstitial deletion of the long arm of chromosome 6 between bands q15 and q21.

Complexity increases with the addition of a second sideline. There are now three abnormal clones in addition to the normal clone.

Stemline (sl) – The t(14;18) translocation is still seen in 17/20 cells, but only four of those 17 cells have it as the sole abnormality. These four cells are still considered the stemline, and will be listed first: 46,XY,t(14;18)(q32;q21.3)[4]. It is not, however, the mainline (most frequent clone) of the karyotype.

First Sideline (sdl or sdl1) – Both the translocation (represented by the sl symbol) and trisomy 12 are present in 13 of the 17 abnormal cells, but eight of these 13 cells have further evolved into a second sideline, leaving 5 cells defined in the first sideline:/47,sl,+12[5].

Second Sideline (sdl2) has the t(14;18) and +12 from the first sideline, and has added a deleted chromosome 6 in eight cells. Because there are no other sidelines, ambiguity is not an issue and the first sideline can still be referred as sdl, but will change to sdl1 in the next scenario. The new event becomes 47,sdl,del(6)(q15q21)[8]. With the highest cell count (8 cells), it also becomes the mainline of the karyotype.

Putting it all together, the karyotype becomes:

46,XY,t(14;18)(q32;q21.3)[4]/47,sl,+12[5]/47,sdl,del(6)(q15q21)[8]/46,XY[3]

Because the use of idem restricts referral to only the first-listed clone (stemline), all events derived from previous sidelines must be repeated; therefore, the +12 must be placed after del(6), following the rule of ascending autosomal numbers, and the karyotype becomes:

46,XY,t(14;18)(q32;q21.3)[4]/47,idem,+12[5]/47,idem,del(6)(q15q21),+12[8]/46,XY[3]

Scenario 4: Expanding on scenario 3 above, five of these 8 cells with the deletion of chromosome 6 now also show a deletion within the short arm of chromosome 17 at p13.

With three sidelines present, each sideline is numbered for clarity; therefore, sidelines will be described as sdl1, sdl2, and so forth. The new stemline-sideline karyotype is written:

46,XY,t(14;18)(q32;q21.3)[4]/47,sl,+12[5]/47,sdl1,del(6)(q15q21)[3]/47,sdl2,del(17)(p13)[5]/46,XY[3]

The use of symbol sl in this example represents the t(14;18); sdl1 adds +12 to the t(14;18); and sdl2 indicates del(6), +12, and t(14;18). Sideline 1 and sideline 3 are both mainlines of this karyotype, because they share the same highest cell count (5), and are thus the clones that are most frequently observed. This does not, however, change the karyotype order, which is still written in increasing complexity, not frequency.

As complexity increases, so does repetitive description with the use of idem.

46,XY,t(14;18)(q32;q21.3)[4]/47,idem,+12[5]/47,idem,del(6)(q15q21),+12[3]/47,idem,del(6)(q15q21),+12,del(17)(p13)[5]/46,XY[3]

The last sideline, for example, must repeat del(6)(q15q21) and +12, in addition to including the new deletion. The initial stemline translocation, t(14;18), is represented by the symbol idem.

8.9.5 Neoplastic polyploidy

Abnormal polyploidy can be a true sideline of neoplasia and not just cultural artifact; therefore, it can be reported if it meets clonal requirements [1, section 11.1.4]. To incorporate the tetraploid clone as a sideline, the $\times 2$ would be written with the sl symbol to represent double the value of the stemline. The next example will demonstrate this.

In 20 cells, a neoplastic specimen was found to have 6 cells with t(14;18)(q32;q21.3). Four tetraploid cells were also analyzed which had two copies of this translocation; the remaining 10 cells were near-tetraploid, with a chromosome count of 93, as a result of an extra copy (five copies total) of chromosome 12, in addition to the two copies of the translocation.

This karyotype would be written 46,XY,t(14;18)(q32;q21.3)[6]/92,slx2[4]/93,sdl,+12[10]. All cells have the translocation t(14;18), making the six diploid cells with the translocation as the most basic cell line, or stemline, and is listed first. The first sideline is tetraploid. The second sideline evolved from the first sideline (either sdl or sdl1 can be used, depending on

laboratory preference), and gained an extra chromosome 12, thus producing a near-tetraploid chromosome count of 93 (see Table 8.3). Using *idem* may be even simpler at this point. (1, section 11.1.4)

46,XY,t(14;18)(q32;q21.3)[6]/92,idemx2[4]/93,idemx2,+12[10]

8.9.6 Multiple stemlines

Multiple, independent, clonal stemlines are differentiated by Arabic numeral suffices, e.g., sl1, sl2, etc., if they too evolve into additional sidelines.

47,XY,+12[6]/47,sl1,del(17)(p11.2)[8]/46,XY,del(20)(q13.1)[2]/47,sl2,+8[4]

Each sideline should follow its stemline in increasing complexity order and must use its stemline's unique indicator (sl1, sl2, etc.) to avoid any ambiguity. Sideline symbols, for example, sdl1, sdl2, etc., however, should not be used in these multistemline karyotypes; instead, all previous sideline details evolving from the same stemline must be repeated [1, section 11.1.4]. The only description that need not be repeated is the stemline's abnormalities. *Idem* can also be substituted [1, section 11.1.4].

8.9.7 Jumping translocations

When one breakpoint reoccurs clonally in translocation with different chromosome partners, called a jumping translocation, the karyotype will be written following similar order priority as those described above for unrelated clones.

46,XY,t(14;18)(q32;q21.3)[11]/46,XY,t(7;14)(q22;q32)[6]/46,XY,t(11;14)(q23;q32)[3]

This example shows three different rearrangements involving the same position on chromosome 14 (14q32). Karyotype order will be listed by clonal frequency [1, section 9.2.17.4]; therefore, the mainline t(14;18) is first with 11/20 cells, t(7;14) is next with 6/20 cells, and the last is t(11;14) with 3/20 cells.

Neoplastic karyotypes can get quite complex, but every attempt should still be made to identify the stemlines and sidelines of an evolving clone. The above scenarios to the novice may have seemed overwhelmingly complex, but in real-time cancer cytogenetics, the examples provided here are straightforward and resolvable. In real life, we only wish that were so. Perhaps the reader can now appreciate the challenge that neoplastic cytogeneticists have faced for decades, trying to make sense out of the chaos that neoplasia brings to the human karyotype.

8.10 Fluorescence in situ hybridization

FISH on interphase nuclei and metaphase chromosomes has become a critical adjunct to chromosome studies. If a constitutional microdeletion or microduplication is suspected but is too small to detect with confidence by routine chromosomal analysis, and the locus sequence is available for fluorescence interrogation, metaphase FISH can verify the presence or absence of the gene. On the other hand, if the goal is examining a large quantity of cells for a specific locus or rearrangement, without being restricted to cells undergoing mitosis, then interphase/nuclear FISH would be the more desirable choice. Some uses for interphase/nuclear FISH include confirming or detecting low-level mosaicism; identifying specific genetic abnormalities when metaphase chromosomes are not available (e.g., uncultured amniotic fluid or paraffin-embedded tumor tissue); monitoring the ongoing effectiveness of a marrow transplant or the presence of residual disease; and searching for a growing list of gains, losses, and juxtapositions that have clinical significance to neoplasia. Choosing the appropriate hybridization method will also depend on what type of abnormality is expected; therefore, understanding the process behind FISH and the symbols representing these processes all affect how the FISH karyotype is written. (See Chapter 16, Fluorescence in situ hybridization, for a more indepth discussion on this topic.)

8.10.1 Probe validation and normal cut-off values

Interphase FISH analysis relies on the presence or absence of specific, colorful signal patterns. Certain situations, however, may produce false-positive signal patterns that can challenge a test's interpretation, such as, coincidental overlap of signal positioning within the nucleus, background noise from an unknown contaminant, or even cross-hybridization with similar genetic sequences. Just as conventional cytogenetics relies on a minimum number of cells before an abnormality can be reported, so too does FISH rely on a limit of occurrences, called normal cut-off values. These values are not universal, but instead are established [33] and periodically updated through a validation process for *each* probe being clinically used at the individual laboratory (see Chapter 16, section 16.11, FISH probe testing and validation).

The validation process involves examining known normal and abnormal specimens, and establishing a reliable rate (normal cut-off) at which false-positive signal patterns can be expected for that probe at that laboratory and for that specific tissue type (see Chapter 19, section 19.3.1, Determining the normal cut-off for FISH validation). Running a normal control slide with each FISH hybridization session will also verify that the session produced reliable results; however, it cannot anticipate the presence of fluorescing debris that had been introduced to individual test slides prior to FISH hybridization. An abnormal FISH result becomes reportable when its frequency exceeds the normal cut-off range for that pattern using the same probe.

The validation process has its limitations. Cancer cells are so unpredictable that novel signal patterns are sometimes observed that were not previously observed on either normal or abnormal control specimens. Any suspicious, repetitive signal pattern should be brought to the attention of the laboratory supervisor or director.

Different tissue sources using the same probe may also require a validation process specific to each tissue, as determined by the laboratory director. Paraffin-embedded tissue, however, must be validated independently from cytogenetically processed nuclei, as recommended in the July 2011 report by a working group of geneticists serving on the American College of Medical Genetics (ACMG) quality assurance committee [33]. Ultimately, accuracy relies on a staff that is competent to perform the tests being assigned and on results that are based on reliable guidelines and in concordance with at least two independent readers [34].

8.10.2 Signal patterns

A probe signal represents a genetic sequence that has been labeled with a fluorescing color to allow a gene's presence, absence, or position to be confirmed via fluorescence microscopy. We will use four letters – R (red or orange), G (green), A (aqua), and F (fusion yellow) – to represent the most commonly used signal colors. The chromatic yellow fusion (F), however, is not a true color. It is actually created by a red and green hue being physically located within the same three-dimensional field as it is being viewed under the fluorescent microscope. In fact, its red and green components can be separately observed by blocking the opposing color. With these four colors, we will explain the basic process behind FISH analysis through a few hypothetical situations.

Using a red-labeled probe to target the Down syndrome critical region (DSCR) on a peripheral blood sample from a newborn referred for Trisomy 21, the presence of two red signals (2R) would indicate that two normal genetic sequences are present, one on each homologue. Three red signals (3R) would indicate the presence of three copies of the gene, thus trisomy for the DSCR region. A loss of one signal (1R), however, could indicate either the loss (deletion) of that sequence (1R), or the loss (monosomy) of the entire homologue. To distinguish these two scenarios, an internal control of a second color, e.g., green, would label either the centromere or a sequence on the opposite arm of the same chromosome. Investigating a sequence on acrocentric chromosomes, however, cannot use the opposite-arm strategy for controls; therefore, the choice internal control in this scenario would be the centromere of chromosome 21. If, however, the interrogated sequence resides near the centromere, a more distal sequence on the same arm would be labeled (see 8.10.4, Metaphase FISH karyotype).

Using these hypothetical probes, the new signal pattern interpretations become:

2R2G = normal pattern

1R2G = gene sequence deletion

1R1G = monosomy

Taking this scenario one step further, three red signals of equal size, but only two green centromeric signals (3R2G), could indicate the presence of an isochromosome for the long arm of chromosome 21, which will also indicate a positive result for Down syndrome. Parental studies would be needed in order to determine if the isochromosome is inherited.

Depending on the situation and specimen type, signal interpretation may also require that a control locus on a second chromosome be targeted. For example, using the same red-labeled probe to target the DSCR region for a prenatal study on amniotic fluid nuclei, but this time using a green signal to target a locus (or centromere) on a second chromosome, for example, chromosome 13, a positive result for Down syndrome would show three red signals for the DSCR locus, but only two green signals for the gene on chromosome 13 (3R2G). A 3R3G signal pattern could indicate trisomy for both targets, chromosomes 13 and 21, but in real life, that situation is unlikely and probably not viable. What is likely and could be viable is that the fetus may have three sets of every chromosome (triploid), which would be verified in a separate hybridized field by examining the signal patterns for the sex chromosomes and autosome 18 (see 8.10.10, Building a string of interphase FISH results).

Sometimes probe selection must be creative in order to confirm a rearrangement. For example, assume that a 3R2G signal pattern is observed in 20% of interphase nuclei, using a red signal to target a sequence on the long arm of chromosome 17, and a green control to target the centromeric region of chromosome 17. If a third color, e.g., aqua (A), is added that targets a locus on the short arm of chromosome 17, and only one signal for the short arm is present (3R2G1A), in addition to three red signals for the long arm of chromosome 17 (3R) and two green signals for chromosome 17's centromeres (2G), the pattern suggests that one homologue has no short arm and two identical copies of the long arm, hence, an isochromosome.

Investigating structural rearrangements, e.g., translocations and inversions, introduces two additional probe strategies, break-apart (BAP) and fusion (F) probes. A BAP probe will label both proximal and distal sequences of a known breakpoint with red and green signal colors, creating two yellow fusion signal colors (2F) when normal sequences are present on both homologues. When the specimen is positive for an inversion, the yellow fusion color will split into individual red and green signals (1R1G1F). The fusion strategy, on the other hand, will start with a normal pattern being separate red- and green-labeled loci (2R2G). When a translocation has occurred, the yellow fusion color will be created by the merge of the two different breakpoints now in juxtaposition. Depending on label positioning, fusion strategies can be single fusion (SF), which will appear 1R1G1F when positive; dual fusion (DF), which will appear 1R1G2F when positive; and extra-signal (ES), which will appear 1R1r1G1F, with the small red signal (r) verifying the fusion signal (see Chapter 16, Figure 16.7).

Some probe signal sizes are expected to vary; others are not. Centromeric probes, for example, target highly repetitive sequences that naturally show variation within the normal population; therefore, size variability is expected with these probes [33]. Paraffin-embedded tissue also poses a challenge, because it is a sliced segment from a tumor block; therefore, signals can become truncated. Unique genetic sequences, however, should have a relatively consistent size for both homologues; therefore, any size variation may require further investigation.

8.10.3 Probe name

The probe name used within the karyotype (which is usually provided by the manufacturer when purchased) can be derived from the following resources:

- **Probe or clone name:** for example, N85A3 [35] targets the subtelomere region on the long arm of the acrocentric chromosome 22 at 22q13.3, the region associated with Phelan–McDermid (PMS) syndrome [36] (see Chapter 9, Table 9.1). It is also used as an internal control when investigating the more proximal DiGeorge (DGCR) critical region at 22q11.2.
- When the clone name is not available, the **designated locus** (in pter to qter order) can be used from either the current UCSC (www.genome.ucsc.edu) or Ensemble (www.ensembl.org/) Genome Browsers [1, section 13.2].
- **Human Genome Organization** (HUGO)'s approved gene name (www.hugo-international.org/): for example, *MYC* (c-myc myelocytomatosis viral oncogene homolog, avian), is located on chromosome 8 at 8q24 [37]; *ABL1* (c-abl oncogene 1, nonreceptor tyrosine kinase) is located on the long (q) arm of chromosome 9 at q34.1 [38]; its *BCR* (breakpoint cluster region) counterpart is on the long arm of chromosome 22 at q11 [39].

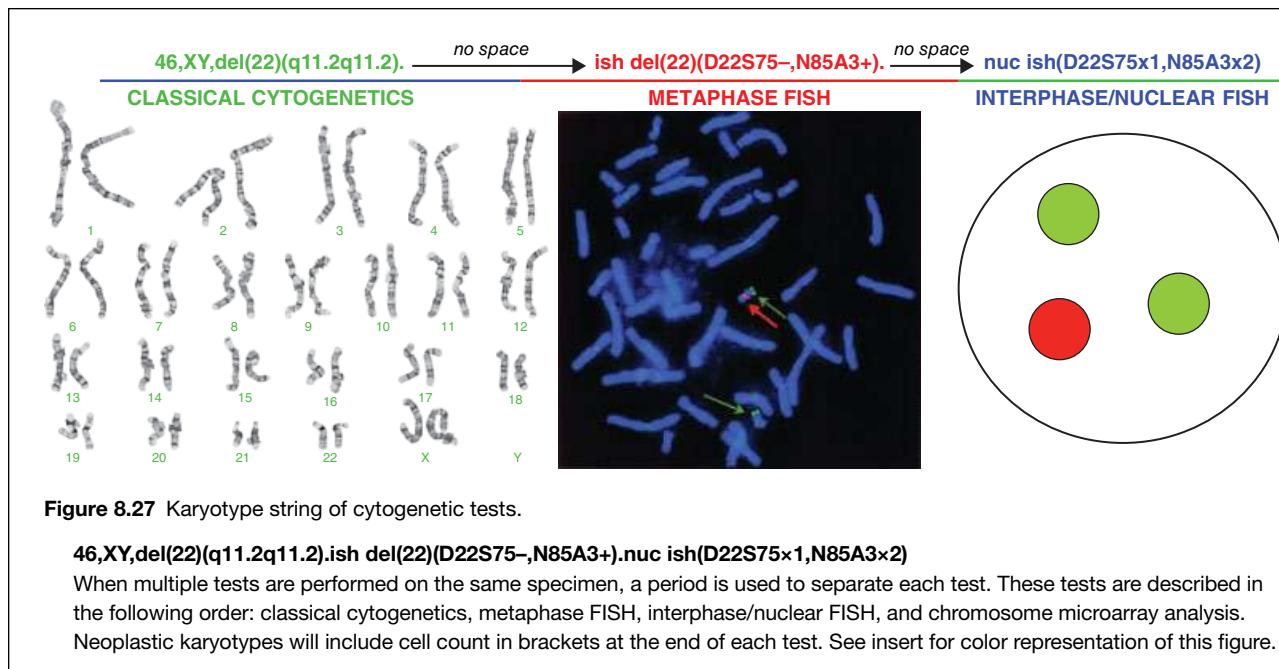
Although the clone name is preferred, any of these naming options (probe name, clone name, gene name, accession number or D-name) can be used at the discretion of the investigator or laboratory director [34]. Unlike human gene names, probe names are not italicized when written in text or in karyotype formula [1, section 13.2]; however, gene names are also not italicized within the karyotype.

When a probe is composed of multiple, contiguous loci, either all probe names can be included in the karyotype in pter to qter order, separated by forward slashes, no spaces, or a single designee can be used in the karyotype to represent all loci within the final report. For example, D21S259/D21S341/D21S342 describes the contiguous genetic sequences from 21q22.13 to 21q22.2, which are targeted in the Abbott Molecular Inc. prenatal assay for aneuploidy detection of chromosome 21 [40]. If, however, probes use HUGO gene names *DSCR3* and *DSCR4* at 21q22.2 [41], a single designee, for example, DSCR for Down syndrome critical regions, can be used in the karyotype, and its full composition would be described in the final report [1, section 13.3].

8.10.4 Metaphase FISH karyotype

Both normal and abnormal metaphase FISH karyotypes start with the ish (in situ hybridization) symbol, either by itself or after a period at the end of a cytogenetic karyotype, with a space following ish, e.g., 46,XY.ish (see Figure 8.27). The following example will use the GDB locus D-name D22S75 to investigate the DiGeorge critical region (DGCR) on chromosome 22 [42] at 22q11.2 [43]. This region is associated with DiGeorge, velocardiofacial (VCF), and conotruncal anomaly face syndromes, also collectively referred by the acronym CATCH22 (cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, hypocalcemia) [35]. Because this acrocentric chromosome is investigating a gene that is located close to the centromere, the internal control will target the N85A3 sequence that is located at a more distal locus (22q13.3) on the same chromosome arm (see 8.10.3, Probe name).

When the targeted chromosome appears *structurally normal*, the metaphase FISH karyotype will describe signal presence using the multiplication (x) sign, followed by the number of signals observed [1, 13.2]. Each probe is described separately, in its own descriptive set (chromosome location, probe name, and number of signals), placed in pter → qter order, with a comma



separating each co-hybridized group, but no spaces. *ISCN 2016* states, “Only the clinically relevant or informative results need to be in the karyotype” [1, section 13.2]; therefore, the control probe for 22q13.3 (N85A3) can be omitted. Either karyotype option is acceptable [1, section 13.2], because the final report will still define all probes that were used.

46,XY.ish 22q11.2(D22S75x2),22q13.3(N85A3x2)

46,XY.ish 22q11.2(D22S75x2)

Both karyotypes show normal metaphase FISH results investigating the DGCR region on chromosome 22. Its co-hybridized control N85A3 can be included (top) or eliminated (bottom). When using both the target and control loci, 22q11.2 (D22S75) is closer to pter than the control 22q13.3 (N85A3); therefore, it will be described first, separated by a comma, no spaces.

When the targeted chromosome appears *structurally abnormal*, the karyotype will use the plus (+) or minus (-) symbols to represent the signals present or missing for the locus being interrogated. The karyotype will define the abnormality found, followed with the probe name and a plus (+) sign for a normal presence, minus (-) sign for absence, and double-plus (++) for duplication. When the number of signals on the structurally abnormal chromosome can be counted, multiple “+” signs may be used [1, 13.2]. Co-hybridized probes are listed in the same parentheses, separated by a comma, no spaces.

46,XY.ish del(22)(q11.2q11.2)(D22S75-,N85A3+)

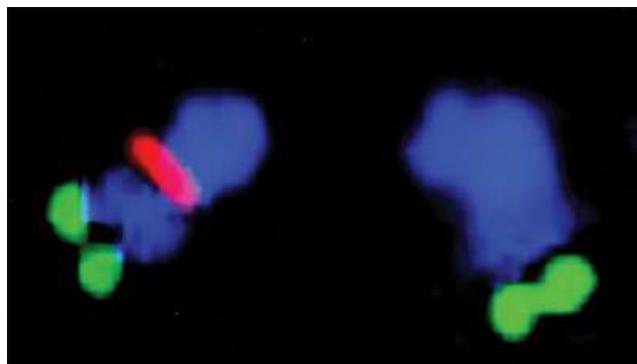
Abnormal FISH results (see Figure 8.28) show that the red investigated sequence (D22S75-) was deleted in one homologue (1R), but the green subtelomere control signal (N85A3+) had a normal presence (2G), verifying that a deletion (1R2G) was present, but not the loss of the entire chromosome (1R1G). This patient is thus positive for the deletion that is responsible for DiGeorge-velocardiofacial syndrome. The normal 46,XY karyotype, even after abnormal FISH results were found, indicates that the microdeletion was not detectable (see 8.5.1, Microdeletion) by routine cytogenetics.

Neoplastic studies must examine a larger number of nuclei and not be dependent on dividing cells; therefore, interphase FISH is generally preferred. We are, however, including the next example in order to demonstrate how FISH neoplastic karyotypes are constructed in a metaphase FISH karyotype. The basic formula is the same as the previous example, except that neoplastic studies will include the total cell count in square brackets, for example, [20], at the end of the karyotype when the same number of signals was observed for all metaphase FISH cells examined, or at the end of each hybridization session if cell counts vary.

46,XX[20].ish 8cen(D8Z2x2),8q24.1(MYCx2)[20]

46,XX[20].ish 8q24.1(MYCx2)[20]

Normal results (2R2G) were found for two different co-hybridized probes, one (2R) targeting the investigating *MYC* region of chromosome 8 at 8q24, and the other (2G) serving as an internal control, targeting the D8Z2 centromeric region of chromosome 8 between 8p11.1-q11.1. The first example lists both probes in pter to qter order, thus placing the investigated



ish del(D22S75-,N85A3+)

Figure 8.28 Metaphase detection for gene locus deletion.

ish del(D22S75-,N85A3+)

Positive results show a deletion for the *DGCR* region on chromosome 22, using both the *D22S75* locus-specific probe (1R) for this region and the control subtelomeric locus *N85A3* in green (2G). See insert for color representation of this figure.

MYC gene after the centromere control probe. The second example can omit the control probe because it adds no additional, relevant information [1, 13.2]. Either option is acceptable.

47,XX,+8[20].ish 8cen(D8Z2×3),8q24.1(MYC×3)[20]

Abnormal metaphase FISH results showed three signals for both the targeted and control loci (3R3G), indicating an extra chromosome 8. Because this metaphase FISH example is defining signal presence on three separate, but *structurally normal*-*appearing* chromosomes 8, the $\times 3$ notation is used instead of ++ [1, 13.2].

Sex chromosome aneuploidy in constitutional metaphase FISH does not describe the extra or missing X or Y chromosome with a plus or minus sign. Using the centromeric probe DXZ1, the metaphase FISH karyotype for a normal female would be written 46,XX.ish Xcen(DXZ1×2). A Turner syndrome metaphase FISH karyotype would be written 45,X.ish Xcen(DXZ1×1), and an extra X would be 47,XXX.ish Xcen(DXZ1×3).

8.10.5 Metaphase fusion FISH strategy

In addition to identifying the absence or presence of a genetic sequence or whole chromosome, different probe architectures have been designed to identify structural rearrangements that are associated with specific neoplastic conditions. These can involve the same chromosome, different chromosomes, or even chromosome homologues. Single- (SF), dual- (DF), and extra-signal (ES) fusion probe strategies target loci that are known under certain neoplastic conditions to become juxtaposed by rearrangement, as via a translocation or inversion.

Single-fusion FISH

Using the t(9;22) *BCR/ABL1* translocation (see 8.5.12, Reciprocal translocation) to demonstrate this FISH strategy, SF FISH (1R1G1F) will generally target the *ABL1* sequence with a red label on both chromosome 9 homologues, and the region proximal to the *BCR* sequence with a green label on both chromosome 22 homologues. Because the proximal green label in SF FISH remains stationary when a translocation has occurred, a cell that is positive for the *BCR/ABL1* translocation will emit one yellow fusion color at the point where the red-labeled *ABL1* gene has become juxtaposed to the green-labeled *BCR* gene on the derivative chromosome 22. The two noninvolved homologues will emit a red (normal chromosome 9) hue and a green signal for the other (normal chromosome 22). The derivative chromosome 22 will show the fused yellow signal (*BCR+*,*ABL1+*), and the derivative chromosome 9 will have no label (*ABL1-*).

46,XX[20].ish 9q34(ABL1x2),22q11.2(BCRx2)[20]

Normal metaphase FISH showed two signal copies for each co-hybridized target (2R2G), indicating normal homologues for both the *ABL1* region of chromosome 9 at 9q34 and the *BCR* region of chromosome 22 at 22q11.2. Autosomal listing follows ascending numerical order, with chromosome 9 being described before chromosome 22.

ish t(9;22)(q34;q11.2)(ABL1-,BCR+,ABL1+)[20]

A SF (1R1G1F) result showed the absence of the *ABL1* signal (ABL1-) on chromosome 9 (left side of semicolon), and the presence of both signals on chromosome 22 (BCR+,ABL1+). Probes are listed in pter to qter order as they appear on chromosome 22, with a semicolon (:) separating the description of the two chromosomes, and a comma separating the translocated sequence on chromosome 22.

***Dual-fusion* metaphase FISH**

DF and ES probe methodologies are predominately used on neoplastic tissue that require a large number of nuclei for detecting low levels of a rearrangement; therefore, the use of metaphase chromosomes are not generally applicable for this FISH strategy. *ISCN 2016* [1], however, provides the capability to report metaphase results; therefore, it is included here.

ish t(9;22)(q34;q11.2)(ABL1+,BCR+,BCR+,ABL1+)[20]

This karyotype demonstrates a positive DF FISH result, with a fusion signal on both translocation partners. The proximal, stationary side of the *ABL1* sequence is listed before the translocated *BCR* segment for chromosome 9; likewise, the stationary *BCR* segment on chromosome 22 at q11.2 is listed before its juxtaposed *ABL1* 9q34 sequence. A semicolon will separate the descriptions of chromosomes 9 and 22.

ish der(9)t(9;22)(q34;q11.2)del(9)(q34q34)(ASS1-,ABL1-,BCR+),der(22)t(9;22)(BCR+,ABL1+)[20]

Tri-color, DF, ES results showed a microdeletion within the 9q34 region of the derivative chromosome 9 that was involved in the t(9;22) rearrangement. Results show the absence of both *ASS1* and *ABL1* sequences on the derivative chromosome 9, but the presence of the translocated *BCR* segment from chromosome 22. Its partner, derivative chromosome 22, is positive for the *BCR/ABL1* juxtaposition. Derivative 22 need not repeat the breakpoints previously defined in the karyotype (see 8.3.8, Repeated description) [1, section 13.2].

8.10.6 Chromosome paints

Unlike locus specific probes, whole chromosome paints (wcp) [1, section 13.2] color the entire chromosome, making a rearrangement involving any part of that chromosome traceable. For example, whole chromosome paints can verify the recipient of an assumed balanced translocation in constitutional studies, especially if one partner is not easily detected.

47,XY,+r.ish +r(21)(wcp21+,DSCR+)

An unidentifiable ring (+r) by routine cytogenetic analysis was determined by whole chromosome paint to be at least in part comprised of chromosome 21. The presence of the signal for the Down syndrome chromosome region confirms three copies of the critical region for Down syndrome.

Other similar FISH options include partial chromosome paints (pcp) [1, section 13.8], subtelomeric FISH [1, section 13.2.2] and multicolor [1, section 13.7] FISH paints, which also provide the ability to track by color the displacement of chromosomal segments. Partial chromosome paints, for example, can verify the presence, absence, duplication or displacement of a micro-dissected region in question, and is written in karyotype format similar to wcp. Multicolor FISH (spectral karyotyping or SKY and multiplex-FISH or M-FISH; see Chapter 17) provides a genome-wide picture of cytogenetic abnormalities in a single experiment, which helps connect the pieces of complex, multiple interchromosomal rearrangements in neoplastic tissue. This technique has no special nomenclature and thus also relies on the wcp format. Subtelomere FISH (ish subtel) targets 41 unique chromosome subtelomeric ends [1, section 13.2.2]. All these tests, and more, have earned their place under the FISH umbrella.

8.10.7 Interphase FISH karyotype

Interphase FISH deals with colored signal patterns in an interphase nucleus, which are, in turn, interpreted as the presence or absence of a genetic sequence or rearrangement. Because visual confirmation of the signal's precise location within a specific chromosome can only be assumed in the interphase cell, *ISCN 2009* [12] introduced a short system for writing the interphase FISH karyotype that eliminates chromosomal description and simply reports the probes used and their signal quantity by

co-hybridization. Each co-hybridized set is separated by a comma (no spaces) from other sets, and all sets are listed in standard karyotype order priority, based on first-listed chromosome order in X before Y before lowest autosome, or pter (p-arm terminal) to qter (q-arm terminal) order when targeting loci on the same chromosome (see 8.3.7, Karyotype order priority). Precise band location for both the investigating probe and its normal cut-off values would be defined separately within the text of the final report. Signal counts are written inside the parentheses when only one probe is being described, or when co-hybridized probes show different signal counts; if the counts are the same and more than one probe is described within the parentheses, the times (x) sign and number of signals is placed outside but adjacent to the closing parenthesis. If results are normal, multiple hybridizations can be combined in a single set of parentheses. [1, section 13.3.1]

nuc ish(D8Z2,MYC)×2[200]

46,XX[20].ish 8cen(D8Z2×2),8q24.1(MYC×2)[20].**nuc ish(D8Z2,MYC)×2[200]**

Normal cytogenetic, metaphase FISH and interphase FISH results (2R2G) are seen for the *MYC* gene located on the long arm of chromosome 8 at 8q24.1, using the centromeric D8Z2 locus as an internal control. Periods separate each different test. Because all probes showed the same 2R2G signal count, the ×2 is written outside the co-hybridization parentheses. Neoplastic FISH karyotypes require total nuclei counted in square brackets at the end.

46,XX,del(22)(q11.2q11.2).ish del(22)(D22S75-,N85A3+).nuc ish(D22S75×1,N85A3×2)

Abnormal cytogenetic, metaphase FISH and interphase FISH results for the DiGeorge microdeletion (1R2G) on the long arm of chromosome 22 at q11.2 shows one signal of D22S75 (1R) for the DiGeorge critical region (DGCS) and two signals for the control N85A3 (2G) targeting the subtelomere region on the same chromosome (see Figure 8.27). Signal counts are placed within the parentheses when they vary between co-hybridized targets. Constitutional studies do not require total cell count in square brackets, unless signal patterns show reportable mosaicism.

nuc ish(ABL1×1,ABL1 dim×1,BCR×2)[115/200]

A variant signal size should not be ignored if the probe does not target an area of highly repetitive alpha-satellite sequences, as in the centromeric regions. If the size variation is repeatedly observed (e.g., 1R1r2G) and considered significant, the diminished (dim) symbol for a potential deletion, or enhanced (enh) symbol for a suspected duplication, would be used. If a second small ABL1 signal is also present (1R2r2G), not necessarily of the same size, for example, nuc ish(ABL1×1,ABL1 dim×2,BCR×2)[115/200], the *ABL1* gene could be partially in translocation with an unidentifiable chromosome [1, section 13.3.1]. Both of these scenarios may benefit from careful cytogenetic analysis.

8.10.8 Interphase fusion karyotype

The fusion probe, and its opposing BAP FISH architecture, must change karyotype format in order to accommodate the description of the formed or broken fusion signal, respectively. An abnormal interphase fusion karyotype places probe names and total signal counts in the first parentheses, followed by an adjoining parentheses that will define how many fusion signals were observed from those independent signals (see Figure 8.29).

Single-fusion interphase FISH

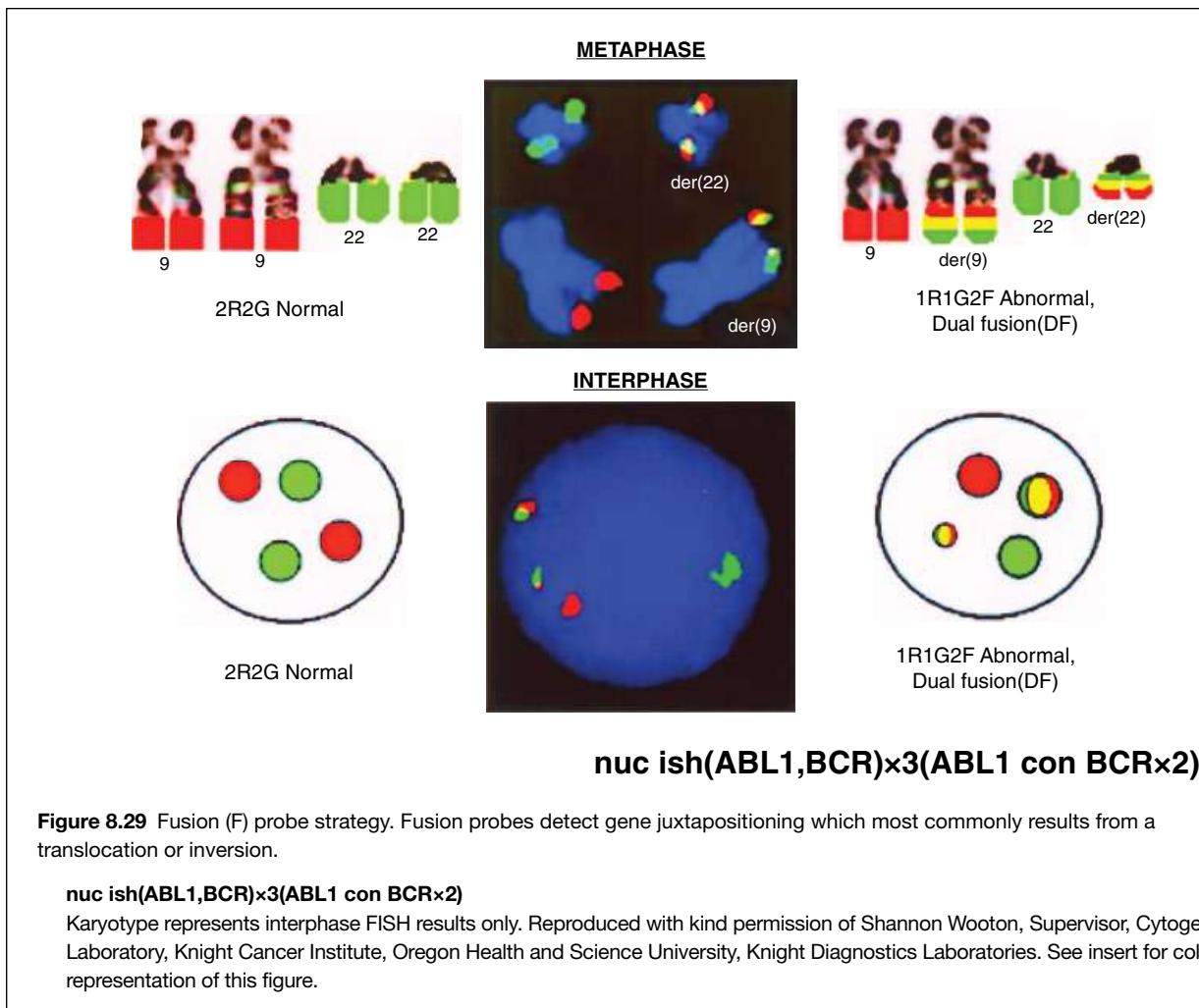
nuc ish(ABL1,BCR)×2(ABL1 con BCR×1)[400]

A positive (1R1G1F) result for the translocation was observed in 400 interphase nuclei in this SF karyotype. The example describes the presence of two separate ABL1 (red) and BCR (green) signals, with one of the red and green signals emitting a fusion yellow signal (ABL1 con BCR×1) at the two interrogated translocation sites. A space is placed both before and after the *con* symbol. Because arbitrary positioning of normal red and green signals within the same fluorescent plane will emit a false-positive yellow hue, single-fusion strategy in interphase nucleic FISH has a lower sensitivity rate for detecting low levels of the *BCR/ABL1* rearrangement and is thus rarely used.

Dual-color, dual-fusion (DCDF) interphase FISH

nuc ish(ABL1,BCR)×3(ABL1 con BCR×2)[400]

Abnormal results using the DF strategy (1R1G2F) will show 3 red and 3 green signals when viewing colors independently; however, two of each color are fused into the chromatic yellow signal as a result of a translocation. The remaining one red and one green signal represent the two normal homologues. Two of the three signals are generally smaller than their normal homologue as a result of the break.



Extra signal, dual-fusion interphase FISH

nuc ish(ASS1×2,ABL1×3,BCR×3)(ABL1 con BCR×2)[400]

The three-color, DF probe strategy labels the *ASS1* gene on the proximal side of the breakpoint on chromosome 9 with a third color, for example, aqua. This strategy allows detection of *BCR/ABL1* rearrangements in patients for which both the distal *BCR* and the proximal *ABL1* are deleted, resulting in a single-fusion abnormal pattern. In almost all *ABL1* deletions that occur during the translocation, the *ASS1* gene is deleted as well. Thus, a juxtaposition artifact (false positive) would show one red, one green, and one fusion, but two aqua *ASS* signals, while a translocation (true positive) with deletions of *BCR* and *ABL1* would show one red, one green, one fusion, and 1 aqua (*ASS*) signal. This design reduces the rate of false positives in single-fusion variant patients, provides visual confirmation of the deleted chromosome 9q, and improves the interpretation of complex rearrangements.

8.10.9 Break-apart probe strategy

The BAP strategy functions in reverse to the fusion probe described above, where the fusion symbol *con* represents the normal probe configuration, and the separation symbol *sep* indicates the abnormal pattern. For BAP probes, the two signals are normally positioned at two adjacent positions on the same chromosome, thus emitting one fusion color under normal conditions. When a separation is observed as a result of a rearrangement, distinct red and green signals will be seen in place of the one yellow fusion color. Viewing a metaphase cell may help determine whether the signals reside on the same chromosome, indicating that an inversion has separated the signals, or whether they appear on different chromosomes, in particular, its homologue, indicating a translocation has occurred.

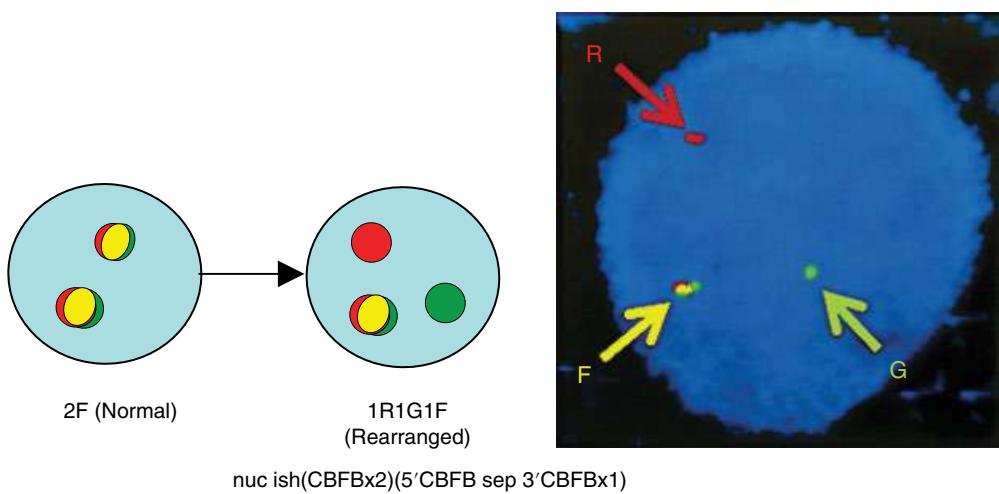


Figure 8.30 Break-apart strategy.

nuc ish(CBFB \times 2)(5'CBFB sep 3'CBFB \times 1)

(Left) Normal pattern for this strategy is two fusions (2F).

(Middle and right) The separation of one yellow fusion signal (1R1G1F) into separate red (1R) and green (1G) signals indicates either a translocation or inversion of those loci. Note: Distance between symbols must also exceed a specific distance (e.g., 1 or 2 signal widths) in order to distinguish potentially normal chromatin duplication from an abnormal rearrangement. See insert for color representation of this figure.

nuc ish(CBFB \times 2)[200]

nuc ish(5'CBFB,3'CBFB) \times 2(5'CBFB con 3'CBFB \times 2)[200]

Normal results show two fused signals (2F) (Figure 8.30) in both the short and detailed format for this type of assay. The detailed example describes probe positions from pter to qter.

nuc ish(CBFB \times 2)(5'CBFB sep 3'CBFB \times 1)[200]

An abnormal disruption of the CBFB region on one chromosome 16 indicates a positive result (1R1G1F) [1, section 13.3.2.4]. No other assumptions can be made as to whether the rearrangement is an inversion or translocation without viewing chromosomes.

When the inversion is verified by both classical cytogenetics and metaphase FISH, the detailed FISH karyotype format is used to describe the chromosome in pter to qter order.

46,XY,inv(16)(p13.1q22).ish inv(16)(p13.1)(5'CBFB+)(q22)(3'CBFB+)[20].nuc ish(CBFB \times 2)(5'CBFB sep 3'CBFB \times 1)[200]

As a result of an inversion, this 3' end of the CBFB probe located at 16q22 will now begin the detailed karyotype. The karyotype must show that the two ends of the probe have separated in order to demonstrate that the inversion has occurred [1, sections 13.2, 13.3.2.4].

Because FISH methodology has been evolving over the last two decades, previous karyotype options may still be used with the director's approval, as long as the format is understandable to geneticists internationally, and all essential information is included within the report. The key to any change is global understanding among geneticists.

8.10.10 Building a string of interphase FISH results

To introduce the concept of writing the interphase/nuclear FISH karyotype using multiple hybridizations, we will demonstrate a prenatal FISH panel to detect aneuploidy for the five most common aneuploid syndromes (sex chromosomes X and Y, and autosomes 13, 18, and 21). Hybridization is performed on uncultured nuclei that were separated from amniotic fluid.

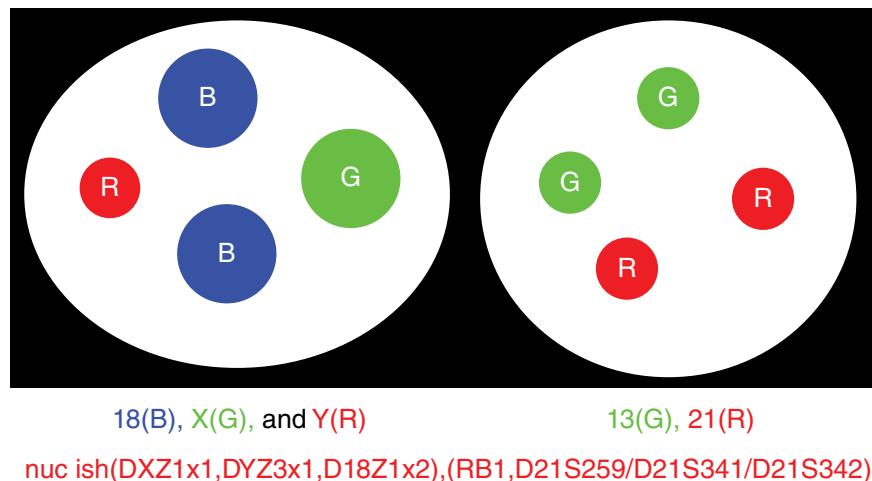


Figure 8.31 Centromeric and gene locus strategy.

nuc ish(DXZ1x1,DYZ3x1,D18Z1x2),(RB1,D21S259/D21S341/D21S342)x2

(Left) Normal male pattern for centromeric probes targeting chromosome 18 (2A), one X (1G) and one Y (1R). If the result is female, the pattern will show no red Y signal and two X green signals (2G). Note: Centromeric probes may show a size difference due to normal heteromeric variation of that repetitive DNA. (Right) Normal pattern for locus specific targets for chromosomes 13 (2G) and 21 (2R). Note: Variation in locus-specific probe sizes could indicate a deletion or duplication and may require further investigation. See insert for color representation of this figure.

Probe order within a co-hybridization set

Two separate hybridizations are demonstrated in Figure 8.31. The first includes three centromeric regions: DXZ1 (X centromere in green), DYZ3 (Y centromere in red), and D18Z1 (18 centromere in aqua). Centromeric probe sizes can vary, due to their highly repetitive DNA structure, and may also appear diffuse; therefore, unusually small or large signal sizes can still be normal heteromorphisms. Probes used in this co-hybridized set will be listed in standard karyotype order, that is, X before Y before autosome 18.

The second set will use two targeted genetic sequences, *RB1* (13q14) in green that will target chromosome 13 at q14, and three contiguous probe loci in red (D21S259/D21S341/D21S342) to target the Down syndrome critical region from 21q22.13 to 21q22.2 (see 8.10.3, Probe name). Arranged in increasing autosomal number order, chromosome 13's *RB1*-targeting signal will be listed before chromosome 21's contig loci, for example, (RB1,D21S259/D21S341/D21S342)x2, with the signal quantity (x2) described outside the parenthesis when all probes listed inside the parentheses showed the same number of signals per probe.

Following standard karyotype order priority, based on the first-listed chromosome within each co-hybridization group, the set that has the X sex chromosome probe as first-listed would be described before the co-hybridization set with the first-listed probe targeting the locus on chromosome 13 (*RB1*). Concurrent parentheses in a string of hybridizations are separated by commas, but no spaces, similar to a string of cytogenetic events (see 8.3.7, Karyotype order priority). The two co-hybridized sets will therefore be placed in the following order: (DXZ1,DYZ3,D18Z1),(RB1,D21S259/D21S341/D21S342). Karyotypes described below show some of the potential normal and abnormal results that can be observed with this probe set.

nuc ish(DXZ1x1,DYZ3x1,D18Z1x2)(RB1,D21S259/D21S341/D21S342)x2	normal male
nuc ish(DXZ1x2,D18Z1x2),(RB1,D21S259/D21S341/D21S342)x2	normal female
nuc ish(DXZ1x2,DYZ3x1,D18Z1x2),(RB1,D21S259/D21S341/D21S342)x2	47,XXY Klinefelter
male nuc ish(DXZ1x1,D18Z1x2),(RB1,D21S259/D21S341/D21S342)x2	45,X Turner female
nuc ish(DXZ1x1,DYZ3x1,D18Z1x3),(RB1,D21S259/D21S341/D21S342)x2	Trisomy 18 male
nuc ish(DXZ1x2,D18Z1)x2,(RB1x2,D21S259/D21S341/D21S342x2)	Trisomy 13 female
nuc ish(DXZ1x2,D18Z1)x2,(RB1x2,D21S259/D21S341/D21S342x3)	Trisomy 21 female
nuc ish(DXZ1x2,DYZ3x1,D18Z1x3),(RB1,DSCR)x3	Triploid male

When classical cytogenetics, metaphase FISH and interphase FISH studies are all reported in one karyotype, periods are used to separate the three test strings.

46,XX,t(9;22)(q34;q11.2)[20].ish t(9;22)(ABL1+,BCR+;BCR+,ABL1+)[15/20].nuc ish(ABL1,BCR)×3(ABL1 con BCR×2)[190/200]

8.10.11 Paraffin-embedded malignant tissue

Identifying amplification of the *ERBB2* gene (also referred to as *HER2* or human epidermal growth factor receptor 2) in breast cancer patients is critical to choosing the right patient for trastuzumab-containing therapy. On November 1, 2013, the American Society of Clinical Oncology (ASCO), along with the College of American Pathologists (CAP), published improved guidelines for interpreting *HER2* testing, with the intention to reduce the number of equivocal results and repeat testing [44]. In addition to refining how the tumor is to be scored, these new guidelines also define interpretive ranges for IHC (immunohistochemistry) and single-color ISH (which does not include a ratio value). We will only concentrate on dual-color FISH in this chapter.

Dual-color probes target the *ERBB2* gene on chromosome 17 at band q12, and use D17Z1 (CEP17) as a copy number control by binding to the centromeric region on chromosome 17. The 2013 ASCO-CAP guidelines use both the ratio of the two signals and/or the average number of *ERBB2* signals per nucleus independently to determine a tumor's classification:

Negative (nonamplified) = *HER2/CEP17* signal ratio <2.0 (previously ≥ 2.2) **and** average *HER2* signal <4.0 .

Equivocal = *HER2/CEP17* signal ratio <2.0 **and** average *HER2* signal ≥ 4.0 and <6.0 .

Positive (amplified) = *HER2/CEP17* signal ratio ≥ 2.0 , **or** average *HER2* signal ≥ 6.0 , **or** both. These guidelines would thus include the rare situation where the *HER2/CEP17* signal ratio is ≥ 2.0 , **BUT** the average *HER2* signal is <4.0 , as being positive for *HER2* amplification.

Because the *HER2/CEN17* ratio is reported with only one decimal point, rounding rules will depend on the second and subsequent decimal digits.

- If the second decimal digit is <5 , leave the first digit as is ($1.94999 = 1.9$).
- If the second decimal digit is >5 , increase to the next digit ($1.96000 = 2.0$). Similarly, if the second digit is 5, but it is followed with any integer having a value other than zero, increase to the next digit ($1.95001 = 2.0$).
- If the second digit = 5, and there are no digits following or the digits following are zeros, the first decimal digit is rounded to the nearest **even** number; therefore, 1.95000 would become 2.0, but 1.85000 would remain 1.8.

The report should include the total mean number per probe and their comparative signal ratio.

nuc ish(D17Z1,ERBB2)×2[20]

HER2/CEP17 ratio = 1.0

HER2 ratio per cell = 2.0

Two copies of both signals were seen in all 20 nuclei examined within the targeted tumor regions. Following p to q order, the centromere of chromosome 17 comes before 7q12; therefore, the centromeric probe (D17Z1) for chromosome 17 would be placed before the targeted *ERBB2* locus at 17q12. Since both signal patterns show the same number of targets, the number can be placed outside the parenthesis. The *HER2/CEP17* ratio of 1.0 falls within the nonamplified range, which has a 2.0 cut-off for amplification. This ratio alone, however, cannot provide an interpretation by current guidelines, because a *HER2* range of ≥ 4.0 and <6.0 would bump the diagnosis up to equivocal, even if the *HER2/CEP17* ratio is <2.0 . In this example, however, the *HER2* ratio is 2.0, and the specimen is negative for amplification.

nuc ish(D17Z1×2~4,ERBB2×10~20)[20]

HER2/CEN17 ratio = 5.2

HER2 ratio per cell = 15

This second example shows ranges for the two signals, where two to four signals were seen for the centromere probe of chromosome 17, and approximately a fivefold increase (1–20) of *ERBB2* signals were seen in the same nuclei. Although the karyotype indicates that the lowest number of *HER2* signals was 10, which clearly exceeds the ASCO-CAP guideline of six signals per nucleus, the ratios are still necessary for the diagnosis. The *HER2/CEN17* ratio of 5.2 (cutoff being <2.0) and the *HER2* ratio of 15 signals per nucleus (cutoff being 6) both verify that the result is positive for *HER2* amplification.

nuc ish(D17Z1,ERBB2)×6[20]

HER2/CEN17 ratio = 1.0

HER2 ratio per cell = 6.0

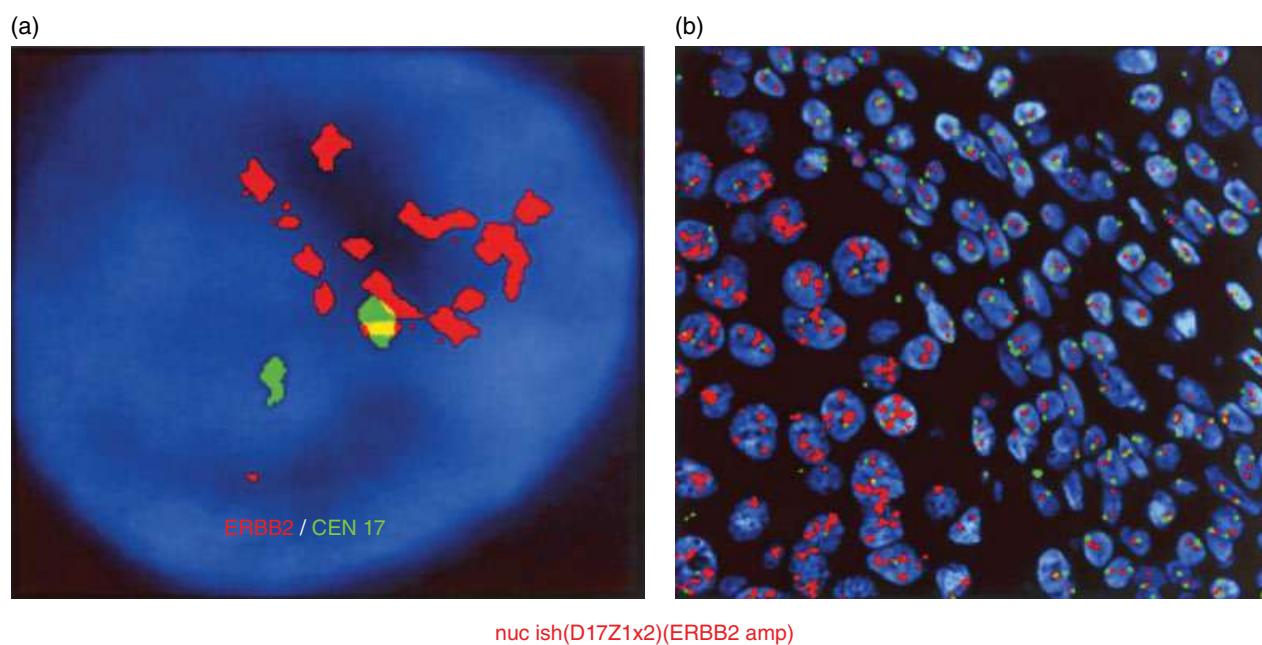


Figure 8.32 Paraffin-embedded breast tissue.

nuc ish(D17Z1x2)(ERBB2 amp)

(a) Amplification of the *ERBB2* locus with two centromeric D17Z1 signals (green) of chromosome 17. (b) Invasive breast tumor with amplified ERBB2 signals alongside nonamplified tissue. See insert for color representation of this figure.

Without a range, this karyotype states that every nucleus examined showed six signals for both the centromere of chromosome 17 and the targeted gene locus *ERBB2* at 17q12. This translates to a 1.0 ratio of D17Z1 to ERBB2 signals, which falls within the nonamplified range (<2.0); however, according to current ASCO-CAP 2013 guidelines, the 6.0 ratio of HER2 signals per nucleus falls within the amplified range (cutoff ≥6), making this specimen positive for amplification [44].

nuc ish(D17Z1x2-4)(ERBB2 amp)[20]

HER2/CEN17 ratio = 8.0

HER2 ratio per cell = 10.0

The amplification symbol (amp) [1, section 13.3.1] is used when nuclei show so many copies of a single locus that its copy number cannot be reliably quantified (see Figure 8.32). Although most commonly associated with an abnormal ERBB2 signal presence in paraffin-embedded breast tumor tissue, it has also been identified with other neoplastic situations [1, section 13.3.2]. This example is the only karyotype in this genre that provides a level of interpretative conclusion within the karyotype, but the report must still confirm all results by including all required ratios.

nuc ish(D17Z1,ERBB2)x2-8[20]

HER2/CEN17 ratio = 2.1

HER2 ratio per cell = 6.0

This last example demonstrates the importance of making no assumptions from the karyotype alone. Both signal patterns independently show the same range values. This range, however, does not imply that every cell showed the same matching pattern. We don't know how many of the 20 cells showed eight signals and how many showed two signals; therefore, signal ratio is critical to the interpretation. The HER2/CEP17 ratio of 2.1, and mean HER2 ≥6 both indicate a result that is positive for amplification. If the ratios for that same karyotype had been: HER2/CEN17 ratio = 1.7 and mean HER2 signals per cell = 3.2, interpretative results would have been very different.

In situ FISH analysis on paraffin-embedded tissue depends heavily on the reader's capability to select malignant nuclei among potentially normal surrounding tissue; therefore, special standards apply for these tests, including the pathologist's involvement

in pinpointing the targeted regions. Loss of tissue topography also complicates the relationship between the fluorescing image and the H&E (hematoxylin and eosin) stained slide. Because of these drawbacks, chromogenic (CISH) and silver-enhanced (SISH) *in situ* hybridization alternatives have been developed that use dual color under brightfield microscopy. Advantages of these procedures include the use of in-house bright-field microscopes rather than fluorescence equipment, and permanently stained and mounted slides [45].

8.10.12 Bone marrow transplant chimerism

A bone marrow transplant karyotype uses its own unique format for reporting recipient//donor cell comparisons. The recipient's karyotype is placed to the left of the double slash (//), without spaces, and the donor's karyotype is placed to the right of the double slash [1, section 4.1]. The following examples assume that a male recipient with a t(9;22) translocation received a bone marrow transplant from a female donor.

46,XY,t(9;22)(q34;q11.2)[20]//– only the patient's cells carrying the t(9;22) translocation were present.

46,XY,t(9;22)(q34;q11.2)[1]/46,XY[5]//46,XX[14] – three cell lines were found: one cell showed the patient's (recipient) male karyotype with the t(9;22); five cells showed normal male cells, also of the patient; and 14 cells were normal female donor cells. The t(9;22) was only seen in one cell, which does not meet reportable standards by itself (see 8.8, Random versus reportable); however, because the translocation was previously reported in the patient's bone marrow, it would not only be reported, but also would be listed first, even though it was only seen in one cell. This single cell could reflect residual disease [1, section 11.1.6].

//46,XX[20] – only female donor cells were found. Because there were no recipient cells present, the karyotype begins with the slashes.

FISH karyotypes follow the same format.

nuc ish(DXZ1,DYZ3)×1[25]//(DXZ1×2)[375]

In this example, 25 cells from the XY patient (recipient) were seen in 400 total nuclei scored. The remaining 375 were female donor nuclei.

//nuc ish(DXZ1×2)[400]

All 400 scored nuclei showed a female karyotype.

nuc ish(DXZ1,DYZ3)×1[400]//

Results placed only to the right of the double slashes represent the recipient's cells. There were no donor cells present.

8.11 Microarray (arr) and region-specific assay (rsa)

The study of the human molecular genome, including microarray or array comparative genomic hybridization (aCGH), is probably the fastest growing field of genetics today. It has added valuable insight into a rapidly growing list of neoplastic and prenatal investigative platforms. Databases have been collecting genomic definition and refinement for over a decade, and are building reliable reference libraries of normal variant versus abnormal sequences. This manual is dedicated to cytogenetic, not molecular, protocols, but we add this section as a brief overview so that those becoming involved in this arena will be able to understand the most basic karyotype formations. Because the karyotype symbols were modified in *ISCN 2016* [1], we will update these basic examples to conform with *ISCN 2016* standards.

Symbols used in microarray are relatively simple, as compared to Table 8.1. New symbols not generally applicable to routine cytogenetics include heterozygosity (htz), homozygous (hm), cx (complex rearrangements), ct (chromothripsis, i.e., complex patterns of alternating copy number changes), GRCh (Genome Reference Consortium human: human genome build or assembly), pos (positive), neg (negative), rsa (regional-specific assay), underscore (which indicates the range of nucleotide positions) [1, section 3]. Results can also be reported in a list rather than a string. Two different karyotype systems can be used: [1] – a short system which describes only the abnormal nucleotides, and [2] – a detailed system which describes both the abnormal nucleotides and their bordering normal nucleotides [1, section 14.1].

The general rules for the microarray karyotype include:

1. If results are normal, the karyotype will list autosomes first, followed by sex chromosomes, separated by a comma. Copy numbers are placed outside the parentheses. For example, a normal female would be written **arr(1-22,X)×2** and a normal male **arr(1-22)×2,(X,Y)×1**.

2. If results are abnormal, only aberrations are listed, from lowest to highest chromosome, with sex chromosome abnormalities listed last. Aberrant nucleotides will follow pter to qter order.
3. When defining nucleotide boundaries, *ISCN 2016* (1) removed commas within the nucleotide number, and replaced the dash with an underscore (_) for indicating the span of affected nucleotides. As explained in *ISCN 2016*, these changes are “in line with the molecular genetic nomenclature outlined by Human Genome Variation Society (HGVS) recommendations (www.HGVS.org/varnomen)” [1, Section 14.1, p. 115].
4. When nucleotide numbers are used to define an abnormal result, the specified genome build, e.g., GRCh38, which stands for Genome Reference Consortium Human Build 38 assembly, is placed directly behind the array symbol in brackets, e.g., arr[GRCh38], generally followed by a space. The specific genome build is not necessary, however, when describing a normal male or female result or an aneuploidy result that has been described by the short method.
5. If the abnormality requires an inheritance symbol (dn, mat, pat, inh), a space will precede that symbol, except when following a parenthesis in the detailed format.
6. When written with other cytogenetic or FISH karyotypes, the array karyotype will be separated from the preceding test by a period; a space will separate the symbol arr from a succeeding chromosome number or letter, but not if followed by a parenthesis or square bracket.
Microarrays can only demonstrate gains or losses of DNA; therefore, FISH or karyotype analysis is necessary to demonstrate the structure of most abnormalities. The arr karyotype may include chromosome band positions if used in conjunction with chromosomal analysis (1, section 14.2).
7. Karyotypes for mixed cell populations may include the estimated proportion within brackets following the copy number. For example, arr(X)x1[0.3] shows the loss of a single copy of the X chromosome in 30% of the cell population analyzed. There is no need to show normal results or the specific genome build [1, section 14.1] when describing aneuploidy by the short description.

The front cover of this manual demonstrates the investigative advancement that has moved cytogenetics into the era of cytogenomics. The four figures all contribute to the cytogenomic picture: a G-banded cell and its subsequent karyogram, FISH detection for the microdeletion, and both array (see Figure 8.33) and SNP (single nucleotide polymorphisms) array to qualify the molecular extent of the deletion and identify any cryptic zygosity, whether homozygous (hmz) or heterozygous (htz). Each test brings with it insight into the mysteries of the cytogenome.

Abnormal karyotypes will list only the aberrations, defining the extent of a loss or gain in relation to its relative nucleotide borders. “Multiple nucleotides may be listed, separated by commas, or an underscore may be used to indicate that the gain or loss encompasses the segment between the listed clones” [1, section 14.2, p. 116].

arr[GRCh38] 21q22.3(44775092_48090352)x1

Chromosome 21 shows a deletion within the telomeric region of one homologue by chromosome analysis and was confirmed by microarray. Aberrant nucleotides are written from pter to qter, following the order of public databases for current genome builds, in this example UCSC (www.genome.ucsc.edu). A space follows the end-bracket of the genome build when succeeded by a number. For a pictorial example of this karyotype, see the images on the front cover.

Region-specific assays (rsa) are used to quantify the number of copies for a particular locus, or limited set of loci, rather than the entire genome. An rsa may include multiplex ligation-dependent probe amplification (MLPA), quantitative fluorescent or real-time PCR-based assays, or some bead-based assays. These molecular-based assays determine the copy number of a chromosome or chromosomal region, or can verify an abnormality detected by another test. If a kit is used and the genomic coordinates are not known, the kit name can be used; however, it is more precise to provide nucleotide numbers.

46,XX.ish del(22)(q11.2q11.2)(D22S75x1,N85A3x2).rsa 22q11.2(D22S75)x1

A normal female karyotype by cytogenetic analysis was found abnormal by a region-specific assay, and confirmed by metaphase FISH for detection of the DiGeorge critical region at 22q11.2. If a kit were used, its name could be used in the abnormal karyotype, before the abnormal copy number result, e.g., **rsa 22q11.2('kit name')x1**.

Translocations can be written with the same double-colon (break::reunion) symbol used in the detailed cytogenetic karyotype. Using the 9;22 translocation, a positive result by a region-specific assay would describe the rearrangement as **rsa(BCR::ABL1)pos**.

We will conclude this section by creating a karyotype for the case depicted on the front cover of this manual. G-banded chromosome analysis showed a suspicious, potential deletion within the long (q) arm of chromosome 21, but it was too cryptic to report without verification. Using a BAP (break-apart fusion-yellow) probe (VIIyRM2029) to target the subtelomeric 21q22.3 region under investigation, and an aqua control probe (RUNX1) to label a locus on the proximal side of the target at 21q22.2, metaphase FISH verified that the targeted region (fused-yellow signal) on one homologue was deleted. Nuclear interphase FISH verified that

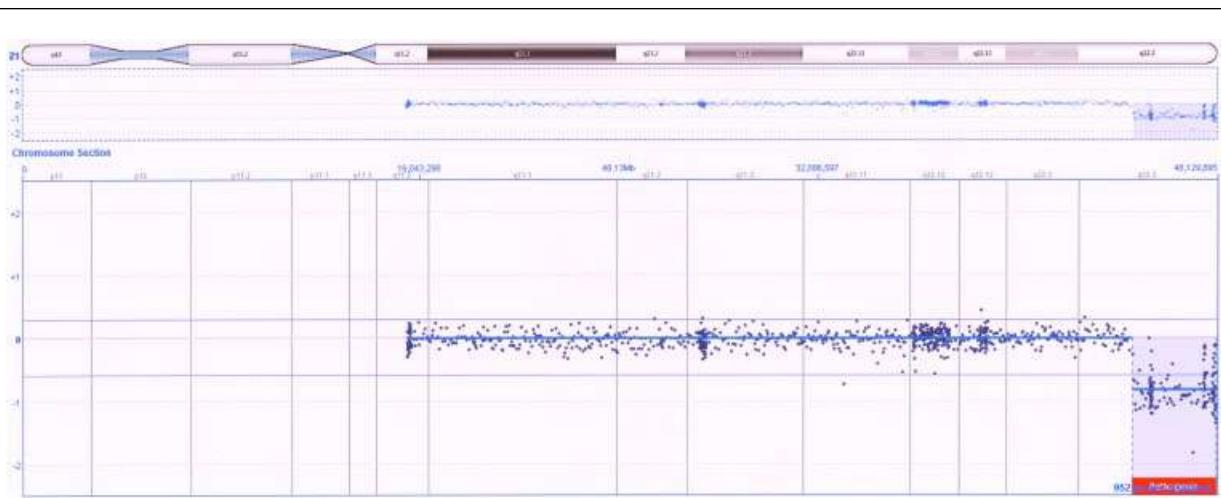


Figure 8.33 Microarray identifies a 3.3 Mb deletion of the subtelomeric region of chromosome 21 at q22.3. Harrison, Rikki. Brain Tickler. *JAGT*, 2nd Quarter 2016; 42[2], p.18. See insert for color representation of this figure.

there was no mosaicism present. Microarray identified the missing nucleotides, and estimated that the deletion was 3.3 Mb, a size that is difficult to detect with routine cytogenetics (see Figure 8.33). With these confirmations, the deletion could be reported.

If the deletion had not been initially detected by cytogenetic analysis, the cells would be revisited. After a closer look, if the technologist determines that the deletion is recognizable, it would be reported in the cytogenetics karyotype or could be amended if previously reported as normal. However, if after revisiting, it still could not be detected, the deletion would not be reported in the cytogenetics section of the karyotype.

Although small, the deletion in our example was recognizable with G-banding (see front cover); therefore, it would now be included in the karyotype. On the other hand, because nuclear interphase FISH did not detect mosaicism, its inclusion does not offer any new information and thus would not be included in the karyotype, but would be mentioned in the report. It is worth noting, however, that by current standards, it is not technically "wrong" if one chose to include it within the karyotype; it would be placed after the metaphase FISH results.

The final karyotype is written like this:

46,XY,del(21)(q22.3).ish del(21)(q22.3)(RUNX1+,VIJyRM2029-).arr[GRCh37] 21q22.3(44775092_48090352)×1

In today's "user-friendly" environment, final FISH reports may rely less on ISCN and more on conformity with other laboratory report formats that are more familiar to the nongenetics physician. But for the geneticist, one line of ISCN formula provides all the necessary information that would take a full page to describe for nongenetic professionals.

8.12 Conclusion

To learn a new language, one must understand the symbolic alphabet and its vocabulary in order to effectively communicate in that language. So too is ISCN a series of descriptions that are formed by symbols and formulae (see Table 8.5). Knowing how to write a karyotype takes practice, but if the technologist can remember the picture being described, he or she may realize that this language is as logical as its original image.

Acknowledgments

The creators of ISCN were amazingly progressive. We are forever grateful to these intuitive geneticists and to the March of Dimes Foundation for supporting a once fledgling specialty into a core arm of medicine. We also express our sincerest appreciation to S. Karger publishers for providing a portion of the proceeds from ISCN book sales to help fund committee meetings and work associated with preparing the new editions of ISCN. We thank Su Yang for his kind review, Ricki Harris for her interesting case, and Helen Lawce with her OHSU cytogenetic team for sharing some of the remarkable examples that grace the pages of this chapter. Finally, we thank Charles Dana Bangs for numerous, critical readings of this chapter. Your insightful contributions were greatly appreciated.

Table 8.5 Karyotype formulae. A review of some of the intrachromosomal and interchromosomal karyotypes discussed in this chapter

Intrachromosomal	Event	Formula	Critical points
46,XY,add(9)(p12)	Additional material of unknown or unidentifiable origin	one chromosome; one break	Monosomy for p arm segment of chromosome 9 from p12 → pter; although the additional material may involve one or more chromosomes and breakpoints, its identity is unknown. If additional material is found on both terminal ends, the derivative karyotype should be used.
46,XY,del(2)(q35)	Deletion, terminal	one chromosome; one break	A break has occurred within the long arm of chromosome 2 at band q35, indicating monosomy for 2q35 → 2qter.
46,Y,fra(X)(q27.3)	Fragile site	one chromosome; one break	Fragile site on chromosome X at q27.3; structurally abnormal X is written after the normal sex chromosome.
46,XY,hsr(9)(p13)	Homogenously staining region	one chromosome; one break	Homogenously staining, additional material of unknown origin is repeatedly duplicated at band p13 on chromosome 9.
46,XY,i(21)(q10) 45,XY,i(21)(q10) 47,XY,i(X)(q10)	Isochromosome	one chromosome; one break	(1) Trisomy for the q arm of chromosome 21; loss of the NOR region and p arm; q10 represents the arm that is present; 46-chromosome count indicates patient has Down syndrome. (2) 45-count indicates the patient is normal but can only produce Down syndrome offspring. (3) The extra isochromosome creates tetrasomy for the long arm of the X chromosome; no plus sign is used in constitutional studies but the structural rearrangement will be placed after the normal Y sex chromosome.
46,X,idic(X)(q13)	Isodicentric	one chromosome; one break	Duplication of the short arm of chromosome X and a segment of the long arm from Xq10 → Xq13; monosomy for the long arm of X from q13 → qter; structurally abnormal chromosome is placed after normal X; chromosome has two centromeres and both are active.
46,X,psu idic(X)(p11.2)	Pseudoisodicentric	one chromosome; one break	Trisomy for X segment from p11.2 → qter and monosomy for p11.2 → pter; two symbols in succession are separated by a space; although both centromeres are present, only one is active.
46,XY,del(7)(q22q34)	Deletion, interstitial	one chromosome; two breaks	Interstitial deletion leaves chromosome 7 monosomy for the segment between 7q22 and 7q34.
46,XY,del(17)(p11.2p11.2)	Deletion, micro	one chromosome; two breaks	Using the same breakpoints in the karyotype formula indicates a microdeletion. This microdeletion leaves chromosome 17 monosomy for a segment within the p11.2 region (Smith Magenis syndrome).

(Continued)

Table 8.5 (Continued)

Intrachromosomal	Event	Formula	Critical points
46,XY,dup(1)(q21q32) 46,XY,trp(1)(q21q32) 46,XY,qdp(1)(q21q32)	Duplication, direct	one chromosome; two breaks	Duplication, triplication, and quadruplication for segment 1q21 → 1q32; segment is in its natural (direct) order.
46,XY,dup(7)(q32q22)	Duplication, inverted	one chromosome; two breaks	Trisomy for segment 1q21 → 1q32; duplicated segment is inverted.
46,XY,dup(7)(q22q22)	Duplication, micro	one chromosome; two breaks	Similar to a microdeletion, using the same breakpoints in the karyotype formula indicates a microduplication. This leaves chromosome 7 trisomy for a segment within the q22 region.
46,XY,inv(11) (q21q23)	Inversion, paracentric	one chromosome; two breaks	Breakpoints occur within the same arm; segment inverts and reattaches; no loss or gain but there is a positional change.
46,XY,inv(7)(p14q34)	Inversion, pericentric	one chromosome; two breaks	Breakpoints occur within opposite arms; segment inverts and reattaches; no loss or gain but there is a positional change.
46,XY,rec(7)dup(7p) del(7q)inv(7)(p15q32) pat	Recombinant	one chromosome; two breaks	Trisomy for chromosome segment pter → p15 and monosomy for q32 → qter, as a result of unequal crossing-over during meiosis between a rearranged chromosome 7 that is inherited from the patient's father, and its normal counterpart.
1) 46,XY,r(18) (p11.3q23) 2) 49,XY,+r1x2,+r2	(1) Ring (2) Ring of unknown origin (markers use similar format)	(1) one chromosome; two breaks (2) unknown	(1) Monocentric ring chromosome involving a break and reunion at or near the terminal ends, at p11.3 and q23 on chromosome 18. (2) Two different ring formations of unknown origin: r1 showed 2 copies per cell, and r2 had only one copy per cell. Rings are described after derivatives with an unknown centric identity and before markers.
46,XY,ins(7) (q36p13p15)	Insertion, direct	one chromosome; three breaks	Segment 7p13 → 7p15 is inserted into the long arm at 7q36; segment is in its natural (direct) order; no loss or gain but there is positional change.
(1) 46,XX,2~15dmin[20] (2) 46,XX,~15dmin[20]	Double minutes	unknown	(1) A range from 2 to 15 double minutes are present; no plus signs are used with dmins and chromosome count does not change; (2) Approximately (mean range) 15 double minutes were seen per cell. The [20] for total cell numbers investigated is placed after mosaic or chimeric cell lines, and both neoplastic and FISH karyotypes, when applicable.
46,XY,upd(15)pat	Uniparental disomy	one chromosome	Homozygous homologues; both are inherited from the father.

Table 8.5 (Continued)

Interchromosomal	Event	Formula	Critical points
45,XY,dic(17;20) (p11.2;q11.2)	Dicentric	two chromosomes; two breaks	Because both centromeres are present in this dicentric chromosome, chromosome 17 is monosomy for most of its p arm (p11.2 → pter), and chromosome 20 is monosomy for most of its q arm (q11.2 → qter).
45,XY,psu dic(20;17) (q11.2;q11.2)	Pseudodicentric	two chromosomes; two breaks	Same effect as the dicentric, but one centromere has been inactivated.
47,XY,+dic r(7;18) (p11.2q32;p11.2q22)	Dicentric ring	two chromosomes; four breaks	Additional dicentric ring chromosome showing a trisomy for the q arms of chromosomes 7 and 18, including the p11.2 region adjacent to the centromere of both chromosomes.
46,XY,t(17;20) (p10;q10)	Translocation, whole arm	two chromosomes; two breaks	Chromosome exchange at the centromere translocates the p arm of chromosome 17 with the q arm of chromosome 20, and visa versa for the opposite arms; no loss or gain but there is a positional change.
46,XY,ins(17;X) (q21;q22q26)	Insertion, direct	two chromosomes; three breaks	Chromosome X segment q22 → q26 is inserted into the long arm of chromosome 17 at q21; segment is in its natural (direct) order; no loss or gain but there is positional change; karyotype does not follow standard karyotype order because the receiving chromosome must be listed first.
46,XY,der(2)t(2;5) (p23;q35)inv(2) (q21q31)	Derivative, multiple event	two chromosomes, four breaks	The derivative chromosome 2 was derived from a translocation between chromosome 2p23 and 5q35, and an inversion of chromosome 2 long arm between 2q21 and 2q31. Events are placed in pter → qter order; therefore, the inversion in the q arm is written after the translocation involving the p arm of chromosome 2.
46,XY,t(5;12;8;21) (q31;p13;q22;q22)	Translocation, multichromosomal	four chromosomes; four breaks	First-listed chromosome will meet the highest priority in karyotype order – that is, X before Y before lowest numerical autosome; remaining chromosomes will be determined by which one receives the previous chromosome's segment; no apparent loss or gain but there is positional change.
47,XY,t(9;22) (q34;q11.2),+der(22) t(9;22)[20]	Derivative	two chromosomes, two breaks	Balanced translocation between the long arm of chromosome 9 at q34 and the long arm of chromosome 22 at q11.2; also known as the Philadelphia (Ph) chromosome. Extra derivative 22 uses the abbreviated repeated description. The [20] indicates total number of cells; it is used at the end of mos/chi cell lines, neoplastic and FISH karyotypes.
45,XY,der(13;21) (q10;q10)	Derivative, acrocentric	two chromosomes, two breaks	Similar to whole arm translocations, where breaks occur at the centromere region and the arms described in the karyotype are the arms present. This karyotype contains the long arms (q10) of chromosomes 13 and 21. Because the short arms of acrocentrics are NOR regions, loss is inconsequential and the carrier will show no phenotypic expression except potential reproductive problems.

Addendum for *ISCN 2016* updates

We are listing below those symbols that were removed in *ISCN 2016* [1].

The previously defined symbol (**hg**) representing a “human genome build or assembly” has been removed. In its place, **GRCh** (Genome Reference Consortium human: human build or assembly) has been added. [1, chapter 3]

The symbol **dir** (meaning direct) has also been removed from the symbol table (chapter 3). Although helpful in the earlier years of cytogenetics, its use has become unnecessary as familiarity with proximal to distal band numbering has become more recognized.

The following (similar) karyotype options were removed, which showed separation of co-hybridized targets into separate parentheses: for example,

nuc ish(ABLx3),(BCRx3),(ABL1 con BCRx2)[400]
nuc ish(ABL1x3),(BCRx3),(ABL1 con BCRx1),(ABL1 con BCR con ABL1x1)[400]

References

1. ISCN (2016): *An International System for Human Cytogenomic Nomenclature*. McGowan-Jordan J, Simons A, Schmid M (eds). Basel: S Karger, 2016.
2. Denver Conference (1960): A proposed standard system of nomenclature of human mitotic chromosomes. *Lancet* 1960; I: 1063–1065.
3. London Conference on the Normal Human Karyotype. *Cytogenetics* 1963; 2: 264–268.
4. Chicago Conference (1966): *Standardization in Human Cytogenetics*. Birth Defects: Original Article Series. 1966. NY: The National Foundation; 2(2).
5. Paris Conference (1971): Standardization in Human Cytogenetics. Birth Defects: Original Article Series. 1972. NY: The National Foundation; 8(7).
6. ISCN (1978): *An International System for Human Cytogenetic Nomenclature*. Birth Defects: Original Article Series. 1978; NY: The National Foundation, 14(8).
7. ISCN (1981): *An International System for Human Cytogenetic Nomenclature – High Resolution Banding*. Birth Defects: Original Article Series 1981; NY: March of Dimes Birth Defects National Foundation, 17(5).
8. ISCN (1985): *An International System for Human Cytogenetic Nomenclature – High Resolution Banding*. Harnagen DG, Klinger HP (eds). Birth Defects: Original Article Series. 1985; NY: March of Dimes Birth Defects National Foundation, 21(1).
9. ISCN (1991): Guidelines for Cancer Cytogenetics, Supplement to *An International System for Human Cytogenetic Nomenclature*. Mitelman F (ed). Birth Defects: Original Article Series 1991; Basel: S Karger.
10. ISCN (1995): *An International System for Human Cytogenetic Nomenclature*. Mitelman F (ed). Basel: S Karger, 1995.
11. ISCN (2005): *An International System for Human Cytogenetic Nomenclature*. Shaffer LG, Tommerup N (eds). Basel: S Karger, 2005.
12. ISCN (2009): *An International System for Human Cytogenetic Nomenclature*. Shaffer LG, Slovak ML, Campbell LJ (eds). Basel: S Karger, 2009.
13. ISCN (2013): *An International System for Human Cytogenetic Nomenclature*. Shaffer LG, McGowan-Jordan J, Schmid M (eds). Basel: S Karger, 2013.
14. Schwartz S, Palmer CG. High-resolution chromosome analysis: I. Applications and limitations. *Am J Med Genet* Oct 19 1984; 2: 291–299. <http://www.ncbi.nlm.nih.gov/pubmed/6542308>.
15. Weissenbach J, Levilliers J, Petit C, Rouyer F, Simmler M-C. Normal and abnormal interchanges between the human X and Y chromosomes. *Development 101 Supplement* 1987; Great Britain: 67–74.
16. Genetics Home Reference: Your guide to understanding genetic conditions. National Library of Medicine. <http://ghr.nlm.nih.gov/chromosome/21>. Retrieved 3/13/13.
17. Hiller B, Bradtke J, Balz H, Rieder H (2004). CyDAS: a cytogenetic data analysis system. *Bioinformatics* 2005; 21(7): 1282–1283.

18. Ballif BC, Wakui K, Gajecka M, Shaffer LG. Translocation breakpoint mapping and sequence analysis in three monosomy 1p36 subjects with der(1)t(1;1)(p36;q44) suggest mechanisms for telomere capture in stabilizing de novo terminal rearrangements. *Hum Genet* 2004; 114: 198–206.
19. Slater HR, Bailey DK, Ren H, Cao M, Bell K, Nasioulas S, Henke R, Choo KHA, Kennedy GC. High-resolution identification of chromosomal abnormalities using oligonucleotide arrays containing 116,204 SNPs. *Am J Hum Genet* 2005; 77: 709–726.
20. University of Nevada School of Medicine Genetics Program, Director: Colleen A Morris, MD, Genetics Division of the Department of Pediatrics. unr.edu/geneticslk.html. <http://www.medicine.nevada.edu/dept/genetics/williams.html>. Retrieved 2-14-2013.
21. The Neuropathology Learning Program for Residents & Medical Students, Department of Pathology, University of Oklahoma Health Sciences Center. <http://moon.ouhsc.edu/kfung/jty1/neurohelp/ZNP2IE05.htm>. Retrieved February 14, 2013.
22. 22q11.2 deletion syndrome. Genetics Home Reference, U.S. National Library of Medicine <http://ghr.nlm.nih.gov/> <http://ghr.nlm.nih.gov/condition/22q112-deletion-syndrome>. Retrieved 2-14-2013.
23. Bi W, Park SS, Shaw CJ, Withers MA, Patel PI, Lupski JR. Reciprocal crossovers and a positional preference for strand exchange in recombination events resulting in deletion or duplication of chromosome 17p11.2. *Am J Hum Genet* 2003; 73(6):1302–1315.
24. Delicado A, Escribano E, Pajares IL, de Bustamante AD, Carrasco S. A malformed child with a recombinant chromosome 7, rec(7) dup p, derived from a maternal pericentric inversion inv(7)(p15q36). *J Med Genet* 1991; 28: 126–127.
25. Makishima H, Maciejewski JP. Pathogenesis and consequences of uniparental disomy in cancer. *Clin Cancer Res*, 2011; 17(12): 3913–3923. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3523887/pdf/nihms269876.pdf>
26. Haase D, Schwartz S. Genetic Biomarkers Revealed: Unraveling the Complexities of Cancer Genomes in Blood Malignancies. Webinar presentation. <http://webinar.sciencemag.org/webinar/archive/genetic-biomarkers-revealed>. Retrieved 2/5/2013.
27. Bacher U, Haferlach C. der(9;18)(p10;q10). Atlas Genet Cytogenet Oncol Haematol. September 2006. <http://AtlasGeneticsOncology.org/Anomalies/der918p10q10ID1418.html>. Retrieved 2/14/2013.
28. Hsu LY, Kaffe S, Jenkins EC, Alonso L, Benn PA, David K, Hirschhorn K, Lieber E, Shanske A, Shapiro LR, Schutta E, Warburton D. Proposed guidelines for diagnosis of chromosome mosaicism in amniocytes based on data derived from chromosome mosaicism and pseudomosaicism studies. *Prenat Diagn* 1992; 12(7): 555–573.
29. Hsu LY, Benn PA. Revised guidelines for the diagnosis of mosaicism in amniocytes. *Prenat Diagn* 1999; 19(11): 1081–1082.
30. Ing PS, Van Dyke DL, Caudill SP, Reidy JA, Bice G, Bieber FR, Buchanan PD, Carroll AJ, Cheung SW, DeWald G, Donahue RP, Gardner HA, Higgins J, Hsu LY, Jamehdor M, Keitges EA, Laundon CH, Luthardt FW, Mascarello J, May KM, Meck JM, Morton C, Patil S, Peakman D, Pettenati MJ, Rao N, Sanger WG, Saxe DF, Schwartz S, Sekhon GS, Vance GH, Wyandt HE, Yu CW, Zenger-Hain J, Chen AT. Detection of mosaicism in amniotic fluid cultures: a CYTO2000 collaborative study. *Genet Med* 1999; 1(3): 94–97.
31. Debacker K, Kooy RF. Fragile sites and human disease. *Hum Mol Genet* 2007; 16(2): 150–158.
32. Mitsui J, Tsuji S. Common Chromosomal Fragile Sites: Breakages and Rearrangements in Somatic and Germline Cells. *Atlas Genet Cytogenet Oncol Haematol* July 2011. <http://AtlasGeneticsOncology.org/Deep/ChromFragSitesID20098.html>. Retrieved 2/14/2013.
33. Mascarello JT, Hirsch B, Kearney HM, Ketterling RP, Olson SB, Quigley DI, Rao KW, Tepperberg JH, Tsuchiya KD, Wiktor AE. A Working Group of the American College of Medical Genetics (ACMG) Laboratory Quality Assurance Committee. ACMG Policy Statements, Standards & Guidelines, Section E9 of the American College of Medical Genetics technical standards and guidelines: fluorescence in situ hybridization. *Genet Med* 2011; 13(7): 667–675.
34. HUGO Gene Nomenclature Committee at the European Bioinformatics Institute at <http://www.genenames.org>
35. http://www.cytocell.co.uk/products/aquarius/microdeletion-syndrome-probes/DiGeorge-VCFS-TUPLE1-and-22q13-3-Deletion-Syndrome-Combination_information.asp. Retrieved 2/14/2013.
36. Phelan MC, Rogers RC, Saul RA, Stapleton GA, Sweet K, McDermid H, Shaw SR, Claytor J, Williw J, Kelly DP. 22q13 deletion syndrome. *Am J Med Genet*, 2001; 101(2): 91–99.

37. HUGO Gene Nomenclature Committee at the European Bioinformatics Institute. www.genenames.org/cgi-bin/quick_search.pl?cgifields=type&num=50&search=MYC&submit=Submit. Retrieved 2/14/2013.
38. HUGO Gene Nomenclature Committee at the European Bioinformatics Institute. www.genenames.org/cgi-bin/quick_search.pl?cgifields=type&num=50&search=ABL1&submit=Submit. Retrieved 2/14/2013.
39. HUGO Gene Nomenclature Committee at the European Bioinformatics Institute. http://www.genenames.org/cgi-bin/quick_search.pl?cgifields=type&num=50&search=BCR&submit=Submit. Retrieved 2/14/2013.
40. *AneuVysion Multicolor DNA Probe Kit*, 05J38, January 2011. Abbott Molecular Inc., Des Plaines, IL. http://www.abbottmolecular.com/static/cms_workspace/pdfs/US/Package_Insert_04_02_2012.pdf. Retrieved 5/11/13.
41. HUGO Gene Nomenclature Committee at the European Bioinformatics Institute. www.genenames.org/cgi-bin/quick_search.pl?cgifields=type&num=50&search=DSCR&submit=Submit. Retrieved 2/19/2013.
42. HUGO Gene Nomenclature Committee at the European Bioinformatics Institute. www.genenames.org/cgi-bin/quick_search.pl?cgifields=type&type=contain&num=50&search=DGCR1&submit=Submit. Retrieved 2/14/2013.
43. HUGO Gene Nomenclature Committee at the European Bioinformatics Institute. http://www.genenames.org/cgi-bin/quick_search.pl?cgifields=type&type=contain&num=50&search=HIRA&submit=Submit. Retrieved 2/14/2013.
44. Wolff AC, Hammond MEH, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JMS, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G, Hayes DF. Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update. *J Clin Oncol* 2013; 31(31): 3997–4014. <http://jco.ascopubs.org/cgi/doi/10.1200/JCO.2013.50.9984>.
45. Reisenbichler ES, Horton D, Rasco M, Andea A, Hameed O. Evaluation of dual immunohistochemistry and chromogenic *in situ* hybridization for HER2 on a single section. *Am J Clin Pathol* 2012; 137: 102–110.

CHAPTER 9

Constitutional chromosome abnormalities

Kathleen Kaiser-Rogers

Pathology & Laboratory Medicine, Pediatrics, Genetics and UNC Hospitals Cytogenetics Laboratory, University of North Carolina at Chapel Hill, NC, USA

9.1 Numerical abnormalities

The role of a clinical cytogeneticist is to detect both structural and numerical abnormalities in our patient population. Although molecular cytogenetic techniques, including fluorescence in situ hybridization (FISH) and chromosome microarrays, are now available, for many years the primary tool used to detect chromosome abnormalities has been karyotyping. The first karyotypes were derived from solidly stained rather than banded chromosomes. While it was possible to count the solidly stained chromosomes and organize them into groups (A through G) based on their size and shape, most structural abnormalities were undetectable in the absence of banding. It is therefore not at all surprising that the earliest constitutional chromosome abnormalities identified in humans were numerical rather than structural.

The first chromosome abnormality was reported in 1959, when Lejeune [1] and Jacobs [2] each independently reported the presence of an extra chromosome 21 in association with Down syndrome. Later that year several numerical sex chromosome abnormalities were also identified, including the 45,X karyotype associated with Turner syndrome, the 47,XXY karyotype associated with Klinefelter syndrome and the 47,XXX karyotype associated with triple X syndrome [3–5]. The numerical abnormalities reported in these early cases would be described as aneuploidies because they involve gains or losses of less than an entire haploid set of 23 chromosomes. While most of the constitutional aneuploidies observed in the human population involve the gain (trisomy) and less frequently the loss (monosomy) of a single chromosome, gains involving multiple chromosomes are also observed.

In contrast to aneuploidy, the term euploidy is used to refer to the loss or gain of one or more entire haploid sets of 23 chromosomes. The typical human somatic cell is diploid and contains 46 chromosomes, while a triploid karyotype contains 69 chromosomes or one extra haploid set and a tetraploid karyotype contains 92 chromosomes or two extra haploid sets. Triploid and tetraploid constitutional karyotypes have only rarely been reported in liveborn individuals. Haploid karyotypes containing a single set of 23 chromosomes are observed in human gametes. Haploid and near haploid karyotypes can also be acquired in association with some forms of cancer. For example, near haploidy (i.e., a karyotype with less than 34 chromosomes) is seen in some patients with acute lymphoblastic leukemia (ALL; see Chapter 11, Hypodiploidy).

Numerical chromosome abnormalities represent the leading cause of reproductive failure and congenital birth defects in humans. Approximately 35–45% of miscarriages, 4% of stillbirths and 0.3% of liveborn individuals have aneuploid or euploid karyotypes [6–8]. The frequency with which these chromosome abnormalities occur in conjunction with their associated high rates of morbidity and mortality has made this an important and active area of study. While much remains to be learned, the research accumulated thus far has demonstrated that there are likely multiple causes for both aneuploidy and euploidy, and that their etiology is complex.

9.1.1 Clinical consequences of numerical aneuploidy

Since the first numerical abnormalities were discovered in 1959, aneuploidies involving each of the 24 unique human chromosomes (22 autosomes, X and Y) have been observed in the various samples that are typically karyotyped in clinical cytogenetics laboratories. However, because of the lethality associated with most aneuploidies, the types of numerical abnormalities observed and their frequencies differ significantly, depending on the sample being examined. For example, among spontaneously aborted pregnancies, particularly those that are lost very early, trisomies involving every one of the 24 human chromosomes have been reported [6,8,9]. In contrast, only a subset of these trisomies is seen in stillbirths, and fewer still are seen in liveborn individuals. In fact, only the nonmosaic trisomies involving chromosomes 13, 18, and 21, as well as three sex chromosome trisomies, XXX, XXY, and XYY, are considered to be compatible with survival. Additionally, even among these survivable nonmosaic trisomies, in utero death is common [6]. For example, in utero death is estimated to occur in at least 50% of XXY, 80% of trisomy 21, and 97% of trisomy 13 conceptions. For monosomy X, the only complete nonmosaic monosomy that is compatible with survival, greater than 99% of all conceptions die in utero. These data involving the frequency and viability of various aneuploidies illustrate that while numerical abnormalities are often lethal, the loss of genetic material is much more detrimental to normal growth and development than the gain of genetic material. This appears to be true whether one is discussing complete chromosome monosomies and trisomies or the partial imbalances associated with structural abnormalities (see 9.2, Structural rearrangements).

As expected, given the high degree of lethality associated with the gain and especially the loss of an entire chromosome, aneuploidy is one of the leading causes of reproductive failure; aneuploidy is observed in approximately 35% of all spontaneously aborted pregnancies, 4% of all stillbirths and 0.3% of all livebirths [6,7]. The consequence among liveborn children of a nonmosaic aneuploid karyotype involving one of the autosomes is typically intellectual disability, birth defects, and dysmorphic features (see Table 9.1 and Figure 9.1).

In contrast to the severe phenotype associated with the viable autosomal trisomies, the phenotype associated with each of the sex chromosome trisomies is typically mild and often considered to be within normal limits. In fact, it is likely that many individuals with a sex chromosome trisomy have never been karyotyped because there is no reason to suspect a chromosome abnormality. As a group, these individuals are typically tall, and although they do not have intellectual disability, their IQ is approximately 10 points lower than that of their siblings and learning difficulties are fairly common. Because they are infertile, males with Klinefelter syndrome are probably the most likely of this group to be karyotyped. Often these males are only identified when, in an attempt to understand their childlessness, they refer themselves to infertility clinics.

The only complete monosomy capable of surviving to birth involves the X chromosome; however, more than 99% of all 45,X conceptions die in utero. While a 45,X or Turner syndrome karyotype results in infertility, this is not a particularly common reason for ascertainment. The majority of nonmosaic individuals are instead identified prenatally or as newborns because of features associated with edema (puffy hands and feet, cystic hygroma/redundant neck skin; see Figure 9.2) in conjunction with typical cardiac and/or renal findings; in mid-childhood because of short stature; or in adolescence because of failure to enter puberty [10]. While intellectual disability is typically not present and as a group, women with Turner syndrome excel at verbal skills, they do manifest math, visuospatial, and executive function deficits [11]. Given the incongruity between the high rate of lethality associated with Turner syndrome and the mild phenotype observed in many individuals with this syndrome, it has been proposed that the majority of viable pregnancies are actually mosaics. Presumably a second sex chromosome is required for intrauterine survival and confers a milder phenotype, especially if it is present in a cell line containing a normal chromosome complement. Because the presence of a cell line bearing Y chromosomal material has been associated with an increased risk for gonadoblastoma, additional cytogenetics studies are typically performed on Turner syndrome cases to address this issue. Based on the Laboratory Guidelines for Turner syndrome that are currently being drafted by the American College of Medical Genetics (ACMG) Laboratory Quality Assurance Committee, if a 30-cell G-band analysis reveals a nonmosaic 45,X karyotype, a minimum of 200 interphase cells should be examined with X and Y chromosome centromere fluorescence *in situ* hybridization (FISH) probes to address the presence of occult Y chromosome mosaicism. If no such mosaicism is detected but the suspicion is high because of virilization, an attempt should be made to obtain a second tissue type for analysis (see ACMG website for guideline standards) [12].

9.1.2 Mechanisms of aneuploidy

Under normal circumstances meiosis, which occurs solely in our germ cells, involves a single replication event followed by two divisions in which homologous chromosomes (meiosis I) and then sister chromatids (meiosis II) segregate equally to separate cells (Figure 9.3). Specifically, during the synthesis or “S” stage of meiosis each chromosome replicates itself such that it is composed of two sister chromatids, rather than only one. Like or homologous chromosomes then pair and exchange genetic material in a process referred to as crossing over or meiotic recombination (see Chapter 1, section 1.2.4,

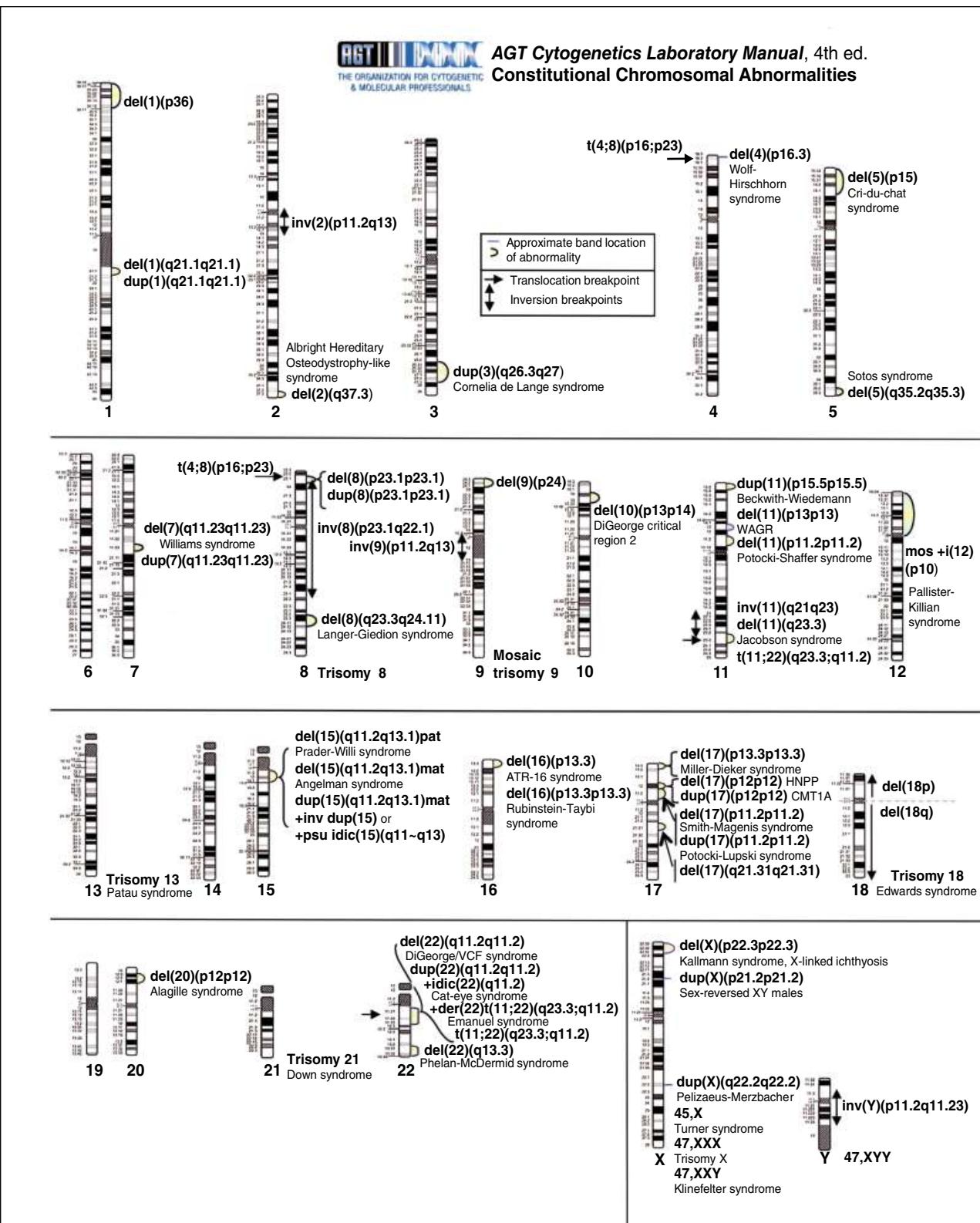


Figure 9.1 Mapping recurrent constitutional gains and losses. For the clinical description of these syndromes, see Table 9.1. Idiograms have been resized and are not to scale. ISCN diagram of human idiograms at the 850-band level (©2009 Nicole Chia) used by permission of the artist. Reproduced from *ISCN 2013: An International System for Human Cytogenetic Nomenclature* 2013. Shaffer LG, McGowan-Jordan J, Schmid M, eds. S. Karger Publishers, Basel.

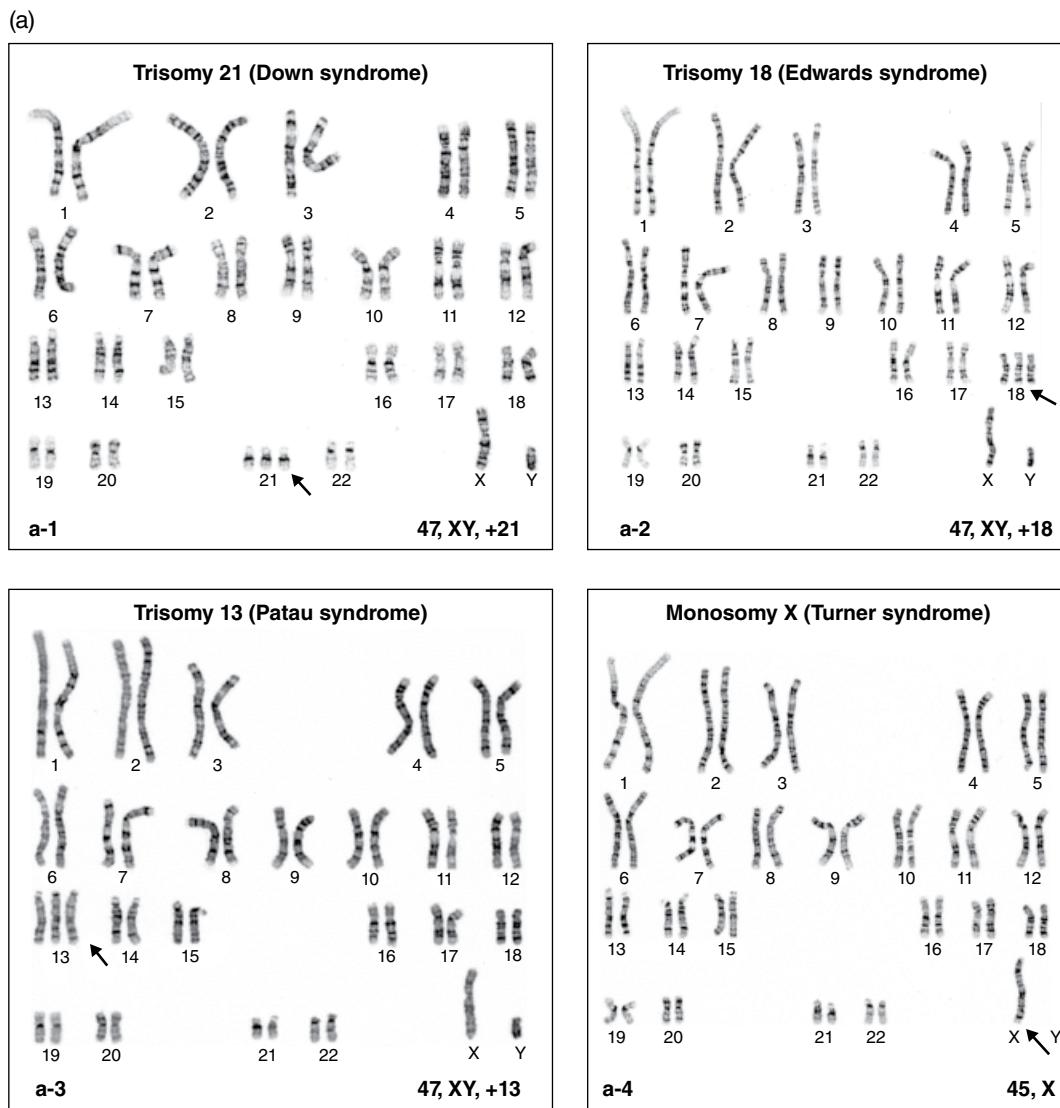


Figure 9.2 First aneuploidies identified. Karyograms (a) and selected clinical features (b) are demonstrated for four of the earliest aneuploid syndromes identified: trisomy 13, 18, 21, and monosomy X.

1) Mikael Häggström, Brushfield eyes.jpg by Erin Ryan (Public Domain), en.wikipedia.org/wiki/File:Brushfield_eyes_magnified.jpg, retrieved 6-16-2010; 2) Courtesy of Dr. Cindy Powell, Departments of Pediatrics and Genetics, UNC at Chapel Hill; 3) Courtesy of Sean and Janine George, who provided photographs of their son Ethan. We thank them for their contributions. See insert for color representation of Figure 9.2b.

Zygotene stage). Next, the paired homologous chromosomes align themselves at the metaphase plate during metaphase I, each homologue is separated from its mate by the meiotic spindle apparatus during anaphase I, and finally cytokinesis or cell division occurs. Meiosis II, which then follows, is essentially a mitotic division during which each univalent chromosome is separated into its two component chromatids, each of which segregates to a separate cell. Initially each univalent chromosome aligns itself on the metaphase II plate, sister chromatids are then separated and pulled to separate poles during anaphase II, and cytokinesis completes the process. The end product of each meiotic event is a single haploid oocyte and two polar bodies in the female or four haploid spermatocytes in the male. When the chromosomes fail to segregate normally during meiosis, the resulting gametes contain extra or missing chromosomes, and after normal fertilization an aneuploid zygote is formed.

(b)

Trisomy 21 (Down syndrome)

Although described as early as 1838, Down syndrome is named after John Langdon Down who documented the syndrome in 1866. Jérôme Lejeune discovered the extra small acrocentric, "G" group, chromosome in 1959.

Flat nasal bridge,
epicanthal folds (fold
of eyelid skin on the
inner side of the eye),
upturned palpebral
fissures (eye openings)
& protruding tongue¹



Single
palmar crease,
short fingers,
and 5th finger
clinodactyly (incurving)²



b-1
Small, low-set,
malformed ears²



Brushfield spots¹
(white specks in the iris)

**Trisomy 18 (Edwards syndrome)**

Trisomy 18, also called Edwards syndrome, was first reported in 1960 by John H. Edwards.



b-2
Clenched hands with
overlapping fingers²



Rocker bottom feet²
or a prominent
calcaneous (heel bone)

Monosomy X (Turner syndrome)

Turner syndrome is named after Henry Turner, who described this disorder in 1938.



Fetal cystic hygroma²
(fluid filled sac) (above left)
may cause a webbed
neck² (above right)



b-4
Edema² (swelling of
the hands and feet)



Short fourth
metacarpal²

Trisomy 13 (Patau syndrome)

Trisomy 13, also called Patau syndrome, was first described by Thomas Bartholinus in 1656. Its chromosomal cause was reported by Klaus Patau in 1960.



b-3
Cleft lip/palate and microphthalmia³
(small eyes)



Scalp defect²



Clenched fists
and polydactyly³
(extra finger)

Figure 9.2 (Continued)

Abnormal chromosome segregation is thought to occur in one of three ways: classical nondisjunction, nondisjunction secondary to chromatid predivision, and anaphase lag. Classical nondisjunction occurs when the chromosomes fail to separate or disjoin. When nondisjunction occurs during meiosis I, homologous chromosomes fail to separate and segregate to their respective poles (Figure 9.4). Instead, both homologues migrate together to one sister cell, while the other cell receives no copies of the nondisjoined chromosome. When the nondisjunction event occurs during meiosis II, it is the sister chromatids that fail to disjoin and segregate, leaving one daughter cell with an extra chromosome (disomic) and the other with none (nullisomic; see Figure 9.5). While both monosomic and trisomic conceptions occur regardless of whether the nondisjunction

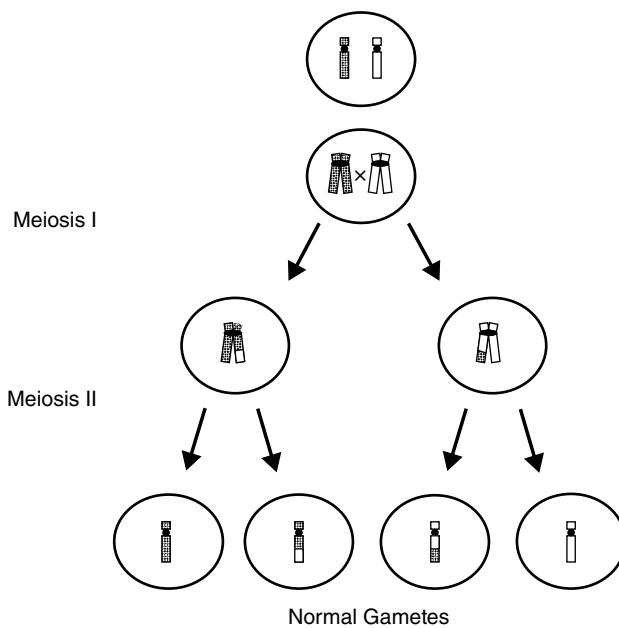


Figure 9.3 Meiosis I occurs solely in germ cells and involves a single replication event, multiple recombination events in prophase of meiosis I (X), and two division events – the first in meiosis I and the second in meiosis II. Four unique sperm are formed from each male meiosis, while female meiosis produces a single egg and multiple polar bodies.

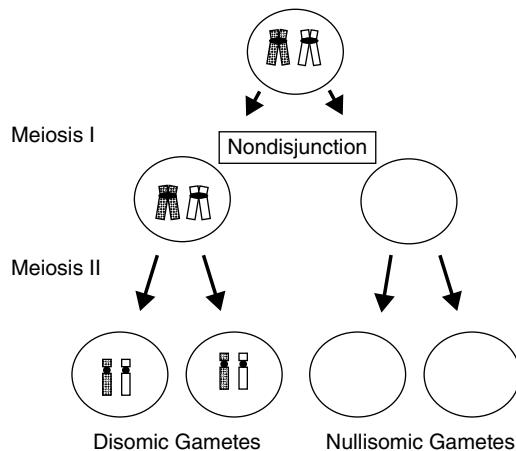


Figure 9.4 Homologous chromosomes fail to segregate in a classic meiosis I nondisjunction event, producing both disomic and nullisomic gametes. Note the chromosomes within the disomic gametes are different or heterozygous along their entire length, because recombination has been omitted for simplicity.

event occurred during meiosis I or II, the genetic composition of the trisomic cells will differ. Note that both the gamete and resulting zygote formed secondary to a meiosis I error contain two different copies of the nondisjoined chromosome; in Figure 9.4 this is denoted by both a white and a hatched chromosome in the disomic gamete. In contrast, the disomic product of a meiosis II error contain two identical copies of the same nondisjoined chromosome; in Figure 9.5 this is denoted by the presence of two hatched chromosomes. This difference in the genetic composition of trisomic conceptions has been exploited to ascertain the meiotic origin of the responsible nondisjunction event. In these studies polymorphic

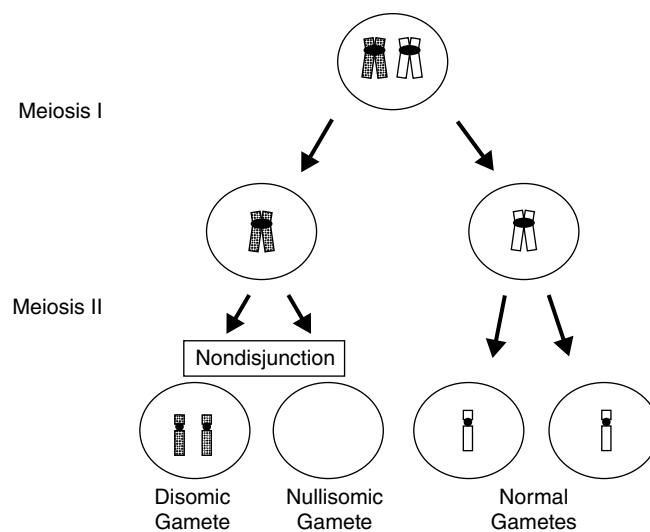


Figure 9.5 Sister chromatids fail to segregate in a classic meiosis II nondisjunction event, producing both disomic and nullisomic gametes. Note the chromosomes within the disomic gametes are identical or homozygous along their entire length, because recombination has been omitted for simplicity.

genetic markers located close to the centromere are used because they are less likely to have undergone recombination than more distally placed markers. If both copies of the nondisjoined chromosome are identical (homozygous) for the pericentromeric markers, then a meiosis II event is most likely responsible. Conversely, if these markers are different, a meiosis I event has likely occurred. By examining the parental origin of these markers, it is also possible to ascertain whether the nondisjunction event occurred during maternal or paternal meiosis. These types of studies have demonstrated that nondisjunction events occur primarily during maternal meiosis I. Exceptions, however, have been documented for particular trisomies (see 9.1.2, Mechanisms of aneuploidy, for the discussion on post-zygotic mitotic segregation errors and 9.1.4, Trisomy).

Angell demonstrated that not all nondisjunction events occur secondary to the classical model described above [13–15]. Their oocyte data suggested that in some cases it is the premature separation of the sister chromatids, rather than the failure of homologous chromosomes to separate during meiosis I, that produces aneuploid gametes (Figure 9.6). Specifically, in many of the oocytes demonstrating abnormal chromosome segregation, they observed prematurely separated chromatids (half univalents) rather than the gains and losses of whole chromosomes (univalents) expected for a classical meiosis I nondisjunction event. Since their original observation, multiple groups have confirmed that nondisjunction secondary to chromatid predision is indeed a common cause for aneuploidy in humans, and the following sequence of events have been proposed [7,16–19]. The chain of events is believed to be initiated when a pair of homologous chromosomes either fails to pair up or they prematurely disjoin during meiosis I, such that two separate univalents are present rather than a single bivalent. One or both univalents are then predisposed to separate into sister chromatids, a process that would normally occur in meiosis II. Each prematurely separated sister chromatid and/or univalent is then free to segregate independently from its mate. Depending on how these chromatids segregate, aneuploidy can result. Presumably premature sister chromatid segregation in meiosis II could also lead to aneuploidy. In this situation, rather than binding to the meiotic spindle apparatus as a single univalent or unit so that each sister chromatid can be parsed to a separate pole, each prematurely separated sister chromatid would instead bind and segregate in an independent fashion. Assuming random segregation of each sister chromatid, approximately 50% of the time an aneuploid gamete would be produced.

Finally, abnormal chromosome segregation and chromosome aneuploidy also appears to occur secondary to a mechanism known as anaphase lag (Figure 9.7). During this process, a chromosome either fails to bind to the spindle apparatus, or it binds but does not migrate to its respective pole. As a result of delayed or absent chromosome migration during anaphase I or II, the lagging chromosome(s) is not included in the reforming nuclear membrane and is lost from one of the daughter cells. In contrast to the meiotic nondisjunction events described above, which yield gametes with either gains or losses, only chromosome loss is seen in association with anaphase lag.

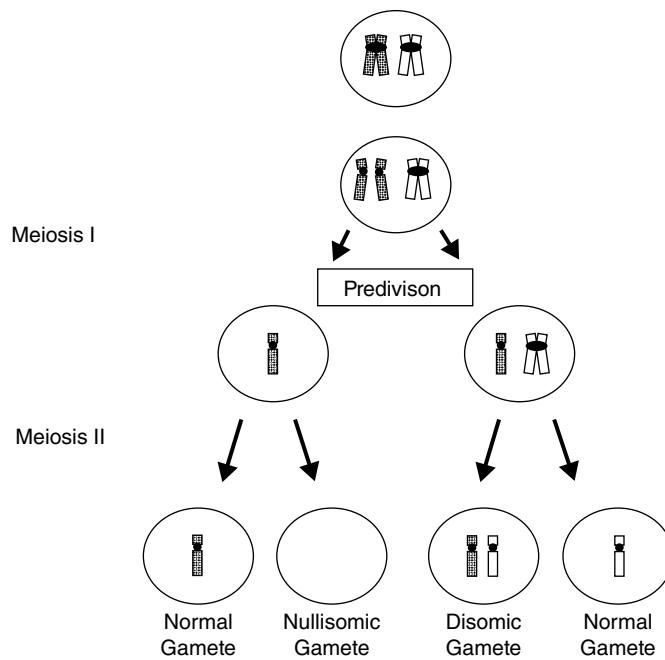


Figure 9.6 Nondisjunction secondary to chromatid predivision, like classic nondisjunction, produces both disomic and nullisomic gametes. Only one of the multiple ways that the prematurely separated sister chromatids can segregate during meiosis I is shown.

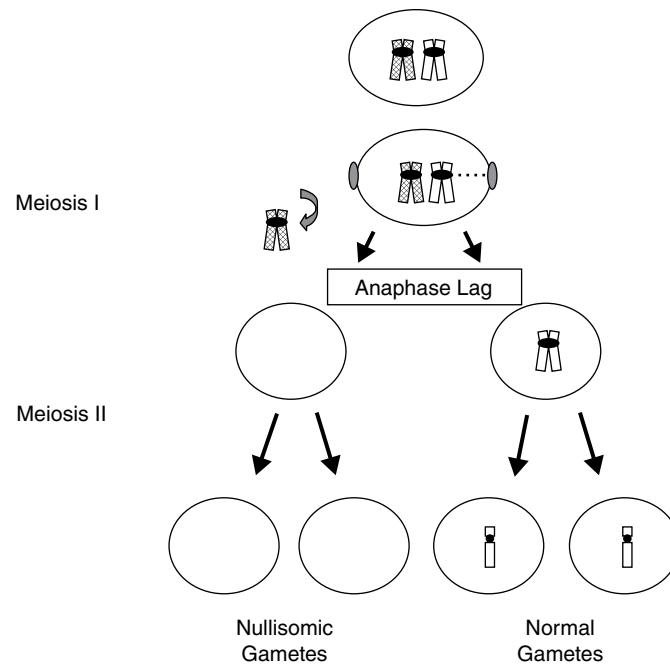


Figure 9.7 Anaphase lag is shown during meiosis I but can also occur during meiosis II. The hatched chromosome fails to attach to the spindle apparatus and is lost from the cell. Nullisomic gametes, but not disomic gametes, result.

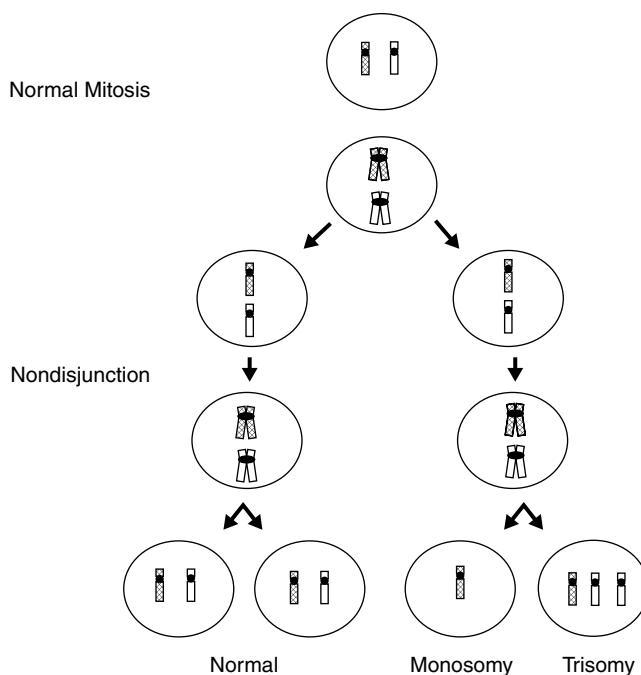


Figure 9.8 Normal mitosis is shown in the upper portion of this figure, while mitotic nondisjunction is seen in one of two sister cells in the lower right portion. If both the resulting monosomic and trisomic cell lines are viable, mosaicism for all three cell lines - normal, trisomic and monosomic, would be present. If neither of the abnormal cell lines is viable, only the normal cell line would survive and continue to divide.

When chromosomes segregate abnormally during meiosis, the ensuing fertilized egg or zygote and all of the cells derived from it, typically carry the same chromosome abnormality(ies). In contrast, chromosome malsegregation during mitosis can result in mosaicism, defined as the presence of more than one cell line, each of which is derived from the same precursor cell. During normal mitosis, each chromosome duplicates itself such that each chromosome is composed of two identical sister chromatids (Figure 9.8). These chromosomes align themselves on the metaphase plate during metaphase, each sister chromatid then segregates to its respective pole during anaphase, and finally a nuclear membrane forms around each complement of chromosomes during telophase to produce two identical daughter nuclei with the same number and kind of chromosomes as the original cell. When chromosome malsegregation occurs via nondisjunction or anaphase lag during any one of the many mitotic cell divisions that take place in the formation of a human being, mosaicism for an aneuploid cell line(s) can occur (Figure 9.8). Not surprisingly, the extent of mosaicism will depend on the timing of the malsegregation event; the earlier the mutation event, the greater the number of affected cells. If, however, the mitotic nondisjunction event occurs early enough and the normal cells are confined to either the extraembryonic or the embryonic tissue, the resulting fetus' karyotype would be completely aneusomic or completely normal, respectively, and no evidence of mosaicism would be observed. In addition to timing, the degree of mosaicism will also be impacted by the survivability of the resulting aneuploid cell(s). Some of the more lethal aneuploid cells may actually undergo apoptosis (cell death) without ever dividing and therefore leave no evidence of their existence. In other situations the aneuploid cell line may grow more slowly than the normal cell line and therefore constitute a smaller percentage of abnormal cells than another less lethal aneuploid cell would. Interestingly, in some cases the degree of lethality and growth characteristics associated with a particular aneuploidy appears to be tissue specific. For example, both trisomy 16 and trisomy 20 have only rarely been detected in PHA-stimulated peripheral blood samples, but have been routinely observed in skin and other tissues [20].

Post-zygotic mitotic segregation errors, in contrast to meiotic errors, have rarely been found in clinically recognized trisomic pregnancies, with two exceptions: approximately 60% of trisomy 7 and 50% of trisomy 8 pregnancies appear to have occurred secondary to post-zygotic errors [21]. Post-zygotic mitotic error is also thought to be responsible for a large proportion of 45,X conceptions [22]. Given the frequent mitotic origin of these three aneuploidies, mosaicism for a normal diploid cell line would be more likely here than with the aneuploidies that are typically meiotically derived. It may therefore be prudent to analyze additional cells for any case in which one of these two trisomies or a 45,X karyotype is suspected based on the clinical or preliminary cytogenetic findings (see 9.1.3, Mosaicism).

While mosaicism is the expected consequence of a mitotic segregation error, uniparental disomy (UPD) (the inheritance of both chromosomes from a single parent) is not. In this situation the trisomic cell line is derived secondary to the doubling of a single chromosome within a normal diploid cell line with biparental inheritance for each chromosome. In contrast, the theoretical risk for UPD secondary to correction or rescue of a trisomy that originated from a meiotic error is 1 in 3. In this situation the trisomy is rescued by loss of one of the three chromosomes, two of which were inherited from a single parent. Only loss of the chromosome contributed as a singleton (1 of the 3 chromosomes) will result in UPD while normal biparental inheritance would arise from loss of either of the two chromosomes (2 of the 3 chromosomes) contributed by the other parent.

9.1.3 Mosaicism

The presence of two or more cell lines within a single individual, each of which is derived from the same original cell line, represents mosaicism. When the abnormal cell line is distributed throughout the body along with a normal cell line, a generalized mosaicism is said to be present and the clinical phenotype is often milder than that seen in association with the comparable nonmosaic aneuploidy. In practice, however, the phenotype associated with mosaicism for any particular aneuploidy is quite variable. This clinical variability is likely a function of the percentage of abnormal cells that are present and how they are distributed within the various tissues of the body or pregnancy. Presumably a more severe clinical presentation occurs when the abnormal cell line is present in high concentrations within the central nervous system and/or other critical organs, and a less severe presentation develops if the abnormal cell line represents a minority of the cells in a less critical tissue, such as that of the gonads or the extraembryonic tissues of a pregnancy. For example, individuals with gonadal or germline mosaicism are phenotypically normal but are at high risk for having clinically affected offspring with a nonmosaic form of the chromosome abnormality they carry in their gonads. Additionally, in the 1–2% of pregnancies in which confined placental mosaicism is identified, the outcome for the pregnancy is typically good. However in a small percentage of cases, the chromosomally normal fetus's growth and development appears to be compromised by a poorly functioning mosaic placenta.

In rare cases, the phenotype of a mosaic aneuploidy may also be influenced by the presence of UPD (the inheritance of both chromosome homologues from a single parent) within the seemingly normal diploid cell line. Most commonly, UPD occurs secondary to rescue of a trisomic conception via mitotic nondisjunction or anaphase lag. In this scenario, one chromosome is lost, leaving behind a diploid cell line in which one pair of chromosomes was inherited from a single parent. Assuming normal recombination occurred during meiosis I, uniparental heterodisomy should be present in the resulting UPD cell line. In other words, the two uniparental chromosomes should be genetically different or heterozygous rather than genetically identical or homozygous. More rarely, a monosomy conception is rescued by a mitotic nondisjunction event involving the monosomic chromosome. As a result, two identical copies of a single chromosome are present in a diploid cell and a complete uniparental isodisomy occurs. While the presence of a UPD effect has been well documented in a number of mosaic aneuploid pregnancies involving some of the more lethal trisomies, for example, chromosomes 14 and 15, there appears to be no UPD effect per se associated with rescue of the common trisomies (13, 18, 21, X and Y). The unmasking of a recessive disorder secondary to complete or partial uniparental isodisomy is always a concern, regardless of whether the involved chromosome manifests a UPD effect or not. Chapter 10 discusses UPD in more detail.

In addition to a milder or atypical phenotype, other signs that mosaicism may be present include body asymmetry caused by different growth rates in the normal and abnormal cells, as well as streaks of skin pigmentation referred to as Blaschko's lines. These lines of Blaschko correspond to the growth patterns seen in the skin and are believed to be the result of differential pigmentation in the normal and abnormal cells of a mosaic individual. The pattern is generally linear on the arms and legs, S-shaped on the stomach and V shaped on the back. A suspicion of mosaicism may also be generated by observations made in the cytogenetics laboratory during routine karyotyping. For example, the presence of one or two abnormal cells in an otherwise normal karyotype could increase our suspicion of a mosaicism, especially if the chromosome abnormality seen is consistent with the clinical findings. In any situation with a high suspicion of mosaicism, the patient workup should be altered appropriately to address this issue.

When mosaicism is suspected, an additional 10–30 cells are typically examined during the karyotyping process. However, even with this more extensive analysis, our ability to detect mosaicism using traditional karyotyping is limited. According to E. B. Hook's data, mosaicism affecting 6% or more of the cells can be ruled out with 95% confidence by counting 50 cells [23]. FISH can also be used to rapidly and efficiently examine a large number of both the dividing metaphase cells and the nondividing interphase cells in a patient suspected of having mosaicism for a specific complete or partial aneuploidy. Depending on whether a monosomy or trisomy is being pursued, the number of cells examined, the type of sample being examined, and the probe combination used to address the presence of low level aneuploidy, mosaicism can typically be detected in the 1–10% range using FISH. Chromosome microarray analysis is also capable of detecting mosaicism, but like both traditional G-banding and FISH, there are limitations with respect to the level of mosaicism that can be detected. Based on dilution studies in which a trisomy 21 cell line was mixed with a normal cell line, mosaic trisomy 21 should be easily distinguished

when present in at least 20% of the cells but is unlikely to be identified when present in 10% or fewer of the cells [24]. Similarly, the lowest levels of mosaicism typically detected by chromosome microarray analysis in actual patients with complete or partial aneuploidies appear to be in the 10–20% mosaicism range [24–26]. As with traditional karyotyping, the strength of chromosome microarray analysis lies in its ability to screen the entire genome for mosaicism without any preconceived notions about what chromosome abnormality is present. Chromosome microarray analysis also has the added benefit of being able to screen the entire genome at a higher resolution than that obtained by traditional karyotyping. Regardless of which of the above methodologies is used, mosaicism detection continues to be a challenge. These individuals are however important to identify, because if the abnormal cell line is also present in their gonads (germline or gonadal mosaicism), they are at risk for having nonmosaic aneuploid offspring.

9.1.4 Trisomy

Over the years many trisomic pregnancies and liveborn individuals have been studied, using first, chromosome polymorphisms and later, molecular marker polymorphisms, to detect both the parental origin and the meiotic stage of the abnormal chromosome segregation event responsible (see 9.2.1, Mechanisms of aneuploidy). Based on these studies, the following generalizations can be made regarding the genesis of human trisomies: (1) greater than 80% of chromosome segregation errors occur during oogenesis; (2) the incidence of segregation errors increases significantly with increasing maternal age, but not paternal age; and (3) meiosis I errors are more common than meiosis II errors [7,27–30]. Despite these generalizations, however, some chromosome specific differences have been noted. For example, while triple X pregnancies, as well as those that are trisomic for one of the acrocentric chromosomes, occur preferentially during maternal meiosis I, trisomy 18 occurs preferentially during maternal meiosis II. Additionally, maternal and paternal errors are equally likely to be responsible for an XXY conception, and as expected, given the paternal origin of the Y chromosome, 100% of XYY conceptions involve a male meiosis II nondisjunction event.

The maternal age effect on trisomy risk is quite striking and rises from an approximate risk of 1/20 for a 20-year-old woman to a risk that approximates 1/3 for a woman in her early to mid-40s. Despite the fact that we have been aware of this effect on trisomies since the early 1930s, exactly why the majority of malsegregation events occur in meiosis I of oogenesis and why they seem to become more prevalent with increasing maternal age remains unknown. It seems likely, however, that this is related, at least in part, to the differences between male and female gametogenesis. In contrast to male meiosis, which begins during puberty and once initiated proceeds to completion in an uninterrupted fashion throughout life, female meiosis is a prolonged and discontinuous process that is initiated in the 11 to 12-week-old fetal ovary, but is not completed until many years later. In fact, meiosis I is arrested in late prophase during fetal development and is not reinitiated until the oocyte is ovulated 10–50 years after birth, while meiosis II is only completed if and when the oocyte is fertilized. While it seems likely that there is something about the long period of time that the oocytes spend arrested in meiosis I that promotes nondisjunction, the specific underlying cause(s) has not been delineated.

Despite the fact that the fundamental causes of the maternal age effect remain unknown, a number of possible associations have been identified. For example, mouse and *Drosophila* data suggests that a reduction in the amount and/or efficacy of cohesion proteins may change with age, thus increasing susceptibility to chromosome malsegregation [31,32]. This deterioration of cohesion molecules over time presumably allows the recombinant homologues that have been paired in meiosis I for decades to dissociate prematurely and segregate randomly, thus producing aneuploidies. Similarly, cohesion deficiencies that impact the quality of sister chromatid pairing during meiosis II and mitosis would also be expected to promote premature segregation and aneuploidy. Other age-related changes that have been proposed to affect chromosome segregation include the gradual deterioration of the meiotic spindle apparatus and/or spindle checkpoint proteins, the increasing rate of oocyte attrition, the accumulation of mitochondrial mutations in the granulosa cells that surround and nourish the oocytes, and changes in hormone levels which alter the environment in which the oocytes are maintained and mature [21,33,34]. Clearly, the relationship between maternal age and aneuploidy is a complex one that likely involves multiple factors.

Other than maternal age, aberrant meiotic recombination is the only other important correlate of nondisjunction that has been identified. Numerous studies using both molecular markers and cytological techniques to map the recombination events that have occurred in association with meiosis I-derived trisomies have demonstrated that abnormal recombination is a contributing factor in all human trisomies. For some trisomies both the frequency and location of the recombination events are altered, while for others only the placement of the exchange is changed. For example, when compared to normal diploid conceptions, the exchange events seen in meiosis I-derived trisomy 16 conceptions appear to occur with equal frequency, but they are more telomeric or distally placed. Among the maternally derived sex chromosome trisomies of meiosis I origin, alterations are seen in both the frequency and placement of the exchanges with a preponderance of recombination occurring within the pericentromeric region rather than the telomeric region as described above for chromosome 16 [35]. The story appears to be most complex, however, for chromosome 21. The number of exchanges between chromosome 21 homologues appears to be decreased in trisomy 21 offspring and the recombination sites have a distal location bias when compared to normal diploid

offspring [36]. Interestingly, however, more recent studies have demonstrated that this distal recombination bias appears to only be a factor for the trisomic offspring of mothers less than 29 years of age; abnormal recombination placement is not observed in the offspring of older mothers who were 35 years of age or older [27,37]. Existing data suggest this apparent disparity does not occur because older women segregate chromosome 21 homologues with a single telomeric exchange better than a younger woman, but rather because an older woman with less efficient meiotic machinery does not segregate chromosome 21 homologues well, regardless of whether the exchanges are optimally placed or not. In other words, with age the amount of nondisjunction attributed to abnormal exchange placement remains elevated but unchanged, while that associated with normal exchanges increases, thus constituting a larger proportion of the total nondisjunction events. While the risk of an meiosis I nondisjunction event imposed by an abnormal exchange event between chromosome 21 homologues appears to be similar regardless of maternal age, the same cannot be said for the much rarer meiosis II nondisjunction events, which interestingly also seem to be initiated by abnormal meiosis I recombination. Specifically, trisomy 21 conceptions of meiosis II origin are associated with a prevalence of pericentromeric meiosis I exchange events, and they are found preferentially in older mothers [27]. The apparent lack of a similar association between maternal age and the location of exchange events along the nondisjoined chromosomes for trisomy 18 and the maternally derived sex chromosome trisomies suggests that chromosome 21 may be unique in this respect [38,39].

Recombination not only ensures genetic diversity by mixing the genetic material located on homologous chromosomes, but as described above it is also required for proper chromosome segregation. It is this second role that is important to consider when thinking about the etiology of aneuploidies. Recombination in conjunction with cohesion molecules serves as a means of tethering homologous chromosomes in the proper orientation such that they can segregate in an organized fashion to opposite poles during anaphase I. Because each pair of homologous chromosomes is tethered or linked by the physical structures of recombination, the chiasmata, each univalent moves in a coordinated fashion to separate daughter cells during meiosis I. In the absence of recombination and sufficient cohesion, each chromosome within the homologous pair would instead be free to act independently of the other, random segregation would occur during anaphase, and approximately 50% of the time the chromosomes would segregate abnormally, resulting in aneuploidy. It is therefore not unexpected that altered meiotic recombination events are seen in trisomic offspring.

As described above, the presence of a single recombination event of suboptimal placement (i.e., too close or far from the centromere) is also associated with an increased predisposition to chromosome malsegregation. It is speculated that the telomeric placement of recombination events may in some cases occur secondary to a decrease in the number and/or quality of the cohesion complex which is tasked with maintaining both sister chromatid associations and chiasmata placement prior to anaphase I. The idea here is that the initial recombination event may have been placed in an ideal medial location, but as a result of decreased cohesion, the chiasmata migrates distally to the chromosome ends. The strength of the force holding the recombinant homologous chromosomes together is insufficient in this situation, allowing the chromosomes to separate prematurely and segregate as independent univalents during anaphase I. Similar to distally placed recombination events, those that are proximally placed have also been proposed to disrupt the cohesion complex, and thus also the sister chromatid associations required for normal chromosome segregation. Alternatively, these pericentromeric recombination events may cause entanglement of the paired homologues such that they fail to separate during anaphase I, and separation instead occurs in anaphase II [40–42].

While maternal age and aberrant meiotic recombination are the only two factors that have clearly been shown to be associated with trisomy formation, the environment is also likely to play a role. It has been difficult, however, to identify predisposing environmental factors for several reasons: (1) the enormity of maternal age effect interferes with ability to detect other more subtle factors; (2) given the long period of time required for female meiosis, it is difficult to assign cause and effect correlations; and (3) there are likely to be multiple nondisjunction mechanisms, and each may respond differently to various predisposing factors. Recently, exposure to bisphenol A (BPA), an estrogenic compound found in many plastics, has been associated with an increased incidence of aneuploidy in mice [43]. Exposure of pregnant female mice to this chemical appears to increase the incidence of aneuploid eggs in their female offspring, but not the pregnant mother herself; this chemical is therefore said to have a grandmaternal effect. More recent studies suggest that the impact of BPA on chromosome segregation is influenced by diet, specifically the concentration of dietary estrogens [44]. Whether human oocytes are similarly affected by BPA and dietary estrogens is not currently known. Other environmental factors, such as periconceptional smoking, especially in association with oral contraceptive use; parity; low social economic status; and exposure to alcohol, irradiation, drugs, or occupational chemicals, have also been implicated as risk factors for aneuploidy [41,45].

Recently, Hultén et al. [46,47], have published data that challenge the current dogma that disomic gametes are typically caused by meiotic nondisjunction events. Their data suggest that trisomy 21 mosaicism in the ovaries of normal female fetuses may instead be responsible. Specifically all eight of the normal fetal ovary samples examined using chromosome 21-specific FISH probes appeared to contain a trisomy 21 cell line in an average of 0.54% of their cells. Additionally the

authors propose that with increasing age, due to both growth and apoptosis differences between the normal and trisomic cell lines, the trisomic cells gradually comprise a larger portion of the ovarian reserve, thus explaining the maternal age effect seen in association with trisomies. Clearly, additional studies will be required to confirm these trisomy 21 results and to address whether maternal gonadal mosaicism is a major causative factor for other trisomies.

In contrast to the plethora of studies that have focused on maternally derived trisomies, only a handful of studies have examined the factors associated with paternal nondisjunction. This disparity is due to the low rate of chromosome segregation errors in spermatogenesis relative to oogenesis and, therefore, a correspondingly lower number of cases for study. Thus far, only chromosome 21 nondisjunction events have been studied in significant numbers, and at this time no paternal age effect or statistically significant changes in recombination patterns have been identified.

9.1.5 Monosomy

While the majority of studies have focused on the origin of trisomies, some data regarding the origin of monosomy X, the only survivable monosomy, is also available. Because the chromosome that failed to undergo proper segregation is missing in these individuals, it is not possible to determine whether the segregation error occurred in meiosis I or II, but one can establish the parent of origin. Examination of over 100 nonmosaic 45,X cases has demonstrated that it is the paternal X chromosome that is lost in 70–80% of these cases [48]. Random post-fertilization loss of one of the two sex chromosomes in normal 46,XX or 46,XY conceptions is thought to best explain the prevalence of 45,X cases in which the maternally derived X chromosome has been maintained. Among the 46,XX conceptions, random loss of the X chromosome would result in equal retention of the male versus the female X chromosome, while random loss of the sex chromosomes in a 46,XY embryo would result in all of the surviving embryos having a maternally derived X chromosome. In other words, because of the lethality associated with a 45,Y karyotype, indiscriminate loss of either sex chromosome would automatically generate more 45,X embryos with a maternal rather than a paternal X.

A mitotic or post fertilization origin for monosomy X conceptions would also explain the discrepancy between the high degree of lethality associated with this karyotype and the relatively mild features that are noted in most liveborn Turner syndrome females. Presumably, those 45,X conceptions that occurred secondary to a post fertilization chromosome segregation error are mosaic for a normal cell line and are therefore more mildly affected and more likely to survive to birth. In contrast, those that were derived from meiotic errors and carry a nonmosaic 45,X karyotype do not survive and represent those pregnancies that are miscarried during early development. Since only approximately 50% of Turner syndrome patients are documented mosaics with both a 45,X cell line and either a normal male or female cell line, or one containing a structurally abnormal sex chromosome, it is assumed that in many cases the mosaicism is cryptic and in some cases may have been confined to extraembryonic membranes [10].

That the random mitotic errors described above are responsible for the majority of 45,X conceptions, rather than meiotic errors, is also suggested by their prevalence relative to the sex chromosome trisomies. The argument here is that if meiotic segregation errors were largely responsible for the generation of monosomy X karyotypes, then the reciprocal trisomies, XXX, XXY and XYY should be seen with an equally high frequency. These trisomies are, however, seen much less frequently than monosomy X conceptions; 45,X conceptions represent approximately 1–2% of all clinically recognized pregnancies, while the combined frequency of the three sex chromosome trisomies is approximately 0.2% [22,49]. In those cases that do arise meiotically, it has been proposed that the predominant loss of the male sex chromosome may be partially attributed to the reduced pairing observed between the X and Y chromosome; pairing between these chromosomes is confined to the pseudoautosomal regions located on the distal short and long arms of both of the sex chromosomes.

In addition to patients with Turner syndrome, sex chromosome loss and less frequently gain is also observed in the blood cultures of older women, while loss of the Y chromosome is seen in the blood and more commonly the bone marrow cultures of elderly men [50–52]. This sex chromosome aneuploidy is thought to be a normal consequence of aging in both sexes, and in women it is the inactive X chromosome that is preferentially lost [53,54]. Because this aging effect appears to be confined to the blood cells, if there is a strong suspicion that true mosaicism rather than an age-related aneuploidy may be present in a patient, a separate tissue can be obtained for karyotyping and/or fluorescence *in situ* hybridization (FISH testing).

9.1.6 Euploidy

A normal diploid karyotype contains 46 chromosomes, including two copies of each of the 22 autosomes and a pair of sex chromosomes. Relative to a diploid karyotype, euploid karyotypes contain gains or losses of an entire haploid set of 23. As described in the introduction, a *haploid* or *near-haploid karyotype* is a rare cytogenetic finding in humans and is seen only in our gametes and in association with particular cancers. In comparison, polyploid karyotypes are more common. Karyotypes

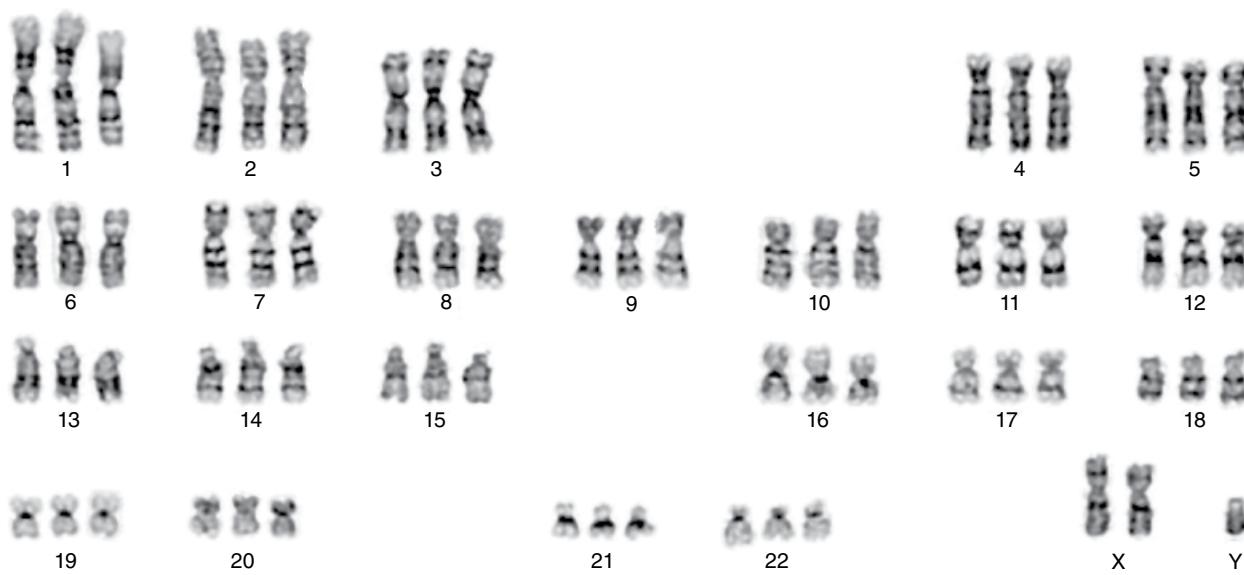


Figure 9.9 A triploid karyogram.

containing one extra haploid chromosome set, or a total of 69 chromosomes, are said to be triploid while tetraploid karyotypes contain 96 chromosomes, secondary to the presence of two extra haploid sets. As you might expect, the presence of an entire extra set or two of chromosomes results in abnormal growth and development; these karyotypes are only rarely seen in liveborn children. They are, however, frequently seen in products of conception samples of spontaneously aborted pregnancies; triploidy is estimated to occur in 6–7% and tetraploidy in 1–2% of these pregnancies [8]. The events that produce polyploid pregnancies are typically thought to represent sporadic events with no significant increased risk of recurrence [55,56].

9.1.7 Triploidy

Triploid karyotypes contain three haploid sets of chromosomes for a total of 69 chromosomes (Figure 9.9). The sex chromosome complement is most often XXY or XXX, and only rarely XYY. In two studies performed on spontaneous abortuses, the ratio of XXY : XXX : XYY triploids was reported to be 92 : 82 : 2 and 36 : 26 : 1 [57,58]. Additional studies, employing either cytogenetic heteromorphisms or DNA polymorphisms, have established that most triploids contain an extra paternal haploid set of chromosomes [59,60]. These diandric triploids are most frequently formed when a single haploid egg is fertilized by two haploid sperm (dispermy) and less frequently secondary to fertilization with a single diploid sperm that has failed to undergo normal meiosis. The rarer digynic triploids contain an extra maternal haploid set of chromosomes and occur most often when a diploid egg undergoes normal fertilization. In this situation, typically either a meiotic nondisjunction event involving the entire set of chromosomes occurs, or instead, the oocyte fails to undergo cytoplasmic division (cytokinesis) despite normal meiotic chromosome segregation. Other more rare mechanisms proposed to yield digynic triploids include retention of the haploid second polar body by the secondary oocyte or egg, fusion of two haploid secondary oocytes, or fertilization of an ovulated primary oocyte [61].

Because the ratio of maternal to paternal genes is critical to normal development of both the embryonic and extraembryonic tissues, the phenotypic consequences of a triploid karyotype depend on the source of the extra haploid set of chromosomes. For diandric triploids, fetal growth is either relatively normal or proportionally stunted, and microcephaly or normal head growth is present. Additionally, the placenta is large and is usually classified as a partial mole because it contains a mixture of hydropic (swollen) and normal appearing villi. In contrast, both the fetus and the placenta of digynic triploids are small, macrocephaly rather than microcephaly is typically present, and the placenta does not show partial mole changes. Features shared by triploids of both types include syndactyly of the fingers and toes, cardiac defects, brain anomalies, hypotonia, and cranial facial abnormalities. Genital anomalies are common in males.

As mentioned above, triploid conceptions typically die in utero or shortly after birth, although a few rare cases of survival for several months have been reported [62]. Interestingly, survival differences have been noted between diandric and digynic

triploids [59]. Diandric triploids are typically aborted at roughly 12 weeks of gestation, while digynic triploids are normally lost either a few weeks earlier at around 10 weeks, or they survive into the mid-second and third trimesters. This differential survival explains some of the inconsistencies noted in the early literature about the percentage of aborted triploid fetuses that are diandric versus digynic. The ratio of diandric to digynic triploids would, for example, be higher in pregnancies that were spontaneously aborted at approximately 12 weeks in comparison to those that survive well into the second trimester.

The longer survival noted in digynic triploids is linked to the fact that it is the male rather than the female centriole (see Chapter 1, The cell and cell division) that replicates during mitosis [63]. In a digynic triploid, the appropriate single male centrosome is present which then replicates to form the bipolar mitotic spindle apparatus necessary for normal and equal chromosome segregation. Because diandric triploids formed secondary to dispermy contain two male centrosomes, a tripolar rather than a bipolar spindle is often produced. In the presence of three poles, chromosome segregation becomes disorganized and mosaicism for multiple aneuploidies occurs. Presumably these chaotic aneuploid embryos are even less viable than an embryo with a standard triploid karyotype.

9.1.8 Mosaic triploidy

The first patient with mosaicism for both a normal diploid cell line and a triploid cell line was described in 1960 by Book et al. [64]. Since this time, at least 25 liveborn cases of mosaic triploidy or diploid/triploid mixoploidy have been reported [65]. Like other types of mosaic individuals, these patients are typically less severely affected than their nonmosaic counterparts, and body asymmetry as well as streaky skin pigmentation (lines of Blascho) has been reported. The actual clinical presentation of any one patient will, however, ultimately depend on the distribution and percentage of abnormal cells. Although the triploid cell line has been detected in both lymphocytes and fibroblasts, the lymphocyte karyotype appears to be normal in approximately 75% of these mosaics. A skin biopsy for fibroblast karyotyping would therefore be recommended for any patient suspected of being a mosaic triploid.

Unlike mosaic aneuploidies, triploidy mosaicism cannot be explained by mitotic nondisjunction. Instead it seems to occur secondary to an abnormal fusion event between a diploid blastomere and either a haploid second polar body or a haploid sperm. For mosaics with a paternally derived triploid cell line, normal fertilization and cell division presumably occur to produce two or more daughter cells or blastomeres. This is then followed by a second fertilization event in which one of the blastomeres fuses with a second sperm to produce the diandric triploid cell line. For maternally derived mosaics, the triploid cell line is formed by an abnormal fusion event involving the second polar body and a blastomere. One instance of chimerism is reported to have occurred secondary to fusion of two separate zygotes or fertilized egg cells, one with a normal diploid karyotype and the other with a triploid karyotype. This mechanism is, however, believed to be very rare since it involves two rare abnormal events, both the formation of a triploid zygote and then the fusion of two independent zygotes.

9.1.9 Tetraploidy

Tetraploid karyotypes contain 92 chromosomes or twice the usual number of chromosomes (Figure 9.10). Complete tetraploidy is more rarely diagnosed than triploidy, and not surprisingly, is rare among liveborn individuals. In the handful of tetraploids that have been examined thus far for mechanism of formation, the majority have had either an XXYY or an XXXX sex chromosome complement, suggesting that mitotic errors that result in a doubling of the chromosomes may usually be responsible. Normal fertilization and zygote formation is believed to occur, but the cell then fails to undergo cytokinesis, the process late in mitosis during which the cytoplasm of a single cell is divided to form two daughter cells. As a result of failed cytoplasmic cleavage, a single cell with a tetraploid chromosome complement is produced rather than two diploid daughter cells. This single tetraploid cell then goes on to divide normally and a nonmosaic tetraploidy results. Mosaicism for both a diploid and a tetraploid cell, also known as diploid/tetraploid mixoploidy, occurs when the failed cytokinesis event occurs sometime after the first cell division. Rarely, tetraploid preimplantation embryos have been observed with an XXXY or XYYY sex chromosome complement, suggesting that other modes of tetraploidy formation occur. Some of the more common scenarios proposed to explain these rare tetraploids include (1) fertilization of a normal haploid egg with three sperm (trispermy), (2) fertilization of a normal haploid egg with two sperm, one of which is haploid and the other diploid, (3) dispermic fertilization of a diploid egg, or (4) fusion of two separate fertilized eggs [66,67].

At least 14 cases of apparent nonmosaic liveborn individuals with tetraploidy have been examined [68,69]. The more common features associated with this chromosome abnormality include intellectual disability, poor growth, hypotonia; structural abnormalities of the hands, feet and limbs; cardiac and urinary tract abnormalities; and craniofacial anomalies. Early death, usually within the first year of life, is typical. Individuals with diploid/tetraploid mosaicism typically share similar features with their nonmosaic counterparts, but are less severely affected. These mosaic individuals can be difficult to identify, because the tetraploid cell line is not always detected in lymphocytes, and there is some data to suggest that the number of tetraploid cells

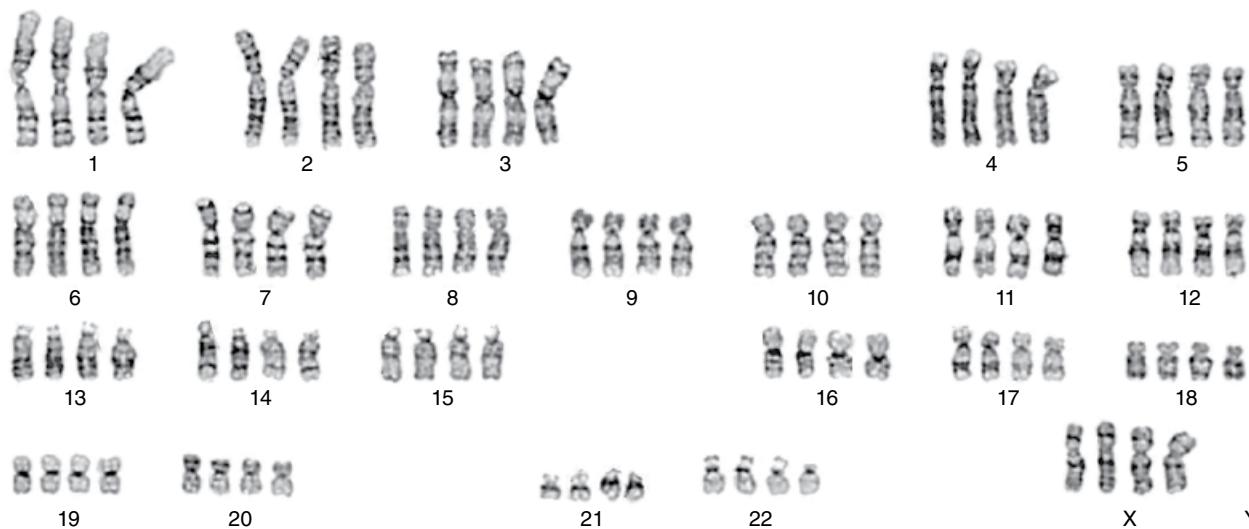


Figure 9.10 A tetraploid karyogram.

decreases in the peripheral blood as a patient ages [70,71]. If possible, fibroblast cells should therefore be karyotyped from any individual for whom tetraploidy is suspected. Mosaicism for a tetraploid cell line can also be difficult to identify during prenatal diagnosis because tetraploid cells are frequently observed in both amniotic fluid and chorionic villus samples [72,73]. Tetraploid cells are therefore typically considered clinically insignificant in these cultures unless they are present at high concentrations and in multiple independent cultures. In this situation, the recommendation is that a high-resolution ultrasound examination be performed to look for features associated with diploid/tetraploid mosaicism.

9.2 Structural rearrangements

In contrast to the limited number of numerical chromosome abnormalities observed in the human genome, the number of potential structural abnormalities is vast. Not only are there multiple types of genomic rearrangements, including duplications, deletions, rings, inversions, insertions, and translocations (Figure 9.11), the breakage and reunion events that yield these structural rearrangements can theoretically occur at any site within the 24 human chromosomes. When examining a sample from a patient with a suspected constitutional abnormality (a mutation present at or before birth), a minimum of 15 colonies or 20 cells are counted to look for numerical abnormalities and at least five cells are analyzed band-by-band to identify structural abnormalities. Changes in the Giemsa- or G-banding pattern, the position of the centromere, and/or alterations in the short (p) to long (q) arm ratio are the indicators used to identify and characterize a structural rearrangement. When these indicators are missing, a rearrangement is said to be cryptic, or submicroscopic, and molecular techniques such as multiplex fluorescence *in situ* hybridization (M-FISH) and/or chromosome microarray analysis are needed for their identification.

As you might expect, numerical abnormalities have traditionally been found more frequently than cytogenetically visible structural abnormalities. Numerical abnormalities are estimated to occur in approximately 5–7.5% of recognized conceptions and 0.3% of live births while structural abnormalities are seen in approximately 0.5% and 0.25% conceptions and live births, respectively [7,74]. The higher incidence of reported numerical abnormalities likely reflects the fact that it is easier to detect the loss or gain of entire chromosomes than to identify structural changes, especially subtle ones. Furthermore, individuals with losses and/or gains of entire chromosomes are more likely to be karyotyped because they have well-characterized, recognizable phenotypes, and are often severely affected. In contrast, individuals with unbalanced structural abnormalities have only partial monosomies and/or trisomies that may affect small chromosome segments. These individuals may therefore be less severely affected than those with full monosomies or trisomies. Moreover, because the majority of structural rearrangements are unique to a particular individual or family, the phenotypes associated with them are often unique and uncharacterized. Those with truly balanced rearrangements and therefore no net gain or loss of critical genetic material, would be even less likely to be karyotyped than those with unbalanced structural rearrangements, because they would be expected to be phenotypically normal. When these individuals are karyotyped, however, it is typically because of a suggestive reproductive or family

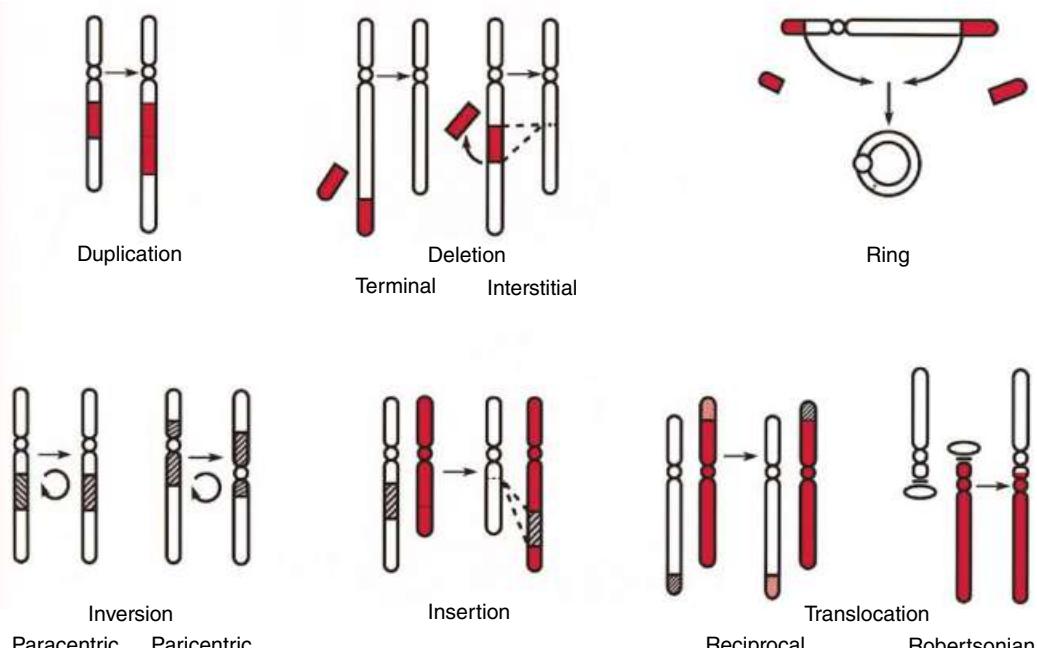


Figure 9.11 The common structural rearrangements observed in human karyotypes include duplications, terminal and interstitial deletions, rings, paracentric and pericentric inversions, insertions and both reciprocal and Robertsonian translocations. See text for details about each of these structural rearrangements.

history. As the use of chromosome microarray analysis becomes more common in cytogenetic and molecular laboratories, many of the subtle structural abnormalities that were previously missed by G-band analysis will be readily identified. As a result, we may find that structural abnormalities actually represent the majority rather than the minority of chromosome rearrangements in man.

A cytogeneticist's first job is to determine whether a clinically relevant chromosome abnormality is present in a particular patient. As discussed above, numerical abnormalities are more easily identified than structural abnormalities, especially very subtle ones. When changes in the G-band pattern, the arm ratio or the centromere position are altered, the initial question asked is whether this change(s) represents normal chromosomal variation or a relevant change with clinical and/or reproductive consequences. This distinction can be difficult when the change has occurred in a region that demonstrates a large degree of banding variation. Some regions of the human genome, such as the short arms and satellites of the five pairs of acrocentric chromosomes (13, 14, 15, 21, and 22), the pericentromeric heterochromatic regions within chromosomes 1, 9, and 16, and the heterochromatic region within the distal long arm of the Y chromosome, all demonstrate size and G-band pattern variations that make these regions difficult to scrutinize for clinically relevant changes. It can also be difficult to detect subtle structural chromosome abnormalities within the sex chromosomes because of the absence of a homologous chromosome for banding comparisons. In females, the active and inactive X chromosomes often have a slightly different appearance because the inactive one is more condensed, making band-by-band staining comparisons more difficult than for homologous autosomes. Hemizygous males completely lack a homologous sex chromosome to serve as an internal control for banding within each cell.

Once a structural rearrangement is detected, the next step is to determine its potential clinical significance, which at times may be difficult. Although individuals who carry clearly unbalanced rearrangements are generally phenotypically abnormal, there are exceptions. Gardner and Sutherland list a number of identified chromosome duplications and deletions involving euchromatic material that do not appear to be associated with clearly abnormal phenotypes [75]. The benign nature of these duplications and deletions has been inferred from the observation that the same apparent rearrangement was inherited from a clinically normal parent. Assuming that the normal parent truly carries the same unbalanced rearrangement as the child, rather than a partially cryptic balanced rearrangement, and the region in question is not imprinted, the most likely explanation for the absence of an abnormal phenotype would be the absence of critical dosage sensitive genes in the involved regions. It can also be difficult to make accurate phenotypic and reproductive predictions about cytogenetically "balanced" rearrangements with no apparent loss or gain of genetic material, especially if they represent new (*de novo*) mutations. Given the 3–5 megabase

(Mb) (3–5 million base pairs) resolution of traditional G-banded karyotyping, a rearrangement that appears to be balanced may, in fact, contain a large loss and/or gain of genetic material at the molecular level. Alternatively, the rearrangement may be truly balanced, but a critical gene(s) has been interrupted secondary to the chromosome breakage events involved. Given the existence of these cytogenetically balanced but molecularly unbalanced rearrangements, as well as the benign duplications and deletions involving euchromatic regions described above, it is important to evaluate parental chromosomes before definitively assigning clinical significance to a small, unique rearrangement. In addition to being helpful for assessing the clinical significance of a unique chromosome rearrangement, parental chromosome analysis should also be pursued so that accurate information about recurrence risks can be conveyed. In the case of a de novo structural rearrangement, the parents would be given a low recurrence risk, usually less than 1%. If instead a parent is found to be a balanced rearrangement carrier, the risk for having unbalanced offspring would typically vary from less than 1% to approximately 50%, depending on the type of rearrangement and the chromosome regions involved. In the rare case of a balanced homologous Robertsonian translocation carrier, for example, a Robertsonian 21;21 translocation carrier, the risk for an abnormal liveborn child would approach 100%. In a few very rare cases, the translocation trisomy pregnancy of such a carrier has been corrected by post-zygotic loss of the free homologue [76]. Since this correction event produces a balanced cell line with UPD, only those pregnancies involving homologous Robertsonian translocations for chromosomes that have no UPD effect (i.e., chromosomes 13, 21, and 22) will result in a potentially normal pregnancy. Correction of a trisomy occurring secondary to a Robertsonian 14;14 or 15;15 translocation would yield offspring with a UPD syndrome.

9.2.1 Mechanism for structural rearrangements

In contrast to full trisomies, which are typically maternal in origin, it has been estimated that 75–80% of large classical structural rearrangements and virtually all of the very complex rearrangements are paternal in origin [77–79]. The preponderance of paternally derived structural rearrangements is thought to reflect the numerous mitotic divisions involved in the continuous production of sperm from puberty until death, compared to the limited number of mitoses in females; it is estimated that the average male undergoes several hundred mitotic events, each with the potential to accumulate a mutation, while females undergo only 20–30 events [80]. Although structural rearrangements as a group are more likely to be paternal in origin, there are some exceptions. For example, approximately 90% of homologous Robertsonian translocations and 80% of terminal chromosome 1 deletions are maternal in origin, while many of the microdeletion syndromes are equally likely to be of maternal or paternal origin [81,82].

Recombination events involving the same position or locus on a pair of sister chromatids or homologous chromosomes are said to be “allelic.” This type of recombination is a normal and necessary occurrence in both somatic and germ cells. Allelic recombination is required for some repair processes and, during meiosis, for normal chromosome segregation, and to ensure mixing of the genome such that no two gametes have the same genetic makeup. In contrast to the positive roles attributed to allelic recombination, nonallelic recombination produces both balanced and unbalanced structural rearrangements. While in theory nonallelic recombination events can occur at any point within the genome, certain regions of the genome appear to be more susceptible to breakage and rearrangement than others. Recent evidence gathered by looking at the breakpoints regions for multiple recurring and nonrecurring rearrangements suggests that the presence of DNA sequences that are repeated elsewhere in the genome (e.g., low copy repeat (LCR), high copy number repeat sequences like *Arthrobacter luteus* (Alu), and long interspersed elements (LINE) sequences) fragile sites such as topoisomerase II or DNase I hypersensitive sites, scaffold attachment regions (SARs) and/or a particular secondary DNA structure, increase the likelihood of a structural rearrangement. The degree of physical proximity between two genetic loci involved in a rearrangement also appears to impact the likelihood of a nonallelic recombination event [83]. Sequence homology and close proximity are presumed to encourage pairing between nonallelic regions, while the fragile sites and secondary structures present at particular sites promote the chromosome breakage and replication fork stalling events needed to initiate the formation of structural rearrangements.

The rearrangement mechanism that is currently best understood is referred to as nonallelic homologous recombination (NAHR) [84]. As the name implies, these types of rearrangements involve recombination events between regions of shared homology that are located at different sites within the genome. Presumably the process is initiated by a double-stranded break and then healed secondary to alignment between homologous nonallelic sequences and subsequent recombination. Although high copy number repeat sequences, like Alu sequences, have been reported to mediate NAHR, LCR sequences appear to be responsible for the majority of these events [85,86]. The LCR sequences identified thus far are typically 10–500 kilobases in size, share greater than 97% sequence identity and, while they are distributed throughout the genome, are enriched in the pericentromeric and subtelomeric regions [87].

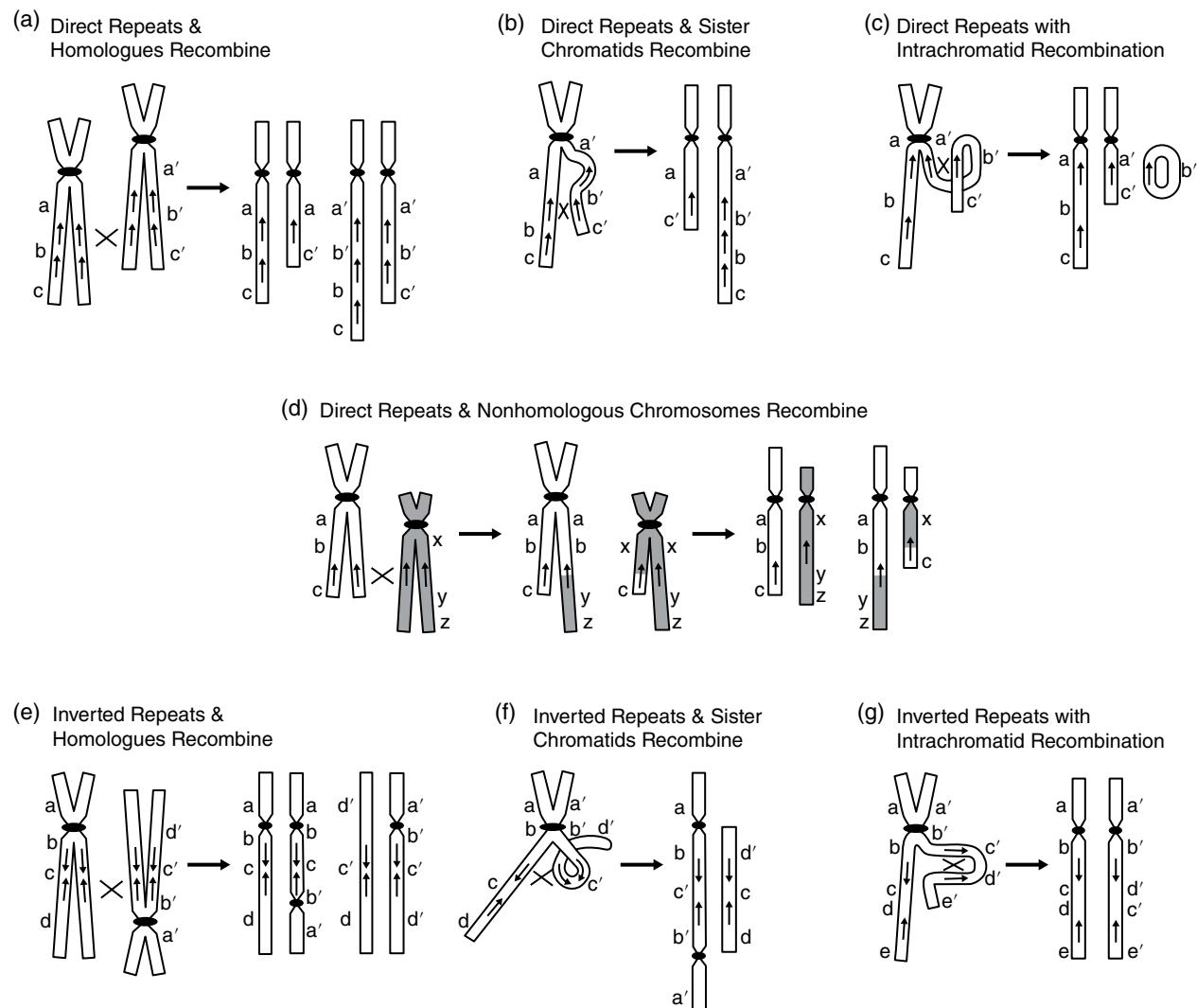


Figure 9.12 Nonallelic homologous recombination (NAHR; denoted by “X”) can produce a variety of structural abnormalities, depending on the location and orientation of the repeat sequences (arrows) that mediate these events. NAHR events involving homologous chromosomes or sister chromatids that are mediated by repeat sequences positioned in a direct (head-to-tail) orientation can produce complementary duplications and deletions (a and b). Complementary linear and ring chromosomes, both of which contain partial deletions, are produced by NAHR events between direct repeat sequences located on the same chromatid (c). Additionally, direct repeats located on different chromosomes can mediate translocations (d) as well as other rearrangements. In contrast NAHR events between inverted (head-to-head or tail-to-tail) repeats located on homologous chromosomes (e), nonhomologous chromosomes (not shown) and sister chromatids (f), can produce complementary dicentric and acentric chromosomes. When NAHR occurs between inverted repeat sequences located within a single chromatid, inversions can occur (g).

The type of rearrangement that results from NAHR will depend on the location and orientation of the repeat sequences mediating the recombination event (Figure 9.12). Direct (same orientation) LCR sequences, when located on the same chromosome, can mediate complementary deletions and duplications. This type of recombination event is also referred to as “unequal crossing over” and is said to be “intrachromosomal” when two sister chromatids are involved and “interchromosomal” when two different homologous chromosomes are involved (Figures 9.12a,b). Deletion and ring chromosomes, but not duplication chromosomes, are formed secondary to nonallelic recombination events involving direct LCR sequences within the same sister chromatid (intrachromatid; Figure 9.12c). Depending on whether the intrachromatid breakpoints involve both chromosome arms or are confined to a single arm, the resulting linear deletion chromosome or the ring chromosome,

respectively, will be acentric. Nonallelic intrachromatid recombination events involving inverted (opposite orientation) LCR sequences result in the formation of an inversion (Figure 9.12g), while comparable interchromatid events can produce dicentric and acentric chromosomes (Figure 9.12f). Translocations and other more complex rearrangements can occur when NAHR events are mediated by LCR sequences located on different homologous or nonhomologous chromosomes (Figures 9.12d,e). Recombination events involving repeat sequences that are separated by large spans of DNA or located on different chromosomes can, of course, result in large, often visible rearrangements involving multiple genes and potentially large imbalances. Included in this group would be the recurring microdeletion and microduplication syndromes, the recurring 4;8 translocation, and many nonrecurring rearrangements [88–90]. Conversely, recombination events involving repetitive sequences separated by shorter distances will result in smaller, sometimes single, gene rearrangements. Hemophilia A, for example, represents a single gene disorder that is often caused by one of two recurring inversions which disrupt the factor VIII gene. These inversions are mediated by inverted LCR sequences located approximately 500 kb apart on the same sister chromatid; the LCR positioned within exon 22 of the factor VIII gene recombines with one of two LCRs located upstream of this gene [91].

Recent evidence suggests that at least some of these LCR mediated NAHR events are facilitated by heterozygosity for overlying submicroscopic inversions [92,93]. Inversion heterozygosity has thus far been documented in the transmitting parent of some children with Williams, Angelman, Sotos and deletion 17q21.31 syndrome. Inversion heterozygosity has also been seen in 100% of the transmitting mothers examined thus far who had a child with the recurring 4;8 translocation [t(4;8)(p16;p23)] and either the recurring inverted duplicated deleted 8p [der(8)del(8)(p23.1)dup(8)(p23.1p11.2)], or its reciprocal product as a supernumerary chromosome [+inv dup(8)(p23.1pter)] [88,94,95]. In the case of the recurring 17q21.31 deletion, the inversion converts an indirect LCR to a direct one that is then able to mediate the NAHR event between the normal and inverted chromosome 17 homologues responsible for the deletion [93]. Presumably, the inversions noted in association with the other recurring rearrangements mentioned above also promote NAHR by improving in some way upon the involved LCR substrates.

While NAHR has been implicated in many recurring and some nonrecurring structural rearrangements, the majority of nonrecurring rearrangements and a few recurring ones appear to occur secondary to breakage and reunion between nonallelic regions that do not share large regions of homology. This mechanism of producing chromosome rearrangements is referred to as nonhomologous end joining (NHEJ) [84,96,97]. Our current knowledge about this mechanism is based largely on information obtained from examination of the breakpoints involved in the recurring 11;22 translocation (see 9.2.10, Reciprocal translocations and Table 9.1). The breakpoints within both partner chromosomes contain palindromic sequences which are inverted complementary regions of DNA (e.g., TAATCG-CGATTA) that are capable of forming secondary structures, such as hairpins or stem-loops. It is hypothesized that these secondary structures, which form following disruption or melting of the double-stranded DNA, are susceptible to nucleases that produce nicks near the center of the palindrome or hairpin. These nicked DNA structures then serve as substrates for additional nucleases that are responsible for the production of double-stranded breaks and further deletion within the palindromic sequence. These double-stranded breaks then serve as substrates for a repair mechanism that carries out NHEJ. While palindromic sequences have now been observed at the breakpoint regions of several recurring and nonrecurring rearrangements, their presence does not appear to be required for NHEJ to occur. In some cases, topoisomerase II cleavage sites, DNaseI sensitive sites, and scaffold attachment regions (SARs), all of which are predicted to form chromatin structures that are vulnerable to cleavage and the formation of double-stranded breaks (or other damage), have been mapped to the breakpoints [83]. The double-stranded breaks at each of these sites are also repaired by NHEJ in a fashion similar to that predicted for breaks occurring within the structures formed by palindromic sequences.

Recently, evidence of a third mechanism for generating nonrecurrent chromosome rearrangements has emerged, which involves DNA replication errors rather than NAHR or NHEJ. The “Fork Stalling and Template Switching (FoSTeS) model” has been proposed to explain the complex duplication and deletion rearrangements associated with Pelizaeus–Merzbacher disease and other nonrecurring rearrangements [84,98,99] (Table 9.1). This model proposes that complex and/or unusual genomic architecture causes the replication machinery to stall, dissociate from the strand that is currently being replicated and invade another region within the same fork or a completely different active replication fork. Replication would then proceed from this second replication region, and potentially subsequent replication regions, until eventually returning to the original replication fork, where replication would again proceed normally. Because the invaded replication forks are predicted to be in physical proximity, but separated by variable and possibly sizable linear distances, duplications and/or deletions result. The orientation of each duplicated segment would depend upon whether the leading or lagging strand is invaded and which direction both the invading and invaded forks are progressing. Because each replication fork moves bidirectionally and replication always progresses in a 5' to 3' direction, invasion of the leading strand moving in one direction would produce a direct duplication, while invasion of the leading strand moving in the opposite direction would produce an inverted duplication. Likewise, the directionality of the invaded lagging strand would dictate whether a direct or an inverted duplication was produced.

Both NHEJ and replicative mechanisms such as FoSTeS have recently been identified to play a role in chromothripsis [100–103]. The word chromothripsis is derived from the Greek words “chromos” (for chromosome), and “thripsy” (for shattering into pieces) and is used to describe a particular type of complex chromosome rearrangement. These very complex rearrangements occur secondary to imperfect repair following a single, localized, catastrophic event, that is confined to a single chromosome, chromosome arm, or in rare cases a few chromosomes. Interestingly, in constitutional cases chromothripsis often results in a relatively balanced state despite the presence of multiple DNA breaks. This is likely due to strong selection against large imbalances. NHEJ appears to be responsible for the majority of constitutional chromothripsis events identified thus far. In these cases, a clustered chromosome region(s) appears to be shattered into multiple pieces that are then randomly reassembled using NHEJ to form a new, highly rearranged, chromosome(s) with deletions, inversions and/or translocations, but generally no copy number gains. In other cases of constitutional chromothripsis a replicative repair mechanism such as FoSTeS appears to be involved. Presumably one or more replication forks within a specific chromosome region(s) collapse and multiple template switching events occur to form a highly rearranged chromosome(s). Because it is possible through template switching to replicate a particular region of the genome more than once, copy number gains can be seen in the complex chromosome rearrangements formed by this mechanism.

9.2.2 Deletions

Some of the earliest identified structural chromosome rearrangements involved large deletions, many of which came to represent some of the classical deletion syndromes recognized by the cytogenetics community including cri-du-chat (5p–), Wolf-Hirschhorn (4p–), and 18p– syndrome (Table 9.1). Given the improvements in banding techniques over the years, much smaller deletions can now be detected. The deletions currently detected by high resolution cytogenetic banding are typically ~3–5 Mb or larger in size. Much smaller deletions, in fact those involving single genes or portions of genes, are now identified using various molecular techniques, such as chromosome microarray analysis or FISH. Deletions produce monosomies that typically result in significant pathology. This pathology is characteristically explained by the presence of only half the normal level of one or more of the gene product(s) encoded within the deleted region (haploinsufficiency). In situations involving sex chromosome deletions in males, complete absence of the deleted gene products rather than just haploinsufficiency would be expected. Complete absence of a gene product in a deletion patient could also occur secondary to unmasking of an autosomal recessive disease allele on the undeleted chromosome. In this situation, the affected patient would be missing the gene in question from the deleted chromosome and contain a mutated copy of the gene on the homologous undeleted chromosome. In other deletion situations, an abnormal phenotype may be due to altered expression levels of a gene or group of genes that remain on the deleted chromosome secondary to position effects; when a gene is placed in a new chromosomal environment, its expression may be altered by its new surroundings.

In theory, the larger the deletion, the greater the number of genes affected and the more severe the resulting phenotype. However, the function or critical nature of the gene(s) within the deleted segment influences phenotype, as well. This is illustrated by the presence of benign deletions, some of which appear to involve significant amounts of euchromatic (expressed) DNA. Included among the list of apparently benign deletions reported by Gardner and Sutherland are those involving the 3p25.3-pter, 5p14, 11p12, 13q21, and 16q21 regions, among others [104]. Each of these presumed benign deletion regions are composed primarily of G-dark material, which is known to carry fewer genes relative to the G-light bands. Given the absence of a clinically recognizable phenotype associated with these deletions, it seems likely that the few gene products that are encoded by these regions are present in excess such that a partial reduction does not obviously affect normal growth and development.

Cytogeneticists classify deletions into one of two types (Figure 9.11). When a deletion occurs secondary to a single breakpoint that removes all of the material distal, or telomeric, to that breakpoint, the deletion is said to be “terminal.” An example of a terminal deletion involving the entire short arm of chromosome 18 that results in 18p– syndrome is shown in Figure 9.13 (see Table 9.1). A deletion is referred to as “interstitial” if it formed secondary to two intrachromosomal breakpoints, with loss of the intervening segment and preservation of the distal chromosome arms. While some deletions can be clearly identified as interstitial, such as the chromosome 13 long arm deletion shown in Figure 9.14, others with very distal breakpoints can be difficult to classify. In fact, molecular studies suggest that 7–25% of apparent terminal deletions actually represent interstitial deletions [105–107].

The microdeletion syndromes represent a well-characterized category of interstitial deletions. These deletions are generally small, approximately 1.5–4 Mb in size, and because they recur in the population, they are associated with recognizable phenotypes (Table 9.1). Given that many of these microdeletions are difficult to detect using traditional G-banded karyotyping, cytogeneticists have relied heavily on the clinicians to identify these patients, so that the relevant chromosome regions could be closely scrutinized using high resolution banding and/or FISH. Examination of the breakpoints involved in these recurring deletions has demonstrated that they occur primarily via NAHR, mediated by LCR sequences on sister chromatids or

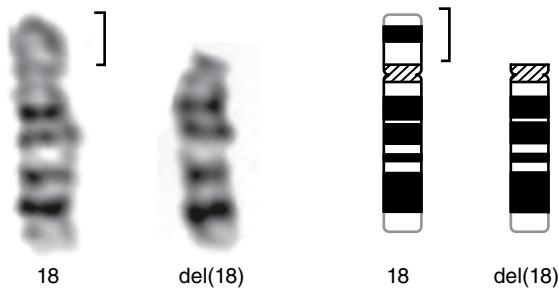


Figure 9.13 A terminal deletion involving most, if not all, of the long arm of one chromosome 18 homologue [del(18)(p11.2)]. See Table 9.1 for a description of the features associated with 18p- syndrome.

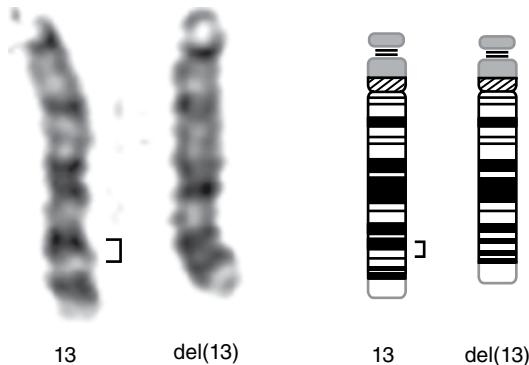


Figure 9.14 An interstitial deletion involving the distal long arm of chromosome 13 [del(13)(q31.3q32.3)].

homologous chromosomes (see 9.2.1, Mechanisms of structural rearrangements and Figure 9.12). Misalignment and recombination between nonallelic homologous LCR sequences is expected to produce not only interstitial microdeletions, but complementary interstitial microduplications, as well (Figures 9.12a–b). Although complementary duplications have currently been identified for only a small number of microdeletion regions, it is assumed that they exist for most, if not all, of the recurring microdeletion syndromes. The fact that some of the complementary duplications have not yet been identified may reflect the presence of an extreme presentation, either a lethal outcome or a very mild phenotype considered to be within the normal range (see 9.2.3, Duplication).

While chromosomes with interstitial deletions retain their telomeres, this is not the case with true terminal deletions. To maintain its stability, a terminally deleted chromosome must therefore acquire a new telomere [(TTAGGG)] to protect its ends from degradation. One method of telomere acquisition, referred to as “telomere healing,” involves the synthesis of an entirely new telomere at or near the breakage site [108–110]. Specifically, a recognition site is identified by the telomerase enzyme and a new telomere is synthesized on the leading strand in a 5' to 3' direction. Synthesis of the complementary lagging strand is then accomplished using traditional replication proteins. Deletions that are healed by this telomere regeneration process represent “true” deletions. Other apparently terminal deletions may represent derivative chromosomes that have acquired a replacement telomere from another chromosome [111–113]. These “telomere capture” events are predicted to occur secondary to telomerase-independent recombination and/or repair procedures involving the deleted chromosome and a sister chromatid end, or a homologous or nonhomologous chromosome end.

9.2.3 Duplications

Rearrangements that result in an extra copy of a chromosome region are referred to as “duplications.” The duplicated material can be located in tandem (side-by-side) as illustrated in Figure 9.15, or it can be positioned at a separate site either within the same chromosome or within a different chromosome. When the orientation of the duplicated material is retained relative to its original position with respect to the centromere, it is said to be a *direct* duplication, and when the orientation is reversed,

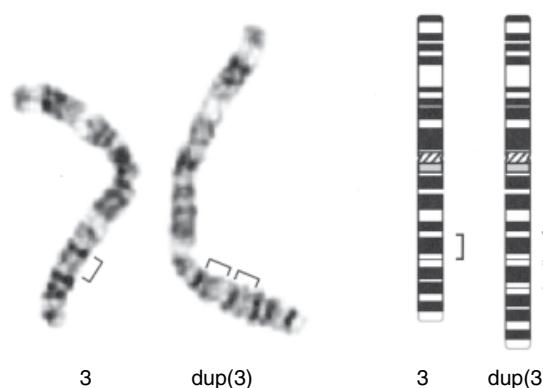


Figure 9.15 A direct (head-to-tail) tandem (side-by-side) duplication of chromosome 3q (long arm) material involving the 3q23 to 3q25.3 region [dup(3)(q23q25.3)].

it is called an *inverted* duplication. Regardless of position or orientation within the genome, pure duplications with no other imbalances will yield partial trisomies. The resulting duplication phenotype will largely depend upon the region involved. When an imprinted region is involved, the parent of origin for the duplication will also affect the phenotype. For example, maternally inherited proximal 15q duplications involving the Prader-Willi/Angelman syndrome region result in an abnormal phenotype that includes autism and intellectual disability (Table 9.1). However, the comparable paternally inherited duplication on the proximal chromosome 15 typically has no clinical consequences. There would, however, be reproductive consequences for females who have inherited such a duplication from their father; the risk of transmitting a maternal duplication would be 50% for each pregnancy (see Chapter 10, Genomic imprinting).

While a large number of recurring deletion syndromes have been identified, described and named, the identification of recurring duplications has proven to be more enigmatic. With the exception of Beckwith-Wiedemann and a Cornelia De Lange-like syndrome associated with duplication of 11p15.5 and 3q26.3, respectively, few recurring duplication syndromes had been recognized and described until recently. A handful of duplications involving the same chromosomal segments that are deleted in microdeletion syndromes have now been documented. These complementary microdeletions/microduplications reflect the reciprocal products of recurring nonallelic NAHR events mediated by LCR sequences (see 9.2.1, Mechanisms for structural rearrangements and Figure 9.12).

Although complementary microduplications have been identified for only a handful of the microdeletion syndrome regions, including Williams, Prader-Willi/Angelman, Smith-Magenis, hereditary neuropathy with liability to pressure palsies (HNPP), DiGeorge, and the 17q21.3 deletion syndrome, it is assumed that additional complementary microduplications exist. The fact that some have not yet been identified is thought to reflect the absence of a defined phenotype that clinicians can use to identify these patients for a focused analysis of the relevant region, and/or the fact that these individuals are likely to have mild phenotypes that do not necessarily warrant a genetic evaluation. Although it is also possible that some of the complementary microduplications have not been detected because they result in early lethality, this seems less likely, given that duplications are generally less detrimental than deletions.

Although the recurring tandem microduplications are predicted to occur secondary to misalignment of nonallelic LCR sequences followed by recombination (i.e., by NAHR), the mechanism(s) responsible for the nonrecurring duplications are less well understood. The FoSTeS model has, however, been proposed to explain the complex duplication events associated with Pelizaeus-Merzbacher disease and other nonrecurring rearrangements (Table 9.1) [84,98].

9.2.4 Inversions

Inversions are intrachromosomal rearrangements in which the material located between two breakpoints is rotated 180 degrees and reinserted in the opposite orientation. These rearrangements are divided into two groups, depending on the position of the breakpoints. A *pericentric* inversion is formed by breakpoints on either side of the centromere, one in each arm (Figures 9.11 and 9.16). These types of inversions can be detected secondary to an altered banding pattern and/or a changed short arm to long arm ratio created by a different centromere position. In contrast, a *paracentric* inversion is

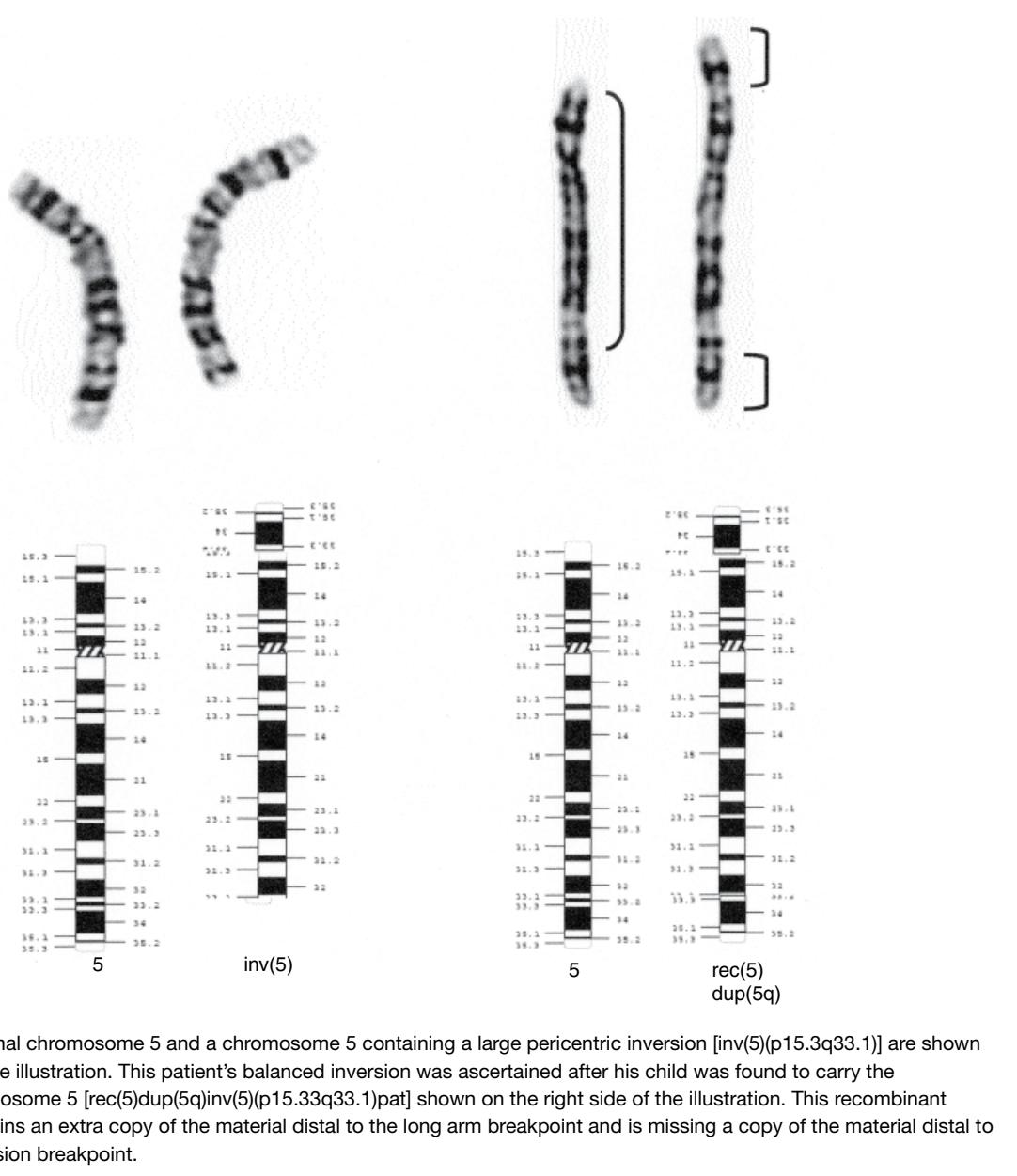


Figure 9.16 A normal chromosome 5 and a chromosome 5 containing a large pericentric inversion [inv(5)(p15.3q33.1)] are shown on the left half of the illustration. This patient's balanced inversion was ascertained after his child was found to carry the recombinant chromosome 5 [rec(5)dup(5q)inv(5)(p15.3q33.1)pat] shown on the right side of the illustration. This recombinant chromosome contains an extra copy of the material distal to the long arm breakpoint and is missing a copy of the material distal to the short arm inversion breakpoint.

produced by breakpoints confined to only one chromosome arm (Figures 9.11 and 9.17). Because an altered banding pattern is the only cytogenetic clue that a paracentric inversion is present, these rearrangements were not detected until the advent of chromosome banding. The subtle nature of paracentric inversions may also account for their lower detection rate. Excluding the most common polymorphic inversions, which typically involve the heterochromatic regions of chromosomes 1, 9, 16 and Y, the estimated frequency of pericentric inversions is 0.12–0.7%, while paracentric inversions are estimated to occur with a frequency of 0.1–0.5% [114].

Inversions are often identified in balanced form as incidental events within an individual who is being karyotyped for reasons other than an abnormal phenotype. It is therefore not surprising that approximately 85–90% of inversions are found to be inherited from a phenotypically normal parent with the same inversion. In fact, some familial inversions have been segregating in the human population for so long that they are well established in the cytogenetic literature as recurring inversions, and valuable information exists concerning the risks for viable segregants and the expected phenotypes for unbalanced offspring (Table 9.1). One example of such is a chromosome 8 inversion involving breakpoints at 8p23 and 8q22.

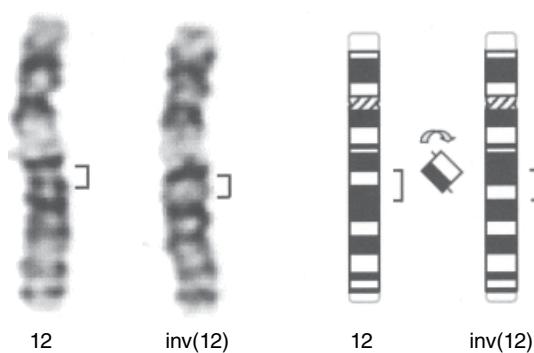


Figure 9.17 A balanced paracentric inversion involving the long arm of chromosome 12 [inv(12)(q15q21.2)].

This recurring pericentric inversion is seen in many families of Mexican-American decent and has been traced back to a single founder living in northeastern New Mexico during the late 1800s [115]. Similarly, a recurring paracentric inversion of chromosome 11 involving breakpoints at 11q21 and 11q23 is common in the Netherlands and among the Canadian Hutterite, and is thought to have originated in the Netherlands as a single mutation [116,117].

Other frequently observed inversions represent recurrent independent events rather than longstanding familial inversions that have been passed from parent to child for many generations. As described for some of the recurring microdeletions, microduplications, and translocations, LCR sequences and breakage-prone regions appear to play a role in some recurring inversions. In fact, the same LCR sequences that mediate some of these recurring deletions, duplications and translocations, have been shown to mediate polymorphic submicroscopic paracentric inversions. Data are now accumulating to suggest that at least some of these submicroscopic inversions, while not directly causing disease themselves, actually increase the probability of nonallelic recombination events that yield deleterious deletions, duplications, and other rearrangements. Such inversions have, for example, been found preferentially in the transmitting parents of children with Williams, Sotos, Angelman, and the 17q21.3 deletion syndrome, as well as those with one of several rearrangements involving the short arm of chromosome 8 [88,92,94,118]. NAHR involving LCR sequences has also been shown to be responsible for several disease-causing paracentric inversions. In approximately 45% of patients with severe hemophilia A, the factor VIII gene is disrupted by one of two intragenic inversions that are mediated by inverted LCR sequences [91]. A similar disease-causing inversion resulting in mucopolysaccharidosis type II (MPS II) is mediated by inverted repeat sequences located within intron 7 of both the iduronate 2-sulfatase gene and a nearby iduronate 2-sulfatase pseudogene [119].

While inversions cause disease by interrupting expression of a critical gene, as described above for some patients with hemophilia A or MPS II, of primary concern is the generation of chromosomally unbalanced offspring secondary to meiotic recombination events. Homologous chromosomes use their shared homology to align themselves during meiosis, usually 2-by-2, to form bivalent structures. When an inversion is present, the two homologous chromosomes continue to form a bivalent, but the actual pairing configuration will be dependent upon the size of the inversion. With small pericentric inversions, the large homologous chromosome regions synapse or pair, but the inverted segment does not (Figure 9.18b). With this configuration, recombination only occurs between paired homologous regions, and no unbalanced recombinant gametes are produced. With larger inversions, one of two pairing configurations form. Full synapsis between the normal and inverted chromosome can be accomplished by forming an inversion loop, or synapsis can be confined to the inversion segment with the ends remaining unsynapsed (Figures 9.18a,c). An odd number of recombination events within the paired inverted segment of either of these two configurations will produce recombinant chromosomes with combined duplications and deletions. The duplications and deficiencies in these recombinant chromosomes always involve the material distal to the inversion breakpoints. An example of a balanced pericentric inversion 5 and a recombinant chromosome 5 are shown in Figure 9.16. This recombinant duplication-deficiency chromosome is lacking the material distal to the 5p breakpoint and contains duplication of the material distal to the 5q breakpoint. The reciprocal recombination product would be expected to demonstrate the complementary deletion (5q breakpoint to 5pter) and duplication (5p breakpoint to 5pter).

Although with small paracentric inversions the inverted regions may remain unpaired, as described above for pericentric inversions, the classic pairing configuration for these inversions is the inversion loop. As shown in the upper portion of Figure 9.19, a single or odd number of recombination events within the inversion loop will create complementary dicentric and acentric recombinant chromosomes containing both deletions and duplications. These recombinant chromosomes are

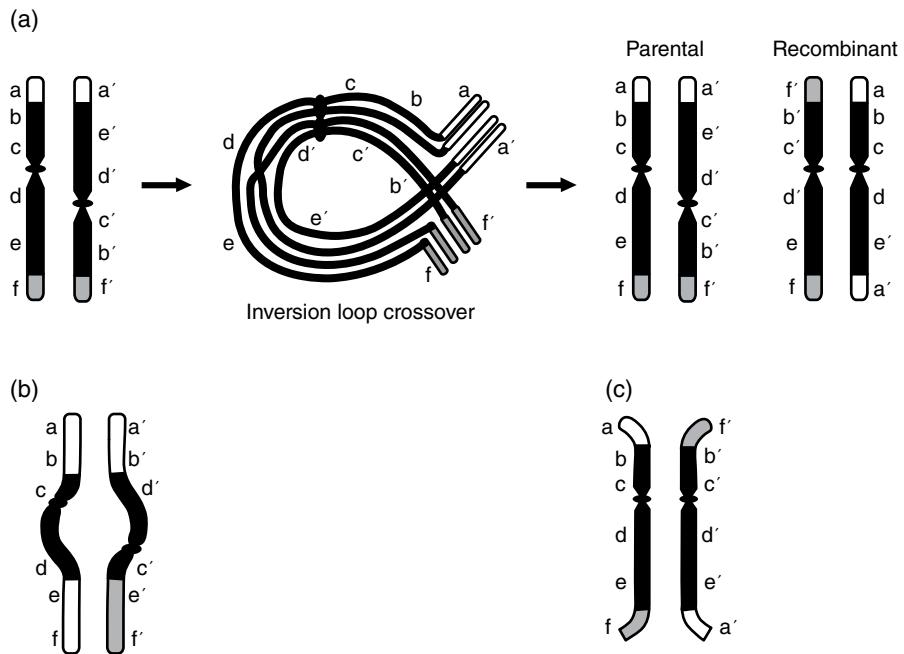


Figure 9.18 Pairing in a pericentric inversion carrier may occur in one of three ways. Pairing is optimized by the formation of an inversion loop (a). Following a single recombination event within the inversion loop, both parental and recombinant products are produced. Note, the recombinant products contain complementary duplications and deletions involving the material that lies distal to the inversion breakpoints. Partial pairing or synapsis can also occur (b,c).

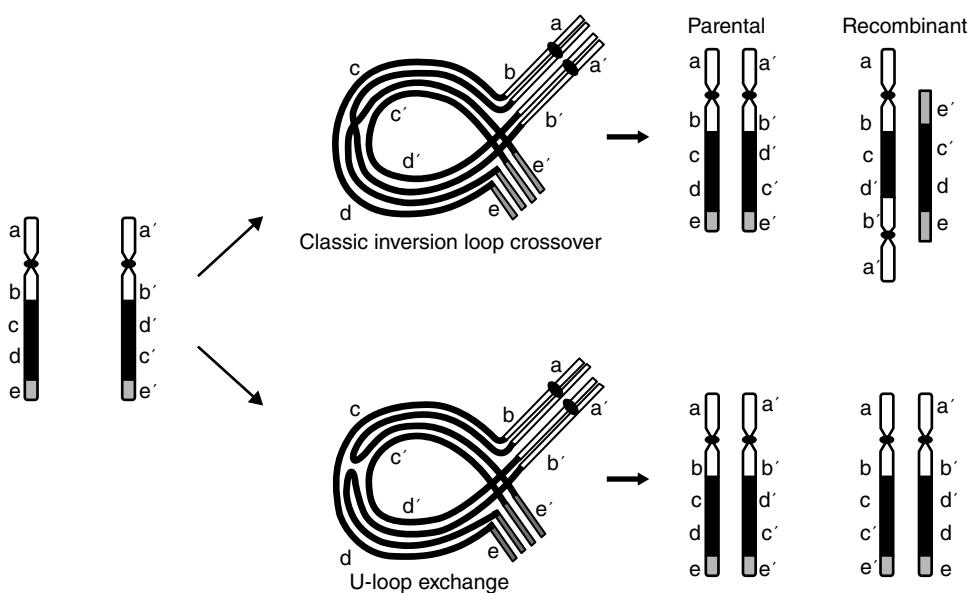


Figure 9.19 Two models for recombination within the inversion loop of a paracentric inversion carrier have been proposed. Following a single classic crossover event (top), acentric and dicentric recombinant chromosomes with duplications and deletions are produced. In contrast, a single U-type exchange event within the inversion loop produces only monocentric recombinant chromosomes (bottom).

rarely seen in liveborn individuals or in the gametes of paracentric inversion carriers [120,121]. This is not unexpected since both dicentric and acentric chromosomes are mitotically unstable. An acentric chromosome lacks the ability to attach to the mitotic spindle apparatus and therefore has a high probability of being lost during each cell division, thus creating a cell with a lethal monosomy. Conversely, the centromeres of a dicentric chromosome are likely to be jointly bound by the spindle apparatus at the two opposing poles. As the dicentric chromosome is simultaneously pulled to opposite poles, it will then either be suspended at the metaphase plate and lost, producing a lethal monosomy, or the chromosome will break, allowing portions of the chromosome to migrate to each pole. Depending on where breakage occurs within the dicentric chromosome, partial monosomies or concomitant partial monosomies and trisomies will be found in the daughter cells. The mitotic stability of the few dicentric recombinant chromosomes that have been reported in the literature has been attributed to suppression of one of the two centromeres.

In addition to the predicted dicentric or acentric recombinant chromosomes which have only rarely been reported in the offspring of paracentric inversion carriers, a handful of U-type exchange recombinants have also been described [120]. As illustrated in the lower portion of Figure 9.19, monocentric recombinant chromosomes that contain both partial duplications and deletions are produced from this atypical recombination event. Because it is often impossible to distinguish between a paracentric inversion and a paracentric (intra-arm) insertion using traditional G-banding, especially if the distance between the excision and insertion site is small, it is possible that some of the recombinant cases attributed to a U-type exchange event in a paracentric inversion carrier may actually be derived from a paracentric insertion carrier. Given that the reproductive risks associated with paracentric insertions are much greater than those associated with paracentric inversions, it is important to attempt to distinguish these two types of rearrangements.

9.2.5 Neocentromeric or analphoid chromosomes

As discussed above in the section on paracentric inversions, chromosomes that truly lack a centromere are mitotically unstable, and therefore are not seen as consistent structures in constitutional karyotypes. That said, a number of rare chromosomes that lack a traditional centromere composed of alpha-satellite DNA sequences, have been reported. These seemingly “acentric” chromosomes contain what is referred to as a “neocentromere” which forms within unmodified interstitial DNA sequences that are not normally associated with centromere formation. Like traditional centromeres, these neocentromeres form constrictions and, with the exception of centromeric protein B (CENPB), which specifically interacts with alpha-satellite DNA sequences, bind the same centromeric proteins [121].

Although neocentromeric chromosomes are rare, more than 90 cases have been reported in the literature. With the exception of chromosomes 7 and 19, neocentromere formation has now been documented in each of the other 24 human chromosomes [122]. The majority are small inverted duplicated chromosomes composed of two mirror image copies of the terminal end of a chromosome. These inverted duplicated chromosomes appear to form secondary to a U-type exchange involving sister chromatids or homologous chromosomes, by crossing over within a paracentric inversion loop, and by other undefined mechanisms (Figures 9.12 and 9.19). Neocentromeres have been documented in ring chromosomes, as well as in linear chromosomes containing deletions but no duplications. Rarely neocentromere formation has been documented in a structurally normal chromosome that also contains its original intact but inactivated centromere [122].

While neocentromeres have occasionally been identified as part of a balanced chromosome rearrangement, in most cases they are associated with an imbalance. The resulting imbalance is most often a partial tetrasomy (85% of cases) or partial trisomy, and rarely a partial monosomy [122]. Patients who carry a neocentromeric chromosome are often mosaics. In some cases the mosaicism may reflect the fact that the neocentromeric chromosome was formed post-zygotically in a pregnancy that was otherwise chromosomally normal. In other cases mosaicism may reflect the presence of an unstable ring chromosome or a neocentromere that functions less efficiently than a traditional centromere. In situations in which loss of the unstable neocentromeric chromosome or ring is associated with a selective advantage (i.e., a smaller genetic imbalance), mosaicism would seem especially likely. For example, loss of a supernumerary inverted duplicated neocentromeric chromosome would convert a partially tetrasomic cell line to a more favorable normal cell line.

9.2.6 Dicentric chromosomes

“Dicentric” chromosomes, as the name implies, contain two centromeres rather than the expected one. These chromosomes are formed when any two centromere-containing chromosome segments are joined. Robertsonian translocations are among the most common dicentric chromosomes in man (see 9.2.11, Robertsonian translocations). While these chromosomes appear monocentric in G-banded preparations, at the molecular level most are actually dicentric and have formed secondary to breakpoints within the short arms of both of the acrocentric chromosomes involved [123–125]. Additionally, the majority of

isochromosomes involving the long arm of the X chromosome, and typically associated with Turner syndrome, are also dicentric at the molecular level [126]. A wide variety of dicentric chromosomes with two cytogenetically distinct centromeres have also been reported [127]. Among the most common of these clearly dicentric chromosomes is the isodicentric chromosome 15 [idic(15)(q11-q13)], also referred to as an inverted duplicated chromosome 15 [128]. This chromosome contains two copies of the short arm, centromere and proximal long arm, it is virtually always of maternal origin, and it is believed to be the byproduct of a trisomy rescue event. Because one of the centromeres is typically inactivated and does not appear constricted, these chromosomes are more aptly called pseudoisodicentric chromosomes. When this supernumerary chromosome contains the Prader–Willi/Angelman critical region in duplicate, the classic idic(15) phenotype results, which includes developmental delay, autistic behaviors, hypotonia, and in some cases mildly dysmorphic facial features (Table 9.1). The smaller idicentric chromosomes lacking the Prader–Willi/Angelman syndrome region are typically benign.

Given the critical role of the centromere during cell division, the presence of two centromeres within a single chromosome can be problematic. Normal chromosome segregation can only occur if both of the centromeres within the dicentric chromosome attach themselves to the spindle apparatus from the same pole. If instead each centromere becomes attached to the spindle apparatus at opposite poles, the dicentric chromosome will be pulled in opposing directions during cell division and numerous outcomes are possible. In some cell divisions the dicentric chromosome may break, thereby creating two independent monocentric chromosomes that are free to migrate to opposite poles. Depending on where the break occurs and the degree of degradation the chromosome undergoes before a new telomeric structure can be formed, the two resulting daughter cells can harbor different chromosomal imbalances. During other cell divisions the dicentric chromosome may fail to segregate to either pole, remaining in transition at the metaphase plate. Given this scenario, the dicentric chromosome may be completely lost during cell division, causing a single or double monosomy, or it may impede cell division (cytokinesis), causing a tetraploid cell to form. Interestingly, some dicentric chromosomes appear to function as stable monocentric chromosomes. In some cases their stability is attributed to the fact that their centromeres are placed in close proximity and therefore function as a single unit, while in other cases a pseudodicentric chromosome, with one active (constricted) and one inactive (unconstricted) centromere, appears to form [126,129–131].

9.2.7 Isochromosomes

“Isochromosomes,” as originally defined, were monocentric chromosomes containing two identical arms joined at the centromere (Figure 9.20). An isochromosome therefore was thought to contain two copies of the genetic material within the duplicated arm and no copies of the material represented in the deleted arm. More recent molecular examination of these chromosomes has demonstrated that, in many cases, breakage and reunion actually occurs within the pericentromeric region, rather than within the centromere [126,132–134]. Many apparent isochromosomes contain two copies of the duplicated arm, as well as two copies of the centromere, and a small amount of proximal material from the “deleted” arm. They would more accurately be referred to as *isodicentric* chromosomes. The stability of these dicentric chromosomes has been attributed to the inactivation of one of the two centromeres or, given their close proximity, a tendency for both centromeres to function as one.

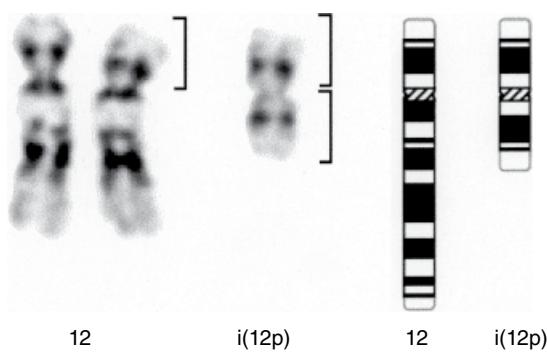


Figure 9.20 A supernumerary isochromosome composed of two copies of the short arm of chromosome 12 [+i(12p10)]. Patients with an extra isochromosome 12p have tetrasomy 12p or Pallister-Killian syndrome. This chromosome abnormality is rarely detected in the metaphase cells from a peripheral blood sample, but can often be detected by interphase FISH or chromosome microarray analysis of this tissue. Alternatively, this chromosome abnormality can often be detected in the metaphase cells obtained from fibroblast cultures.

The original mechanism for isochromosome formation, centromere misdivision, was described by Darlington in 1939 [135]. He proposed that an isochromosome was formed when the centromere underwent a transverse split to separate chromosome arms, rather than the usual longitudinal split to produce two sister chromatids. Given the knowledge that most isochromosomes are actually dicentric chromosomes formed by breakage and reunion events that occur in the pericentromeric regions rather than at the centromere itself, centromere misdivision is no longer touted as the most common mechanism of isochromosome formation. The current belief is that most isochromosomes form secondary to an allelic breakage and reunion event referred to as a U-type exchange (reviewed in [136]). U-type exchange events occurring within the centromere or pericentromeric region would produce monocentric and dicentric isochromosomes, respectively. Nonallelic breakage and reunion events may also be responsible for the formation of some isochromosomes. These NAHR events appear to be mediated by highly homologous inverted LCR sequences (see Figures 9.12e,f) [87,137].

In general, the clinical consequence of an isochromosome is genetic imbalance. In rare cases, a genetically balanced individual carries an isochromosome derived from a single acrocentric chromosome. For example, a balanced Robertsonian 21;21 translocation carrier has 45 chromosomes with an isochromosome for 21q and no free-standing chromosome 21. There are also rare cases of chromosomally balanced individuals with complementary short arm and long arm isochromosomes (e.g., both an isochromosome 2p and an isochromosome 2q) [138–142]. When an isochromosome replaces an existing chromosome within an otherwise normal cell with 46 chromosomes, the individual will be monosomic for genetic material within the deleted arm and trisomic for all of the material within the duplicated arm. Given the lethality associated with large imbalances, especially large monosomies, most of the isochromosomes that fall into this category involve the smaller chromosomes. The chromosomes most frequently involved include X, Y, 13, 18 and 21, each of which yield viable trisomies. The corresponding loss of short arm material from chromosomes X or 18, and either the short or long arm of chromosome Y, would produce a relatively small, viable monosomy. Similarly, acrocentric chromosome short arm loss would be of no apparent clinical consequence given that this region contains redundant ribosomal DNA. Molecular studies demonstrating identical alleles (homozygosity) for all tested loci within an isochromosome that has replaced a normal chromosome, suggest that most have formed secondary to a mitotic recombination event between sister chromatids after formation of the zygote (a post-zygotic error). As expected for a mitotic event, maternal and paternal isochromosomes are seen with equal frequency and their presence is not associated with increased parental age [132,143–149].

In contrast to the monosomies and trisomies produced by isochromosomes within an otherwise normal cell, tetrasomies occur when isochromosomes are present as supernumerary or extra chromosomes. As described above, the majority of these isochromosomes are derived from small chromosome arms because they result in smaller imbalances with greater viability. Some of the more commonly reported supernumerary isochromosomes are derived from 5p, 8p, 9p, 10p, 12p (Pallister-Killian syndrome), 18p, 18q, 20p, and 21q. The recurring supernumerary isochromosome 12 associated with Pallister-Killian syndrome is shown in Figure 9.20 (see also Table 9.1). Most supernumerary isochromosomes are of maternal origin, possibly reflecting the prevalence of maternal nondisjunction errors. In these situations, a meiotic nondisjunction event produces a lethal trisomic conceptus which, in some cases, may be partially “rescued” by early formation of an isochromosome [149,150]. In this scenario a post-zygotic sister chromatid exchange within the pericentromeric region of one of the trisomic chromosomes results in the formation of one isodicentric chromosome and one complementary acentric isochromosome. The isodicentric chromosome then segregates to one of the two daughter cells, while the complementary acentric isochromosome is lost, producing a normal second daughter cell. It is the presence of this normal cell line, albeit in mosaic form, that potentially rescues an otherwise nonviable trisomic pregnancy.

9.2.8 Rings

Ring chromosomes represent one of the most uncommon types of structural rearrangements. Based on prenatal cytogenetic studies and postnatal analyses of consecutive liveborns, the incidence of rings in the human population is estimated to be in the range of 1/25,000 to 1/62,000 [151]. Although the majority of ring chromosomes represent de novo (new) mutation events, in approximately 1% of cases they are inherited from a carrier parent, typically the mother. In familial cases the offspring are typically no more severely affected than their carrier parent; however, in 1/3 of cases the offspring's phenotype is more severe [152]. The more severe phenotype seen in some of these children can be explained by (1) differences in the genetic content of the ring secondary to dynamic mosaicism (see later), (2) the UPD status of the corresponding homologous chromosome pair, (3) the extent of mosaicism, and/or (4) the tissue distribution of the normal versus abnormal cells in parent and child. In rare cases, the ring chromosome is part of a balanced cryptic rearrangement in at least some of the parental cells, while it exists in an unbalanced form in the child [153–155]. Additionally, the offspring of ring carriers may have other chromosome abnormalities involving the same chromosome, including full trisomies, isochromosomes, linear chromosomes with tandem duplications, or rearranged rings [152]. Genetic counseling for a ring carrier is complex and should include a discussion of all

of the possibilities described above. Additionally, the chromosome from which the parental ring was derived should be closely examined in the karyotypes of both the carrier parent and their offspring to look for subtle structural changes that may explain the presence and/or absence of clinical findings. As well, characterization of the ring chromosome using molecular cytogenetic or array-based approaches should be undertaken to clarify its composition.

The largest rings are thought to form secondary to telomere fusion events that result in retention of both subtelomeric and telomeric sequences (Figure 9.21). Other rings are formed when breakage occurs within both arms, distal material is lost, and the broken ends fuse to produce a circular chromosome. Depending on the location of the breakpoints within any given chromosome, the ring size and extent of aneuploidy will vary. In contrast to rings that form secondary to breakage events within both chromosome arms, many of the smaller ring chromosomes appear to be the result of breakage events that are confined to one arm and centromeric or pericentromeric DNA [155]. Still other rare rings are derived from breakpoints that are confined to a single chromosome arm. Although these particular rings lack a conventional centromere, they do contain sequences that are capable of functioning as a centromere (see 9.25, Neocentromeric or analphoid chromosomes). Both these “acentric” ring chromosomes and the more traditional centric ring chromosomes are hypothesized to form secondary to intra-chromatid recombination events that are mediated by repeat sequences (Figure 9.12c). While the majority of ring chromosomes are thought to be derived from breakage events within a normal chromosome, some appear to have been derived from structurally abnormal chromosomes [152,156–158].

The extent and type of aneuploidy demonstrated in an individual with a ring chromosome depends on the content of the ring chromosome(s) and whether the ring chromosome has replaced a normal homologue or represents a supernumerary or extra chromosome. When present as a replacement chromosome in a cell with a complement of 46 chromosomes, a partial monosomy involving the material distal to the breakpoints typically results. Conversely, a trisomy involving the material proximal to the breakpoints is typically seen when a supernumerary ring chromosome is present. It is important to note, however, that in practice, the aneusomies observed in patients with ring chromosomes can be much more complex than eluded to above. Rather than segregating from daughter cell to daughter cell intact, ring chromosomes are often unstable because normal sister chromatid exchange events produce both interlocking chromosomes and dicentric double ring chromosomes that are then resolved in a variety of ways (Figure 9.21). In some cases the centromeres of these interlocked or dicentric double ring chromosomes attach themselves to the mitotic spindles at different poles, such that breakage, end erosion, and ultimately end reattachment occurs, to produce new rings with different compositions. Alternatively, mitotic nondisjunction of the interlocked or double ring chromosomes can occur to produce daughter cells that have either lost the entire ring chromosome or gained an additional copy. The active or dynamic process by which a variety of different cell lines are derived from a single progenitor cell is referred to as *dynamic mosaicism*.

While the abnormal clinical findings observed in patients with a ring chromosome are usually attributed to the presence of a partial aneuploidy, other etiologies have also been documented. For example, UPD, or the inheritance of a pair of homologous chromosomes from a single parent, has been shown to be partially responsible for the phenotype of several individuals with ring chromosomes. Temple et al. describe a patient with a maternally derived supernumerary ring 6 chromosome, paternal UPD for chromosome 6 and the expected transient neonatal diabetes associated with this particular UPD, while Prader–Willi and Silver–Russell syndrome have been reported in patients who carry both a paternal ring chromosome and maternal UPD involving chromosomes 15 and 7, respectively [159–165]. In each case the UPD is thought to have occurred secondary to a trisomy or monosomy rescue event.

In addition to partial aneuploidies and UPD, cell death caused by a lethal genetic imbalance may also be responsible for some or all of the abnormal clinical findings seen in at least some ring chromosome patients. The term “ring syndrome” was

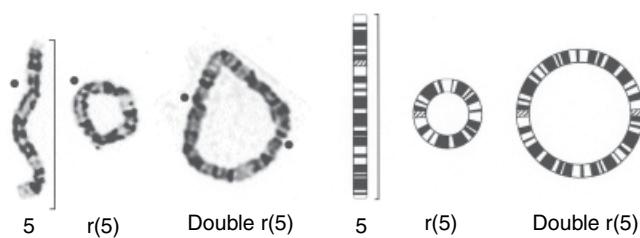


Figure 9.21 A ring chromosome formed secondary to fusion of the very distal sequences on the short and long arms of chromosome 5 [r(5)(p15.33q35.3)]. Some cells containing a double ring chromosome with two copies of chromosome 5 joined in a head-to-tail fashion were also observed; the centromere(s) in each chromosome 5 is represented by a filled circle.

coined by Cote et al. [166], in 1981 to denote the shared phenotype that is observed in patients with 46 chromosomes, one of which is typically a large intact ring. Each of these patients demonstrate severe growth retardation, microcephaly, mild to moderate intellectual disability, and mild anomalies, regardless of which ring chromosome is present. It has been hypothesized that the shared ring syndrome phenotype is the result of dynamic mosaicism involving large imbalances which leads to cell death and a decreased number of viable cells at any given developmental stage. Consequently, overall growth, organogenesis and development are hindered regardless of the particular ring chromosome involved. It has been suggested that this phenomenon may also contribute to the phenotype observed in patients that have ring chromosomes with obvious imbalances in their karyotypes.

9.2.9 Marker chromosomes

The term “marker chromosome” is used to describe any structurally abnormal chromosome that cannot be definitively identified using conventional banding methods [167]. Their incidence in unselected prenatal and consecutively studied postnatal cases is estimated at 0.075% and 0.044%, respectively. As one might suspect, the incidence appears to be higher in pregnancies with ultrasound abnormalities (0.20%) and in developmentally delayed patients (0.29%) [168].

Although marker chromosomes represent a heterogeneous group, some generalization can be made. For example, while the marker chromosomes seen in neoplastic cases can be quite large, those associated with constitutional karyotypes are typically small, usually equal to or less than the size of chromosome 20. The smallest markers, referred to as “minute chromosomes,” can be particularly difficult to identify. Given their small size, their presence can be obscured by the other chromosomes in a metaphase spread, they can be lost during slide dropping, and they can be difficult to distinguish from normal cellular debris in a metaphase cell. While the majority of marker chromosomes contain a traditional centromere with proximal long and/or short arm material, rare marker chromosomes with neocentromeres have been reported [122] (see 9.2.5, Neocentromeric or analphoid chromosomes). These neocentromeric or analphoid markers are usually composed of duplicated distal long arm material, rather than pericentromeric material. Additionally, the marker chromosomes identified in constitutional karyotypes typically represent supernumerary (extra) chromosomes, often referred to as extra structurally abnormal chromosomes (ESACs). The primary exception to this rule involves marker chromosomes derived from the X and Y chromosomes, which are usually found in individuals with 46 chromosomes and a single intact X chromosome. In rare cases, marker chromosomes have been identified as part of a structurally abnormal, but balanced karyotype [122,155].

While marker chromosomes have now been described for all 24 human chromosomes, those derived from the acrocentric chromosomes (13, 14, 15, 21, and 22) appear to be most common. It is estimated that approximately 70–80% of all marker chromosomes are derived from one of the five acrocentric chromosomes and of these, approximately 50% are derived specifically from chromosome 15. Interestingly, although there is no maternal age effect associated with marker chromosomes in general, de novo marker chromosome 15s are associated with increased maternal age. Virtually all marker chromosome 15s that have been characterized molecularly are of maternal origin. Based on these data and the well-established increase in meiosis I nondisjunction events resulting in trisomy 15 associated with increasing maternal age, it has been hypothesized that small marker chromosome 15s represent partial rescue events. By converting one of three chromosome 15s to a small marker chromosome early in pregnancy, a previously lethal chromosome imbalance becomes less severe and the pregnancy survives.

Potentially lethal chromosome imbalances can be made more tolerable not only by decreasing the amount of genetic material that is present in a supernumerary chromosome, but also by decreasing the percentage of cells that carry such a chromosome. Therefore, it is not surprising that mosaicism for a normal cell line is a common finding in patients that carry a small supernumerary marker chromosome; mosaicism has been estimated to occur in 13–50% of these cases [169,170]. Mosaicism is most common in cases involving markers derived from a nonacrocentric chromosome. Approximately 70% of these cases demonstrate mosaicism compared with only 30% of cases that involve an acrocentric chromosome. Among the cases with acrocentric-derived marker chromosomes, the likelihood of mosaicism can be further stratified by chromosome; mosaicism is seen in 50% of cases involving chromosomes 13 or 21, 38% of cases involving chromosomes 14 or 22, and 20% of cases involving chromosome 15.

While the extent of mosaicism varies depending on the chromosome involved in marker formation, so too does the structure of the marker. For example, approximately 85% of marker chromosomes derived from acrocentric chromosomes are pseudodicentric, bisatellited, chromosomes that contain two copies of the proximal long arm, centromere, and short arm. Although these chromosomes are often referred to as inverted duplicated chromosomes based on the initial mechanism proposed to explain their formation, this term is no longer considered accurate. Rather than forming secondary to an inverted duplication, the current belief is that these chromosomes form secondary to a U-type exchange involving either sister chromatids or homologous chromosomes (see 9.2.1, Mechanisms for structural rearrangements). In contrast to the acrocentric-derived marker chromosomes, virtually all supernumerary marker chromosomes derived from chromosome 1, and 80% of

those derived from chromosome 2 are ring chromosomes. For chromosomes 13 and 17, very small supernumerary marker chromosomes, referred to as “minutes,” are most commonly seen, while no clear preference for one specific marker structure is apparent for the remaining chromosomes [171].

Prior to the advent of FISH and other molecular techniques, marker chromosomes were typically characterized based on their size and shape. In recent years, using an array of molecular techniques, it has been possible to further identify and classify many of the marker chromosomes identified in the human genome. However, despite the application of multiple molecular and molecular cytogenetic techniques, it is currently estimated that only 30–40% of markers fall into an identifiable group with a well defined clinical picture [170–174]. Included in this group would be patients with an isodicentric 15, an isochromosome 18, an isodicentric 22 (cat-eye syndrome) or the derivative chromosome 22 from the recurring $t(11;22)$ that is seen in Emanuel syndrome (Table 9.1). For the remaining 60–70% of marker chromosomes, clear genotype-phenotype correlations have yet to be made. While the availability of a variety of high-resolution chromosome microarray platforms will surely improve our ability to identify and characterize marker chromosomes, accurately predicting phenotype will remain difficult because of the multiple factors that must be considered. The clinical manifestations of any marker chromosome will depend not only on the genetic composition of the marker(s), but the extent of mosaicism, the tissue distribution of a mosaic marker chromosome, and/or the presence of UPD. Given this level of complexity, it is not surprising that it is very difficult to predict the clinical outcome of a child who carries a marker chromosome, even when dealing with a familial marker that has been inherited from a normal parent or a well characterized recurring marker such as an $i(12p)$ or $i(18p)$, for which much information exists in the literature.

9.2.10 Reciprocal translocations

Reciprocal translocations form when a two-way or reciprocal exchange of genetic material occurs between different chromosomes. In some cases, these exchanges are mediated by LCR sequences located on different chromosomes in a process known as “nonallelic homologous recombination” (NAHR – see discussion on Mechanisms for structural rearrangements; Figures 9.12a,d). However, most reciprocal translocations appear to be mediated by a DNA repair process known as “nonhomologous end joining” (NHEJ). In this process, a pair of double-stranded breaks, each of which is located on a separate chromosome, is directly joined in an end-to-end fashion in the absence of extensive homology. Once formed, these translocations can be passed intact from generation to generation. In fact, it appears that approximately 70% of reciprocal translocations are familial [175]. In Figure 9.22, an apparently balanced reciprocal translocation involving the long arms of chromosomes 4 and 21 is shown. The phenotypically normal individual who carried this translocation was karyotyped prior to pregnancy because of a family history of a chromosome abnormality. Genetic counseling of this patient was recommended based on the increased risk for miscarriages and/or unbalanced liveborn children among reciprocal translocation carriers.

During prophase of meiosis I, all 23 sets of homologous chromosomes pair two-by-two to form 23 joined linear structures (bivalents) on the metaphase plate. Each of these bivalents eventually separate and the two homologous chromosomes migrate to independent daughter cells. When a reciprocal translocation is present, 21 rather than 23 bivalents are formed, along with a single quadrivalent structure composed of the two reciprocal translocation chromosomes and their normal homologues. The expected quadrivalent and the most common segregants for the reciprocal 4;21 translocation

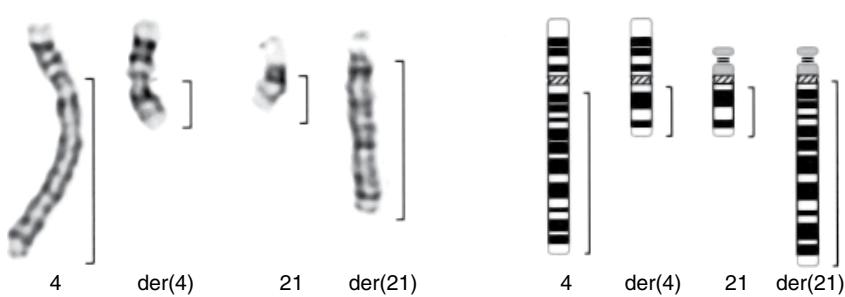


Figure 9.22 A balanced reciprocal translocation involving the long arms of chromosomes 4 and 21 [$t(4;21)(q12;q11.2)$].

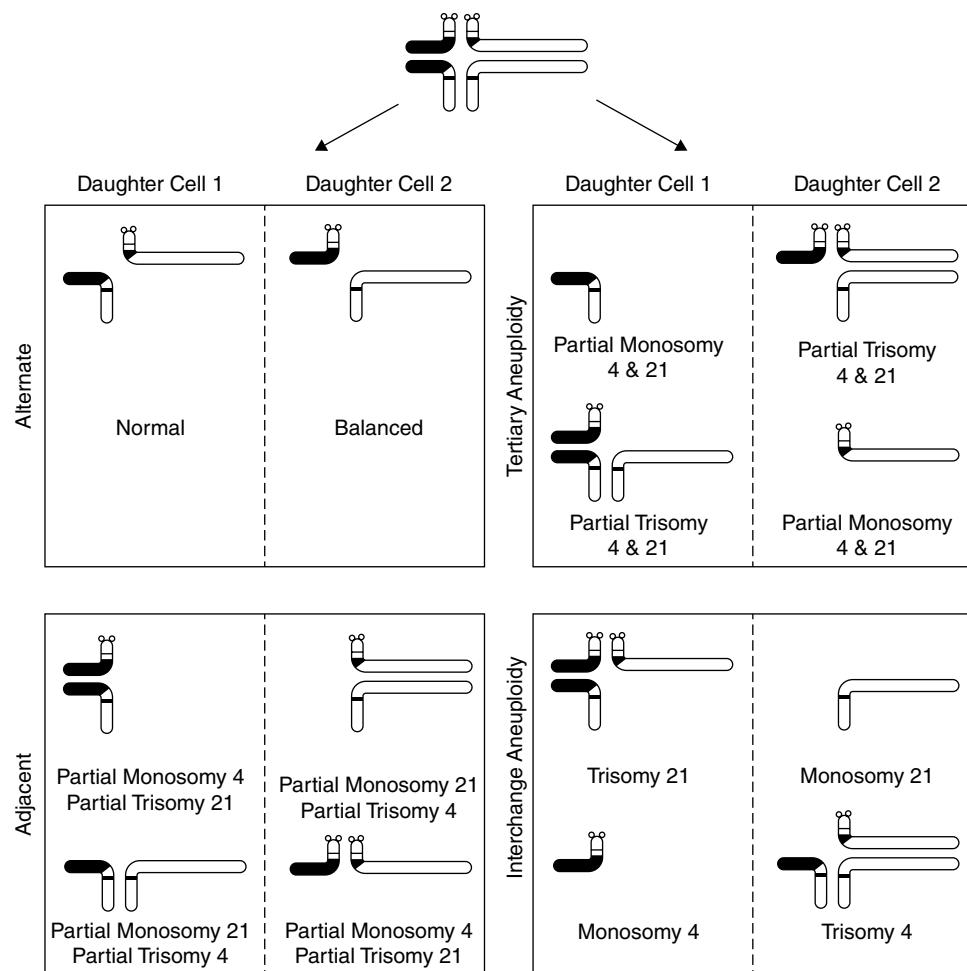


Figure 9.23 The expected meiotic pairing configuration for the reciprocal 4;21 translocation illustrated in Figure 9.22 and the most common meiotic segregants.

are shown in Figure 9.23. As you can see from this illustration, segregation of the chromosomes within a quadrivalent can occur in several ways, most of which will result in unbalanced gametes. Alternate 2 : 2 segregation, in which alternate centromeres within the quadrivalent migrate to the same daughter cell, is the most common form of segregation and the only one that will produce cytogenetically balanced offspring. Theoretically, 50% of the gametes produced from an alternate segregation will carry a normal chromosome complement and the remaining 50% will carry the balanced translocation. Each of the remaining modes of adjacent 2 : 2 segregation, in which adjacent centromeres within the quadrivalent migrate to the same daughter cell, will yield conceptions with partial monosomies and concomitant partial trisomies. Conceptuses with single or double monosomies or trisomies, result from the less common 3 : 1 and 4 : 0 (not shown) modes of segregation. Given the large segments involved in the reciprocal 4;21 translocation used in this example, one would predict that many of this patient's cytogenetically unbalanced pregnancies would be more likely to result in a miscarriage than in a phenotypically abnormal live birth. Of all of the possible unbalanced pregnancy outcomes, a trisomy 21 conceptus derived from one of the 3 : 1 interchange aneuploidy segregants would have the greatest chance of surviving to birth.

Reciprocal translocations represent one of the most common structural rearrangements observed in the human karyotype, occurring in 1/1,000 to 1/673 individuals [176,177]. While most reciprocal translocations are unique to a particular individual or family, a few recurring rearrangements have been reported. The 11;22 translocation, with breakpoints at 11q23.3 and 22q11.2 was the first to be recognized and over 100 families with this translocation have been reported in the

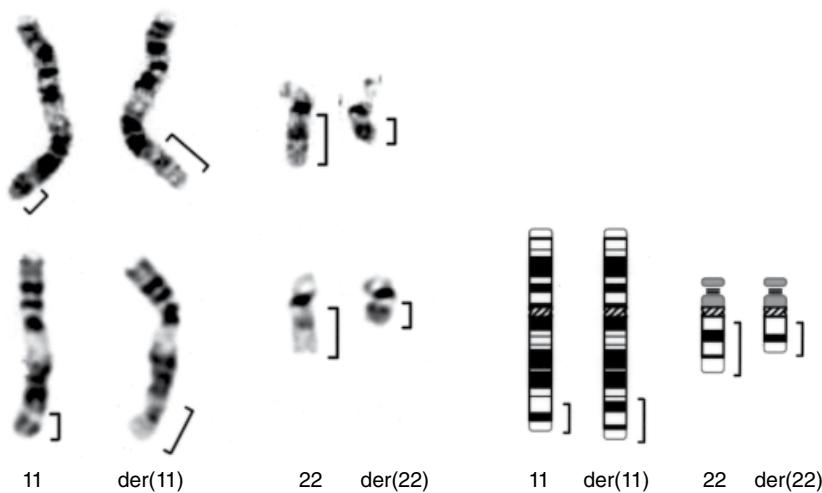


Figure 9.24 A balanced reciprocal translocation involving the long arms of chromosomes 11 and 22 [t(11;22)(q23.3;q11.2)]. This is the more common of several well-documented, recurring translocations reported in man.

literature (Figure 9.24). The unbalanced liveborn children of a balanced 11;22 translocation carrier inevitably have 47 chromosomes, including a supernumerary derivative chromosome 22. Each of these children is partially trisomic for the proximal long arm of chromosome 22 and distal long arm of chromosome 11. A compilation of the major clinical features associated with this karyotype and Emanuel syndrome can be found in Table 9.1. Mapping studies of the breakpoint regions have demonstrated that the recurrent nature of this translocation is a reflection of palindrome-mediated genomic instability. The palindromes, or inverted complementary DNA sequences, located at the breakpoint sites within both chromosomes, form secondary structures susceptible to double-stranded breaks. The recurring reciprocal 11;22 translocation arises when the resulting double-stranded breaks are repaired by the reciprocal end-to-end joining of chromosomes 11 and 22 [178]. Some preliminary evidence suggesting that the same chromosome 22 palindromic sequence involved in the 11;22 translocation may also be involved in a 17;22 translocation reported in at least two families. Each of these translocation carriers has neurofibromatosis type 1 secondary to the 17q11.2 breakpoint involving a palindromic sequence within exon 31 of the neurofibromin gene [179,180].

More recently, a 4;8 translocation involving breakpoints at 4p16 and 8p23 has been reported in over 20 families [88,181–184]. Most of these families were identified following the birth of a child with features of Wolf-Hirschhorn syndrome and the derivative chromosome 4 without the reciprocal derivative chromosome 8 (Table 9.1). This recurring rearrangement appears to be mediated by NAHR between olfactory receptor gene clusters located at the breakpoint regions within both chromosomes. Interestingly, this translocation seems to be facilitated by heterozygosity for two submicroscopic inversions within the involved olfactory gene clusters, one on chromosome 4 and the other on chromosome 8. In each of the five cases examined thus far, the transmitting mother has been heterozygous for both the chromosome 4 and the chromosome 8 inversions [88].

9.2.11 Robertsonian translocations

Robertsonian translocations represent a common structural anomaly with an incidence of 1/1,000 in the general population [185–187]. They occur when the long arms of any two of the five acrocentric chromosomes (13, 14, 15, 21, and 22) join at or near the centromere to produce one large derivative chromosome. When two “D”- or “G”-group chromosomes fuse, a metacentric chromosome is formed, while a submetacentric chromosome is formed following fusion of one “D” and one “G” chromosome. Although the translocation event that produces a Robertsonian translocation is likely a reciprocal one, the complementary short arm product is rarely seen [188]. Given that Robertsonian chromosomes are generally formed secondary to breakpoints within the short arms of both of the involved acrocentric chromosomes, they are typically dicentric, while the reciprocal product is acentric. In the absence of a centromere, this short arm product does not segregate normally during mitosis and is rapidly lost. Balanced Robertsonian translocation carriers therefore have 45 rather than 46 chromosomes and

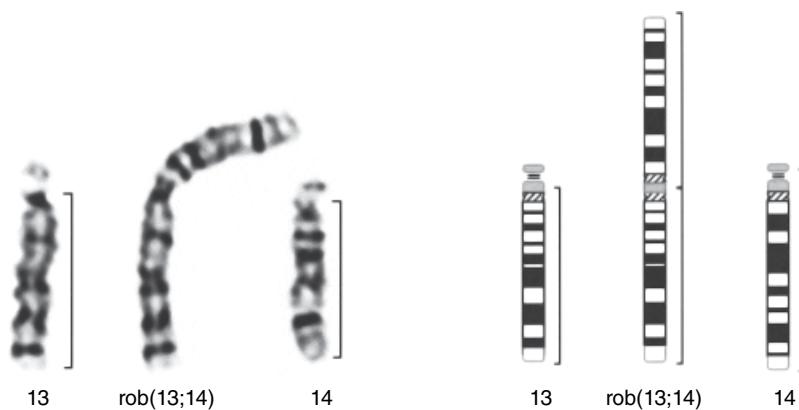


Figure 9.25 A balanced Robertsonian translocation involving chromosomes 13 and 14 [der or rob(13;14)(q10;q10)].

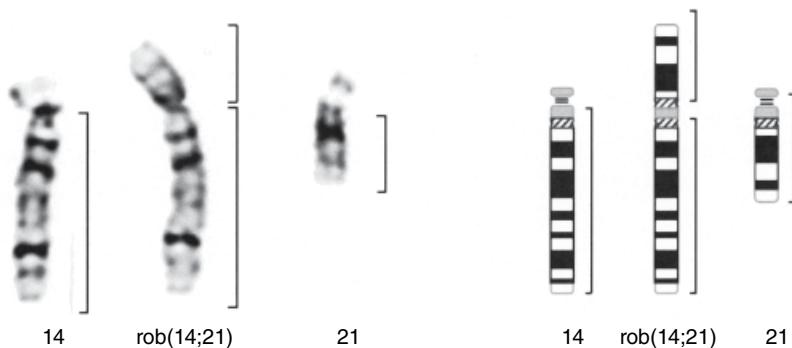


Figure 9.26 A balanced Robertsonian translocation involving chromosomes 14 and 21 [der or rob(14;21)(q10;q10)].

are clinically normal, despite the loss of acrocentric short arm material. Because of the redundant nature of the genetic material within the short arms and satellites of each of the acrocentric chromosomes, its loss from a single pair of chromosomes is benign.

Although a total of 15 different Robertsonian chromosomes are possible, given the pairing of 5 different acrocentric chromosomes, not all of these rearrangements form with equal frequency. Only 5% of all Robertsonian translocations involve fusion of homologous chromosome arms or, more commonly, sister chromatid arms, while the remaining 95% are formed secondary to fusion of nonhomologous chromosome arms. The 13;14 and 14;21 Robertsonian translocations are most common, and represent approximately 75% and 10% of all Robertsonian translocations, respectively (Figures 9.25 and 9.26) [189]. Molecular studies suggest that the high frequency of these two translocations may reflect some underlying feature of chromosomal architecture. Unlike the other 13 Robertsonian translocations in which the breakpoint regions are highly variable, a single pair of breakpoint regions is usually involved in the formation of both of these common nonhomologous translocations. Although the composition of the actual breakpoint regions has not been established, it is hypothesized that homologous inverted repeat sequences shared by these pairs of chromosomes may mediate the recurrent 13;14 and 14;21 Robertsonian translocations. The variable nature of the breakpoints involved in the less frequent Robertsonian translocations suggests that the acrocentric short arms contain multiple sites that are prone to recombination because of their close association in the nucleolus, the role of the nucleolar organizer region in ribosomal RNA synthesis, and/or the underlying architecture of this region.

Although individuals who carry a balanced Robertsonian translocation are phenotypically normal, they are at risk for having children with unbalanced karyotypes secondary to malsegregation of the Robertsonian translocation chromosome

and its homologous chromosomes. Interestingly, the risk for unbalanced liveborn offspring appears to be slightly greater for a female carrier than for a male carrier. Each chromosome malsegregation event will produce monosomic and trisomic conceptions, most of which will die in utero. Because all autosomal monosomies and most trisomies are lethal, miscarriages are common among Robertsonian translocation carrier couples. Only those translocations involving chromosomes 13 or 21, and very rarely chromosome 22, are at risk for producing unbalanced viable offspring secondary to malsegregation. Despite the increased risk of miscarriage and unbalanced progeny, carriers of balanced Robertsonian translocations can and do have children with balanced karyotypes. In fact, a child with either a normal chromosome complement or a balanced Robertsonian translocation is the most likely outcome. This is in contrast to the very high risk for an abnormal outcome that is seen for the balanced carrier of a homologous Robertsonian translocation. A balanced carrier of a Robertsonian 21;21 translocation, for example, can only produce two types of gametes. Approximately half of their gametes will carry the Robertsonian 21;21 translocation, and upon fertilization the resulting zygote will have the three copies of the chromosome 21 long arm material associated with Down syndrome. The remaining gametes will be nullisomic for chromosome 21 and fertilization will result in monosomy 21, a lethal genetic condition. A Robertsonian 21;21 translocation carrier is therefore expected to experience multiple miscarriages and the theoretical risk that a liveborn child will be chromosomally abnormal is 100%.

In addition to an increased risk for having aneuploid pregnancies, Robertsonian translocation carriers are at risk for having viable offspring with balanced karyotypes and abnormal phenotypes secondary to UPD or the inheritance of both homologues from a single parent. In most cases, UPD is thought to arise secondary to the “rescue” of a trisomic conception; when loss of one of the extra free standing chromosomes occurs such that both of the remaining two chromosomes are derived from a single parent UPD results. Less frequently, UPD occurs when a monosomic conceptus is rescued by a nondisjunction event that results in a doubling of the monosomic chromosome. Rarely, a gamete with a chromosome loss from one parent will fuse at fertilization with a gamete containing a complementary chromosome gain from the other parent to form a chromosomally balanced zygote. Given their increased risk for offspring with UPD or unbalanced karyotypes, it is recommended that all Robertsonian translocation carriers be offered genetic counseling and the option of prenatal testing.

9.2.12 Insertions

Insertions, also referred to as *insertional translocations*, are nonreciprocal three-break rearrangements involving two breaks within the “donor” chromosome and 1 break within the recipient chromosome. The segment between the two breaks is excised from the donor chromosome and inserted at the breakpoint on the recipient chromosome. The inserted material produces a *direct insertion* when present in the same orientation with respect to the centromere, and an *inverted insertion* when rotated 180 degrees. Insertions can then be further delineated based on the relationship between the excision and insertion sites. *Interchromosomal* insertions result when two homologous or nonhomologous chromosomes are involved. In instances where both excision and insertion take place within the same chromosome, the rearrangement is referred to as an *intrachromosomal* insertion. Intrachromosomal insertions that involve a single chromosome arm are referred to as within-arm, intra-arm, or paracentric insertions, while those that involve both arms are called between-arm, inter-arm, or pericentric insertions. Direct intra-arm insertions have occasionally been confused with paracentric inversions, especially when the distance between the excision and incision sites is small [120]. Because the reproductive risks associated with these two types of rearrangements are very different, an attempt should be made to distinguish between them using high-resolution cytogenetic banding and FISH.

Although insertions are rare, it is important to identify the families who are segregating these rearrangements, because the risk for having chromosomally unbalanced offspring can approach 50% in some cases. This is especially true if the inserted segment is small and the resulting monosomies and trisomies are well tolerated and, therefore, likely to be seen in liveborn children. Shown in Figure 9.27 is an example of a direct interchromosomal insertion involving chromosomes 1 and 18. This insertion was found in the parent of two children with features of trisomy 18 who had inherited the abnormal or derivative chromosome 1, but not the complementary derivative chromosome 18. Given the relatively small size of this insertion, the viability associated with partial trisomy 18, and the fact that two liveborn children with unbalanced karyotypes secondary to this insertion have been documented in this family, this family would be predicted to have a high risk for future unbalanced offspring.

Chromosomal imbalance in children of insertion carriers results from chromosome malsegregation and recombination events that produce rearranged chromosomes with duplications and/or deletions. With small interchromosomal insertions, the involved chromosomes are predicted to pair with their homologue to form incompletely synapsed meiotic bivalents, as shown in Figure 9.28. Following independent assortment of these chromosomes, four equally likely outcomes would be expected – two cytogenetically balanced and two unbalanced. As illustrated in Figure 9.28, the two unbalanced segregants

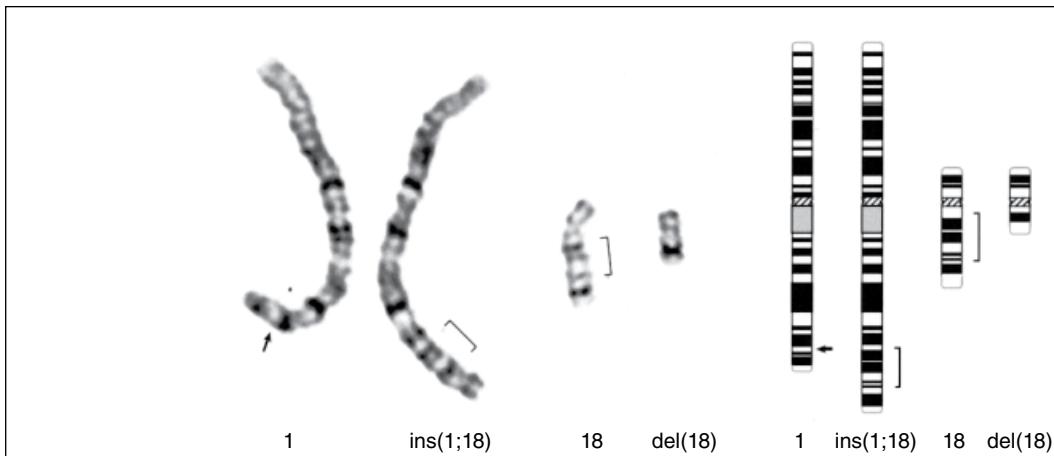


Figure 9.27 A balanced interchromosomal insertion in which approximately 2/3 of the long arm of chromosome 18 has been excised and inserted into the long arm of chromosome 1 [ins(1;18)(q42.1;q11.2q21.3)].

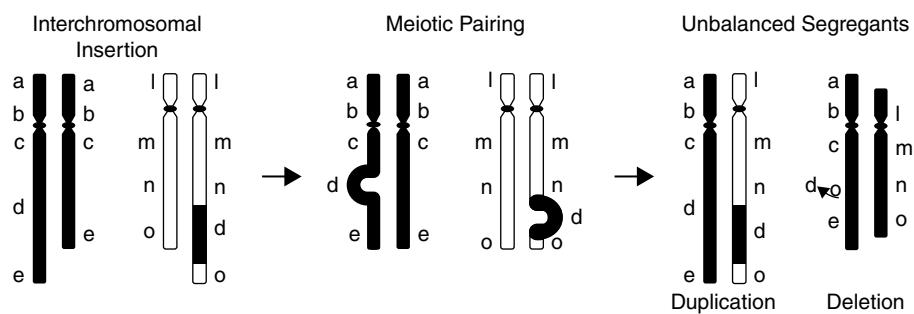


Figure 9.28 An interchromosomal insertion in which the long arm material from region “d” is excised from the black chromosome and inserted into a breakpoint that lies between long arm regions “n” and “o” of the white chromosome (left). Incomplete synapsis of the homologous chromosomes (center) followed by independent assortment would produce either balanced gametes (not shown) or unbalanced gametes with a pure duplication or deletion (right).

would result in gametes with either a pure duplication or a pure deletion involving the inserted segment. The remaining two segregants would produce gametes with a balanced insertion or a normal chromosome complement. Recombination between the paired bivalents would not be expected to alter the segregation outcomes. In situations involving large interchromosomal insertions, a quadrivalent with an insertion loop is expected to form during meiosis I, in contrast to the partially paired bivalents described for small insertions. Although simple malsegregation of this quadrivalent would yield the same four segregants described above, recombination within the insertion loop would yield recombinant chromosomes with both pure and combined duplications and deletions. The resulting unbalanced conceptuses would carry pure trisomies, pure monosomies or a combination of monosomies and trisomies (see Gardner and Sutherland [190] for a detailed description).

As described above for small interchromosomal insertions, small intrachromosomal insertions also typically form incompletely paired bivalents (Figure 9.29). A single meiotic crossover event (or any odd number of events) between the excision and insertion site of such a bivalent would then result in the formation of complementary deletion and duplication chromosomes. In the absence of such a recombination event, no unbalanced recombinant chromosomes would form and the resulting gametes would either carry the balanced intrachromosomal insertion or the normal homologue. The larger insertions are thought to maximize pairing by forming complex double loop structures. Recombination events involving these insertion

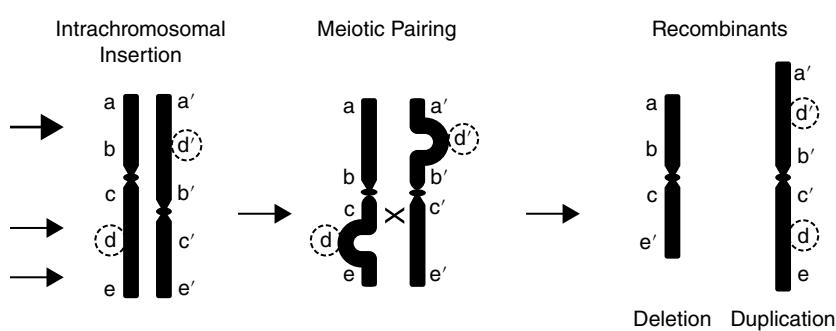


Figure 9.29 An intrachromosomal insertion in which long arm material represented by region “d” is excised (small arrows) from the long arm and inserted (large arrow) into the short arm of its homologue between regions “a” and “b” (left). A single recombination event involving the material between the excision and insertion sites (center) will produce gametes containing either a deletion or a complementary duplication (right).

loops are predicted to form recombination chromosomes with duplications, deletions or both. Survival of the resulting offspring would depend upon the extent of the resulting chromosome imbalance and whether critical dosage sensitive genes are involved. (See Gardner and Sutherland [190] for a description of the pairing options and possible segregants that are seen in association with intrachromosomal insertions.)

9.2.13 Complex chromosome rearrangements

Complex chromosome rearrangements are balanced or unbalanced structural anomalies involving at least two chromosomes and three breakpoints [191]. One of the most complex rearrangements reported to date involves five chromosomes and twenty-four breakpoints [102]. While complex rearrangements are relatively rare, a growing number are currently being reported in the literature. Increasingly, structures that appear to be simple balanced rearrangements based on traditional high resolution chromosome analysis are found to be complex rearrangements upon further characterization using FISH and other molecular techniques. Recently detailed molecular analysis of patients with either cancer or congenital disorders has led, in some cases, to the identification of chromothripsis [100,102,192]. The term chromothripsis is derived from the Greek words for “chromosome” (chromos) and for “shattered into pieces” (thripsis) and is used to describe a single, localized, catastrophic event that upon repair results in a complex chromosome rearrangement involving only one or a few chromosomes. NHEJ appears to be responsible for the majority of constitutional chromothripsis events identified thus far, while replicative repair mechanisms such as Fork Stalling and Template Switching (FoSTeS) appear to be responsible for others. See mechanisms section for more information

In general, the more complex the rearrangement, the greater the number of involved breakpoints and the higher the risk for an abnormal outcome. The abnormal phenotypes associated with some complex rearrangements have been attributed to cryptic duplications and deletions, gene interruptions, and/or the disruption of regulatory regions at one or more of the breakpoints [193–196].

The majority of complex chromosome rearrangements represent de novo events of paternal origin, although in rare instances they are found to be familial. In these familial cases the complex rearrangement tends to be less complicated, and it is inevitably inherited from a female carrier. These observations are in accord with the literature suggesting that approximately 80% of cytogenetically detectable de novo structural rearrangements are paternally derived [197] and that chromosome rearrangements may be more readily tolerated in female meiosis than in male meiosis. All of the chromosomes involved in a complex rearrangement would be expected to unite at the metaphase plate in a single intricate meiotic pairing structure. In theory, the more complex the rearrangement and the greater the number of chromosomes involved, the more intricate the meiotic pairing structure and the greater the number of potential unbalanced segregants. It is therefore not surprising that balanced carriers of a complex chromosome rearrangement have an empiric risk of greater than 50% for an abnormal pregnancy outcome. Interestingly, because the majority of these unbalanced pregnancies appear to end in miscarriage, the empiric risk for an unbalanced liveborn child does not differ significantly from that of a simple balanced reciprocal translocation carrier [198–200].

Table 9.1 Some of the more common recognized chromosomal abnormalities by chromosome number and a brief description of the syndrome's predominant phenotypic characteristics. Not all listed characteristics, however, will be seen in every individual diagnosed with these chromosomal anomalies

Chromosome	Abnormality	Description
1	del(1)(p36)	The most common terminal deletion; the deletion size is highly variable and typically ranges from ~1.5 Mb to ~10 Mb. Associated with intellectual disability, developmental delay, hearing impairment, seizures, growth impairment, hypotonia, heart defects, and dysmorphic features including large anterior fontanelles, microcephaly, brachycephaly, deep-set eyes, pointed chin, and a flat nose and nasal bridge.
2	inv(2)(p11.2q13)	A recurring pericentric inversion with no increased risk for liveborn unbalanced recombinant offspring, although the risk for spontaneous abortions and stillbirths appears to be increased approximately twofold.
	del(2)(q37.3)	Considerable phenotypic variation has been observed in patients with Albright Hereditary Osteodystrophy-like syndrome . The majority of patients have developmental delay, intellectual disability, autistic behaviors, and shortening of the metacarpal bones.
3	dup(3)(q26.3q27)	A few patients have been reported with rearrangements involving this region and phenotypic overlap with Cornelia de Lange syndrome . They have a characteristic facies (low anterior hairline, synophrys, anteverted nostrils, maxillary prognathism, long philtrum, downturned mouth) postnatal growth retardation, intellectual disability, and upper limb anomalies. Mutations in the <i>SMC1A</i> , <i>SMC3</i> , and especially the <i>NIPBL</i> genes are more common etiologies.
4	del(4)(p16.3)	The Wolf-Hirschhorn syndrome patients have deletions of variable size that typically include the 4p16.3 region. The phenotype includes severe growth and intellectual disability, microcephaly, a "Greek Warrior helmet" face, cleft lip and/or palate, cryptorchidism, hypospadius.
	t(4;8)(p16;p23)	One of only a few recurring constitutional reciprocal translocations . Mediated by olfactory receptor gene clusters on chromosomes 4 and 8. Patients with unbalanced karyotypes and the derivative 4 have features associated with Wolf-Hirschhorn syndrome.
5	del(5)(p15)	Cri-du-chat syndrome is named for the characteristic high pitched cry in infants with 5p deletions of variable size that include the p15.31 and/or distal p15.2 region. Microcephaly, severe motor and intellectual disability, round face, hypertelorism, and micrognathia are characteristic.
	del(5)(q35.2q35.3)	The Sotos syndrome phenotype includes overgrowth, macrocephaly, intellectual disability, hypotonia and poor coordination, advanced bone age, and a typical facies (prominent forehead, recessed hairline, long narrow face, pointed chin, hypertelorism and large ears). Deletions involving the <i>NSD1</i> gene are more common in the Japanese population (~50% versus 10-15% in others).
7	del(7)(q11.23q11.23)	The Williams syndrome phenotype includes supravalvular aortic stenosis, intellectual disability, growth restriction, hypercalcemia, an "elfin" facies, stellate iris pattern, a hoarse voice, and a friendly talkative personality.
	dup(7)(q11.23q11.23)	Patients with microduplications reciprocal to the microdeletions associated with Williams syndrome demonstrate mild to moderate intellectual disability, significant speech delay, and autistic behaviors. No strongly reproducible physical features have been identified.
8	del(8)(p23.1p23.1)	A recurring deletion associated with developmental delay, behavioral problems, congenital heart disease, diaphragmatic hernia, and a Frys syndrome-like condition.

(Continued)

Table 9.1 (Continued)

Chromosome	Abnormality	Description
	dup(8)(p23.1p23.1)	These duplications fall into one of two cytogenetically indistinguishable categories . The first category of duplications represents a benign variant, the molecular basis of which is copy number variation involving the beta-defensin gene cluster. The second category of duplications appears to be complementary to the recurring deletion listed above and is associated with a prominent forehead, arched eyebrows, developmental delay, and/or heart defects.
	inv(8)(p23.1q22.1)	This pericentric inversion is typically seen in individuals of Hispanic descent with ancestry from the San Luis Valley of southern Colorado and northern New Mexico. The recombinant chromosome 8 typically seen in their unbalanced offspring contains a duplication of q22.1 to qter material and a deletion of p23.1 to pter material. The associated phenotype includes intellectual disability, heart defects, seizures and a characteristic facies (hypertelorism, thin upper lip, anteverted nostrils, wide face, abnormal hair whorl, low-set ears, downturned mouth, and a low posterior hairline).
	del(8)(q23.3q24.11)	The Langer-Giedion syndrome or tricho-rhino-phalangeal syndrome type II phenotype includes intellectual disability, microcephaly, short stature, exostoses (benign bony growths), cone-shaped epiphyses, sparse scalp hair, large protruding ears, a long nose with a bulbous tip, a long flat philtrum, and thin lips.
	Trisomy 8	Although full trisomy 8 is often lethal, many patients have been reported with mosaic trisomy 8. The phenotype includes mild to moderate intellectual disability, poor coordination, a prominent forehead and ears, deep-set eyes, strabismus, hypertelorism, cleft palate, skeletal abnormalities, and deep palm and sole creases.
9	del(9)(p24)	Clinical findings include intellectual disability, delayed motor development, trigonocephaly (triangular shaped head), small palpebral fissures, a wide nasal bridge, a long philtrum and micro/retrognathia. Male to female sex reversal is thought to reflect haploinsufficiency of one or more of the three DMRT genes within this region.
	inv(9)(p11q13)	A recurring pericentric inversion that is not associated with an increased risk for liveborn unbalanced recombinant offspring, spontaneous abortions or stillbirths.
	Trisomy 9 mosaicism	Phenotype includes severe intellectual disability, growth deficiency, sloping forehead, deeply set eyes, joint contractures, and heart defects.
10	del(10)(p13p14)	Patients with a DiGeorge-like phenotype have been reported with visible partial deletions of 10p. This region is referred to as the DiGeorge critical region 2 (DGCR2).
11	dup(11)(p15.5p15.5)	Cytogenetically identifiable duplications, translocations and inversions involving this region have been seen in a minority of patients with Beckwith-Wiedemann syndrome . More commonly segmental UPD, a gene specific methylation defect, or a point mutation is identified. Clinical features include macroglossia (enlarged tongue), omphalocele (protrusion of abdominal contents through umbilicus), macrosomia (gigantism), ear creases, hypoglycemia, and a predisposition to tumors.
	del(11)(p13p13)	Interstitial deletions of this region are associated with WAGR syndrome , which is associated with Wilms tumor, aniridia (complete or partial absence of the iris), genitourinary anomalies, and intellectual disability (previously referred to as mental retardation).
	del(11)(p11.2p11.2)	Potocki-Shaffer syndrome is associated with multiple exostoses (benign bony growths), an enlarged parietal foramina, craniofacial dysostosis (defective ossification), and intellectual disability.

Table 9.1 (Continued)

Chromosome	Abnormality	Description
11	inv(11)(q21q23)	A recurring paracentric inversion common in the Netherlands and among the Canadian Hutterites. There is no increase in the rate of spontaneous abortions among carriers of the inversion or their partners and recombinant chromosomes arising from the inversion are thought to be rare.
	del(11)(q23.3)	Deletions including 11q23 are associated with Jacobson syndrome . Phenotype includes growth and psychomotor delay, trigonocephaly (triangular shaped head), strabismus, telecanthus, camptodactyly, and thrombocytopenia.
	t(11;22)(q23.3;q11.2)	One of only a few recurring constitutional reciprocal translocations ; mediated by palindromic repeat sequences on both chromosomes. Patients with unbalanced karyotypes inevitably have a supernumerary derivative 22 chromosome and Emanuel syndrome (see below).
12	mos + i(12)(p10)	Tetrasomy 12p secondary to an extra isochromosome of 12 short arm material is associated with Pallister–Killian syndrome . The isochromosome is typically found in fibroblasts, but not in the dividing lymphocytes. Phenotype includes profound intellectual disability, seizures, streaky pigmentation, sparse hair, and coarse facial features in older patients.
13	Trisomy 13 (Patau syndrome)	Associated with growth retardation, macrocephaly, sloped forehead, scalp defects, cleft lip/palate, micro- or anophthalmia (complete absence of the eyes), polydactyly, heart defects, renal anomalies, severe CNS malformation, and intellectual disability.
15	del(15)(q11.2q13.1) pat	Paternally inherited deletions are associated with Prader–Willi syndrome (PWS). Other less common etiologies for PWS include maternal UPD(15) and imprinting defects. The phenotype includes early feeding problems followed by obesity, hypotonia, intellectual disability, short stature, hypogonadism, small hands and feet, and abnormal behaviors.
	del(15)(q11.2q13.1) mat	Maternally inherited deletions are associated with Angelman syndrome (AS). Other less common etiologies for AS include paternal UPD(15), imprinting defects and mutations in the <i>UBE3A</i> gene. The phenotype includes severe motor and intellectual disability, ataxia, hypotonia, epilepsy, absence of speech, a large mandible, and frequent laughter.
	dup(15)(q11.2q13.1) mat	Maternally inherited duplications are associated with autism and intellectual disability. These duplications are complementary to the deletions observed in PWS/AS. A cytogenetically indistinguishable benign variant duplication also exists, the molecular basis of which is the presence of multiple copies of a gene cluster that maps proximal to the PWS/AS region.
16	+inv dup(15) or psu idic(15) (q11 ~ q13)	These small, dicentric chromosomes are typically composed of 2 copies of chromosome 15 short arm material, 2 centromeres and 2 copies of varying amounts of proximal 15q material. If the chromosome contains the PWS/AS region the patient will have intellectual disability and often autism. Those with smaller chromosomes lacking the PWS/AS region can be normal. This is the most common identifiable extra structurally abnormal chromosome seen in the human population.
	del(16)(p13.3)	Patients with very distal deletions involving the alpha hemoglobin loci (<i>HBA1</i> / <i>HBA2</i>) and adjacent genes have alpha-thalassemia/mental retardation (ATR-16) syndrome . Clinical features include alpha thalassemia, intellectual disability, and mild, nonspecific dysmorphic features.
	del(16)(p13.3p13.3)	Patients with more proximal deletions involving the <i>CREBBP</i> gene have Rubinstein–Taybi syndrome . Clinical features includes intellectual disability and growth restriction, broad thumbs and great toes, polydactyly, congenital heart and skeletal abnormalities, and facial dysmorphology including downslanting palpebral fissures, arched eyebrows, beaked nose, thin upper lip, and micrognathia.

(Continued)

Table 9.1 (Continued)

Chromosome	Abnormality	Description
	del(16)(p11.2p11.2)	A recurrent submicroscopic deletion of approximately 600 kb associated with a predisposition to a spectrum of abnormalities ranging from intellectual disability and/or multiple congenital anomalies to autism and learning and speech problems. Several patients with the complementary microduplication and a range of phenotypes including autism have also been reported.
17	del(17)(p13.3p13.3)	Miller–Dieker syndrome is associated with microcephaly, lissencephaly (smooth brain), intellectual disability, an unusual facial appearance, polydactyly, and malformations of the heart and kidney.
	del(17)(p12p12)	Hereditary Neuropathy with liability to Pressure Palsies (HNPP) is observed with deletions involving the p12 region and the <i>PMP22</i> gene. This disease is characterized by painless, episodic, recurrent peripheral neuropathy often preceded by minor trauma or toxic damage. The complementary microduplication causes CMTIA (see later).
	dup(17)(p12p12)	Charcot–Marie Tooth disease (CMTIA) is observed with duplications involving the 17p12 region and the <i>PMP22</i> gene. Common clinical symptoms include distal muscle weakness, wasting and impaired distal sensation in the legs and arms, and reduced or absent reflexes. The complementary microdeletion causes HNPP (see above).
	del(17)(p11.2p11.2)	The Smith–Magenis syndrome phenotype includes intellectual disability and growth restriction, brachycephaly, midface hypoplasia, prognathism, hoarse voice, and behavior problems including self-destructive behavior and sleep disturbances.
	dup(17)(p11.2p11.2)	Several patients with Potocki–Lupski syndrome have now been reported with visible duplication of the same region deleted in Smith–Magenis syndrome (see above). These patients have developmental delay without distinctive physical features.
	del(17)(q21.31q21.31)	A recurring submicroscopic deletion associated with developmental delays, hypotonia, feeding difficulties, seizures, growth retardation, and an abnormal facies (long face, ptosis, bulbous nasal tip, hypoplastic nostrils with a long columella, broad chin, large low-set ears, short philtrum, thin upper lip, and a protruding tongue).
18	del(18p)	Deletion of all or a large portion of 18p is a classic chromosome deletion syndrome associated with mental and growth deficiencies, hypotonia, microcephaly, holoprosencephaly, hypertelorism, epicanthal folds, ptosis, micrognathia, and large ears.
	del(18q)	A classic chromosome deletion syndrome involving relatively large interstitial and terminal deletions. The phenotype includes intellectual disability, short stature, hypotonia, foot deformities, hearing impairment, microcephaly, midface hypoplasia, deep-set eyes and a down-turned mouth.
	Trisomy 18 (Edwards syndrome)	Associated with intellectual disability, failure to thrive, heart malformations, hypertension, clenched fists, “rocker-bottom feet” with prominent heel bone, prominent occiput, micrognathia, low-set malformed ears, and short palpebral fissures.
20	del(20)(p12p12)	Alagille syndrome is characterized by a paucity of intrahepatic bile ducts, posterior embryotoxin (a congenital defect of the eye), and both cardiac and vertebral defects.
21	Trisomy 21 (Down syndrome)	These patients have intellectual disability, hypotonia, cardiac defects, short stature, brachycephaly with a flat occiput, short neck, flat nasal bridge, low-set abnormally folded ears, open mouth with protruding tongue, upslanting palpebral fissures, and epicanthal folds.
22	del(22)(q11.2q11.2)	The DiGeorge/VCF (velocardiofacial) syndrome phenotype is highly variable. Classic features include learning impairment, psychiatric disorders, hypocalcemia, susceptibility to infection due to thymic aplasia, short stature, conotruncal heart and palate abnormalities, malformed ears, and a bulbous nose with square nasal tip.

Table 9.1 (Continued)

Chromosome	Abnormality	Description
	dup(22)(q11.2q11.2)	Small interstitial duplication complementary to the deleted region associated with DiGeorge syndrome. DiGeorge-like features have been reported in some including palate abnormalities, thymic aplasia and heart defects. Their facial features are unique and include widely spaced eyes with superior placement of eyebrows, downslanting palpebral fissures, mild micro-/retrognathia, minor ear anomalies. Some have cognitive defects.
	+idic(22)(q11.2)	Cat-eye syndrome is typically associated with a small, extra, isochromosome and tetrasomy for proximal chromosome 22 material. The phenotype includes mild intellectual disability, coloboma of the iris, downslanting palpebral fissures, preauricular tags or pits, and anal atresia.
	+der(22)t(11;22) (q23.3;q11.2)	This chromosome is seen in the viable offspring of individuals who carry the recurring t(11;22) translocation and results in a double trisomy involving proximal chromosome 22 and distal chromosome 11 long arm material. Emanuel syndrome is associated with intellectual disability, congenital heart disease, malformed ears with preauricular skin tags or pits, a high arched or cleft palate, micrognathia, anal stenosis or atresia, renal aplasia or hypoplasia, and genital abnormalities in males.
	del(22)(q13.3)	The majority of deletions associated with Phelan–McDermid syndrome are terminal. The clinical presentation includes infantile hypotonia, normal growth, global developmental delays, absent or delayed speech, autistic-like behavior, and minor dysmorphic features.
X	del(X)(p22.3p22.3)	Males who carry a submicroscopic deletion of the <i>STS</i> gene have a skin disorder referred to as X-linked ichthyosis . Although mild manifestations are sometimes present in heterozygous females they are not typically affected. These deletions lie distal to those associated with Kallmann syndrome (see below).
	del(X)(p22.3p22.3)	Males with Kallmann syndrome typically carry a submicroscopic deletion involving the <i>KAL1</i> gene and demonstrate hypogonadotropic hypogonadism and anosmia (inability to smell). Carrier females typically have milder manifestations. These deletions lie proximal to those associated with X-linked ichthyosis (see above).
	dup(X)(p21.2p21.2)	Sex-reversed XY males result from duplication of the <i>NROB1 (DAX1)</i> gene. In the presence of this duplication, the male determining function of <i>SRY</i> is suppressed and ovarian development results.
	dup(X)(q22.2q22.2)	A large percentage of patients with Pelizaeus–Merzbacher disease contain duplications involving the <i>PLP1</i> gene and flanking sequences. Typical patients demonstrate nystagmus, spasticity, ataxia, and intellectual disability.
	Monosomy X (Turner syndrome)	These females typically have a 45,X karyotype but a variety of variant karyotypes are also seen. Clinical features include congenital lymphedema, short stature, sexual immaturity and infertility. The majority have normal intelligence but perceptual and spatial thinking is impaired.
	47,XXX (Trisomy X syndrome)	Features include tall stature and an increased risk for motor, speech, and learning difficulties.
	47,XXY (Klinefelter syndrome)	Features include tall, thin stature, small testes, infertility, poor motor development, and an increased risk for learning difficulties with reading and language skills most often affected.
Y	inv(Y)(p11.2;q11.23)	This pericentric inversion typically represents a benign variant; however some have been reported in males with infertility due to a small accompanying deletion. The incidence of this inversion is estimated to be 0.6/1000 males, but a much higher incidence (30.5%) has been reported in the Gujarati Muslim Indian population.
	47,YYY	Features include tall stature and an increased risk for motor, speech, and learning difficulties. Some suffer from attention deficits and impulsive tendencies.

References

1. Jacobs PA, Bikie AG, Court-Brown WM, Strong JA. The somatic chromosomes in mongolism. *Lancet* 1959;1:710.
2. Lejueune J, Gautier M, Turpin R. etudes des chromosome somatiques de neuf enfants mongoliens. *CR Acad Sci Paris* 1959;248:1721–1722.
3. Ford CE, Jones KW, Polani PE, De Almeida JC, Briggs JH. A sex-chromosome anomaly in a case of gonadal dysgenesis (Turner's syndrome). *Lancet* 1959;1:711–713.
4. Jacobs PA, Strong JA. A case of human intersexuality having a possible XXY sex-determining mechanism. *Nature* 1959;183:302–303.
5. Jacobs PA, Baikie AG, Brown WM, Macgregor TN, Maclean N, Harnden DG. Evidence for the existence of the human "super female". *Lancet* 1959;2:423–425.
6. Hassold T, Abruzzo M, Adkins K, Griffin D, Merrill M, Millie E, Saker D, Shen J, Zaragoza M. Human aneuploidy: incidence, origin and etiology. *Environ Mol Mutagen* 1996;28:167–175.
7. Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet* 2001;2:280–291.
8. Benn PA, Hsu LYF. Prenatal diagnosis of chromosomal abnormalities through amniocentesis. In: Milunsky A, ed. *Genetic Disorders of the Fetus: Diagnosis, Prevention, and Treatment*. Baltimore: Johns Hopkins University Press, 2004;214–296.
9. Vicić A, Roje D, Strinić T, Stipoljev F. Trisomy 1 in an early pregnancy failure. *Am J Med Genet* 2008;146A:2439–2441.
10. Sybert VP, McCauley E. Turner's syndrome. *N Engl J Med* 2004;351(12):1227–1238.
11. Bondy CA. Care of girls and women with Turner syndrome: a guideline of the Turner syndrome study group. *J Clin Endo Metab* 2007;92(1):10–25.
12. Wolff DJ, Van Dyke DL, Powell CM; Working Group of the ACMG Laboratory Quality Assurance Committee. Laboratory guideline for Turner syndrome. *Genet Med* 2010;12(1):52–55.
13. Angell RR. Predivision in human oocytes at meiosis I: a mechanism for trisomy formation in man. *Hum Genet* 1991;86(4):383–387.
14. Angell RR. Aneuploidy in older women. Higher rates of aneuploidy in oocytes from older women. *Hum Reprod* 1994;9(7):1199–1200.
15. Angell R. First-meiotic-division nondisjunction in human oocytes. *Am J Hum Genet* 1997;61(1):23–32.
16. Volarcik K, Sheean L, Goldfarb J, Woods L, Abdul-Karim FW, Hunt P. The meiotic competence of in-vitro matured human oocytes is influenced by donor age: evidence that folliculogenesis is compromised in the reproductively aged ovary. *Hum Reprod* 1998;13(1):154–160.
17. Gabriel AS, Thornhill AR, Ottolini CS, Gordon A, Brown APC, Taylor J, Bennett K, Handyside A, Griffin DK. Array comparative genomic hybridization on first polar bodies suggests that non-disjunction is not the predominant mechanism leading to aneuploidy in humans. *J Med Genet* 2011;48:433–37.
18. Pellestor, F, Andreo B, Arnal F, Humeau C, Demaille J. Mechanisms of nondisjunction in human female meiosis: the co-existence of two modes of malsegregation evidenced by the karyotyping of 1397 in-vitro unfertilized oocytes. *Hum Reprod* 2002;17(8):2134–2145.
19. Pellestor, F, Andreo B, Arnal F, Humeau C, Demaille J. Maternal aging and chromosomal abnormalities: new data drawn from in vitro unfertilized human oocytes. *Hum Genet* 2003;112:195–203.
20. Benn PA, Hsu LYF. Prenatal diagnosis of chromosomal abnormalities through amniocentesis. In: Milunsky A, ed. *Genetic Disorders of the Fetus; Diagnosis, Prevention, and Treatment*. Baltimore: Johns Hopkins University Press, 2004;247–249.
21. Hassold H, Hall H, Hunt P. The origin of human aneuploidy: where we have been, where we are going. *Hum Mol Genet* 2007;16(2):R203–R204.
22. Hall H, Hunt P, Hassold T. Meiosis and sex chromosome aneuploidy: how meiotic errors cause aneuploidy; how aneuploidy causes meiotic errors. *Curr Opin Genet Devel* 2006;16:323–329.
23. Hook EB. Exclusion of chromosomal mosaicism: tables of 90%, 95% and 99% confidence limits and comments on use. *Am J Hum Genet* 1977;29:94–97.
24. Ballif BC, Rorem EA, Sundin K, Lincicum M, Gaskin S, Coppinger J, Kashork CD, Shaffer LG, Bejjani BA. Detection of low-level mosaicism by array CGH in routine diagnostic specimens. *Am J Med Genet* 2006;140A:2757–2767.

25. Cheung SW, Shaw CA, Scott DA, Patel A, Sahoo T, Bacino CA, Pursley A, Li J, Erickson R, Gropman AL, Miller DT, Seashore MR, Summers AM, Stankiewicz P, Chinault AC, Lupski JR, Beaudet AL, Sutton VR. Microarray-based CGH detects chromosomal mosaicism not revealed by conventional cytogenetics. *Am J Med Genet* 2007;143A:1679–1686.
26. Shinawi M, Shao L, Jeng LJB, Shaw CA, Patel A, Bacino C, Sutton VR, Belmont J, Cheung SW. Low-level mosaicism of trisomy 14: phenotypic and molecular characterization. *Am J Med Genet* 2008;146A:1395–1405.
27. Oliver TR, Feingold E, Yu K, Cheung V, Tinker S, Yadav-Shah M, Masse M, Sherman SL. New insights into human nondisjunction of chromosome 21 in oocytes. *PLoS Genetics* 2008;4(3):1–9.
28. Allen EG, Freeman SB, Druschel C, Hobbs CA, O’Leary LA, Romitti PA, Royle MH, Torfs CP, Sherman SL. Maternal age and risk for trisomy 21 assessed by the origin of chromosome nondisjunction: a report from the Atlanta and National Down Syndrome projects. *Hum Genet* 2009;125:41–52.
29. De Souza E, Alberman E, Morris JK. Down syndrome and paternal age, a new analysis of case-control data collected in the 1960s. *Am J Med Genet* 2009;149A:1205–1208.
30. Oliver TR, Bhise A, Feingold E, Tinker S, Masse N, Sherman SL. Investigation of factors associated with paternal nondisjunction of chromosome 21. *Am J Med Genet* 2009;149A:1685–1690.
31. Hodges CA, Revenkova E, Jessberger R, Hassold TJ, Hunt PA. SMC1 beta -deficient mice provide evidence that cohesins are a missing link in age-related nondisjunction. *Nat Genet* 2005;37(12):1351–1355.
32. Subramanian VV, Bickel SE. Aging predisposes oocytes to meiotic nondisjunction when the cohesin subunit SMC1 is reduced. *PLoS Genet* 2008;4(11):1–12.
33. Hassold T, Hunt P. Rescuing distal crossovers. *Nat Genet* 2007;39:1187–1190.
34. Hunt PA, Hassold TJ. Human female meiosis: what makes a good egg go bad? *Trends in Genet* 2008;24(2):86–93.
35. Thomas NS, Hassold TJ. Aberrant recombination and the origin of Klinefelter syndrome. *Hum Reprod Update* 2003;9:309–317.
36. Lamb NE, Feingold E, Savage A, Avramopoulos D, Freeman S, Gu Y, Hallberg A, Hersey J, Karadima G, Pettay D, Saker D, Shen J, Taft L, Mikkelsen M, Petersen MB, Hassold T, Sherman SL. Characterization of susceptible chiasma configuration that increase the risk for maternal nondisjunction of chromosome 21. *Hum Mol Genet* 1997;6:1391–1399.
37. Lamb NE, Yu K, Shaffer J, Feingold E, Sherman SL. Association between maternal age and meiotic recombination for trisomy 21. *Am J Hum Genet* 2005;76(1):91–99.
38. Bugge M, Collins A, Petersen MB, Fisher J, Brandt C, Hertz JM, Tranebjærg L, de Lozier-Blanchet C, Nicolaides P, Brondum-Nielsen K, Morton N, Mikkelsen M. Nondisjunction of chromosome 18. *Hum Mol Genet* 1998;7:661–669.
39. Thomas NS, Ennis S, Sharp AJ, Durkie M, Hassold TJ, Collins AR, Jacobs PA. Maternal sex chromosome nondisjunction: evidence for X chromosome-specific risk factors. *Hum Mol Genet* 2001;10:243–250.
40. Bickel SE. Aging (not so) gracefully. *Nat Genet* 2005;37(12):1303–1304.
41. Sherman SL, Freeman SB, Allen EG, Lamb NE. Risk factors for nondisjunction of trisomy 21. *Cytogenet Genome Res* 2005;111:273–280.
42. Warren WD, Gorringe KL. A molecular model for sporadic human aneuploidy. *Trends in Genet* 2006;22(4):218–224.
43. Susiarjo M, Hassold TJ, Freeman E, Hunt PA. Bisphenol A exposure in utero disrupts early oogenesis in the mouse. *PLoS Genet* 2007;3(1):63–70.
44. Muhlhauser A, Susiarjo M, Rubio C, Griswold J, Gorence G, Hassold T, Hunt PA. Bisphenol A effects on the growing mouse oocyte are influenced by diet. *Biol Reprod* 2009;80(5):1066–1071.
45. Pacchierotti F, Adler I-D, Eichenlaub-Ritter U, Mailhes JB. Gender effects on the incidence of aneuploidy in mammalian germ cells. *Environ Res* 2007;104:46–69.
46. Hultén MA, Patel SD, Tankimanova M, Westgren M, Papadogiannakis N, Jonsson AM, Iwarsson E. On the origin of trisomy 21 Down syndrome. *Mol Cytogenet* 2008;1(21):1–10.
47. Hultén MA, Patel S, Jonasson J, Iwarsson E. On the origin of the maternal age effect in trisomy 21 Down syndrome: the Oocyte Mosaicism Selection model. *Reproduction* 2010;139(1):1–9.
48. Jacobs P, Dalton P, James R, Mosse K, Power M, Robinson D, Skuse D. Turner syndrome: a cytogenetic and molecular study. *Ann Hum Genet* 1997;61:471–483.
49. Nussbaum RL, McInnes RR, Huntington FW. *Genetics in Medicine*, 6th ed. Philadelphia: WB Saunders, 2004.

50. Bukvic N, Gentile M, Susca F, Fanelli M, Serio G, Buonadonna L, Capurso A, Guanti G. Sex chromosome loss, micronuclei, sister chromatid exchange and aging: a study including 16 centenarians. *Mut Res* 2001;498(1–2):159–167.
51. Nowinski GP, Van Dyke DL, Tilley BC, Jacobsen G, Babu VR, Worsham MJ, Wilson GN, Weiss L. The frequency of aneuploidy in cultured lymphocytes is correlated with age and gender but not with reproductive history. *Am J Hum Genet* 1990;46:1101–1111.
52. Russell LM, Strike P, Browne CE, Jacobs PA. X chromosome loss and aging. *Cytogenet Genome Res* 2007;116:181–185.
53. Abruzzo MA, Mayer M, Jacobs PA. Aging and aneuploidy: evidence for the preferential involvement of the inactive x chromosome. *Cytogenet Cell Genet* 1985;39:275–278.
54. Surralles J, Jeppesen P, Morrison H, Natarajan AT. Analysis of loss of the inactive X chromosome in interphase cells. *Am J Med Genet* 1996;59(5):1091–1096.
55. Gardner RJM, Sutherland GR. Down syndrome, other full aneuploidies and polyploidy. In: Motulsky AG, Bobrow M, Harper PS, Scriver C, Epstein CJ, Hall J, eds. *Chromosome Abnormalities and Genetic Counseling*. New York: Oxford University Press, 2004;249–263.
56. Warburton D, Dallaire L, Thangavelu M, Ross L, Levin B, Kline J. Trisomy recurrence: a reconsideration based on North American data. *Am J Hum Genet* 2004; 75:376–385.
57. Warburton D, Byrne J, Canki N. *Chromosome Anomalies and Prenatal Development: An Atlas. Oxford Monographs on Medical Genetics*, No 21. New York: Oxford University Press, 1991.
58. Neuber M, Rehder H, Zuther C, Lettau R, Schwinger E. Polyploidies in abortion material decrease with maternal age. *Hum Genet* 1993; 91:563–566.
59. Zaragoza MV, Surti U, Redline RW, Millie E, Chakravarti A, Hassold TJ. Parental origin and phenotype of triploidy in spontaneous abortions: predominance of diandry and association with the partial hydatidiform mole. *Am J Hum Genet* 2000;66:1807–1820.
60. McFadden DE, Jiang R, Langlois S, Robinson WP. Dispermy-origin of diandric triploidy. *Hum Reprod* 2002;17:3037–3038.
61. Rosenbusch BE. Mechanisms giving rise to triploid zygotes during assisted reproduction. *Fertil Steril* 2008;90:49–55.
62. Iliopoulos D, Vassiliou G, Sekerli E, Sidiropoulou V, Tsiga A, Dimopoulou D, Voyatzis N. Long survival in a 69,XXX triploid infant in Greece. *Genet Mol Res* 2005;4(4):755–759.
63. Golubovsky MD. Postzygotic diploidization of triploids as a source of unusual cases of mosaicism, chimerism and twinning. *Hum Reprod* 2003;18(2):236–242.
64. Book JA, Santesson B. Malformation syndrome in man associated with triploidy (69 chromosomes). *Lancet* 1960;1:858–859.
65. Van de Laar I, Rabelink G, Hochstenbach R, Tuerlings J, Hoogeboom J, Giltay J. Diploid/triploid mosaicism in dysmorphic patients. *Clin Genet* 2002;62:376–382.
66. Baumer A, Dres D, Basaran S, Isci H, Dehgan T, Schinzel A. Parental origin of the two additional haploid sets of chromosomes in an embryo with tetraploidy. *Cytogenet Genome Res* 2003;101:5–7.
67. Rosenbusch B, Schneider M. A brief look at the origin of tetraploidy. *Cytogenet Genome Res* 2004;107:128–131.
68. Guc-Scekic M, Milasin J, Stevanovic M, Stojanov LJ, Djordjevic M. Tetraploidy in a 26-month-old girl (cytogenetic and molecular studies). *Clin Genet* 2002;61:62–65.
69. Nakamura Y, Takaira M, Sato E, Kawano K, Miyoshi S, Niikawa N. A tetraploid liveborn neonate. *Arch Pathol Lab Med* 2003;127:1612–1614.
70. Quiroz E, Orozco A, Salamanca F. Diploid-tetraploid mosaicism in a malformed boy. *Clin Genet* 1985;27:183–186.
71. Edwards MJ, Park J, Wurster-Hill DH, Graham JM Jr. Mixoploidy in humans: two surviving cases of diploid-tetraploid mixoploidy and comparison with diploid-triploid mixoploidy. *Am J Med Genet* 1994;52:324–330.
72. Benn PA, Hsu LYF. Prenatal diagnosis of chromosomal abnormalities through amniocentesis. In: Milunsky A, ed. *Genetic Disorders of the Fetus: Diagnosis, Prevention, and Treatment*. Baltimore: Johns Hopkins University Press, 2004;261.
73. Noomen P, Van den Berg C, de Ruyter JL, Van Opstal D, Los FJ. Prevalence of tetraploid metaphases in semidirect and cultured chorionic villi. *Fetal Diagn Ther* 2001;16(3):129–132.
74. Jacobs P. Epidemiology of chromosome abnormalities in man. *Am J Epidemiol* 1977;105(3):180–191.

75. Gardner RJM, Sutherland GR. Variant chromosomes and abnormalities of no phenotypic consequence. In: Motulsky AG, Bobrow M, Harper PS, Scriver C, Epstein CJ, Hall J, eds. *Chromosome Abnormalities and Genetic Counseling*. New York: Oxford University Press, 2004;222–246.
76. Gardner RJM, Sutherland GR. Variant chromosomes and abnormalities of no phenotypic consequence. In: Motulsky AG, Bobrow M, Harper PS, Scriver C, Epstein CJ, Hall J, eds. *Chromosome Abnormalities and Genetic Counseling*. New York: Oxford University Press, 2004;136.
77. Olson SB, Magenis E. Preferential paternal origin of de novo structural chromosome rearrangements, In: Daniel A, eds. *The Cytogenetics of Mammalian Autosomal Rearrangements*. New York: Alan R. Liss Inc., 1988;583–589.
78. Chandley AC. On the parental origin of *de novo* mutation in man. *J Med Genet* 1991;28:217–223.
79. Batista DAS, Pai GS, Stetten G. Molecular analysis of a complex chromosomal rearrangement and a review of familial cases. *Am J Med Genet* 1994;53: 255–263.
80. El-Sawy M, El-Sayed N. Cell division. *Ain Shams J Obstet Gynecol* 2005;2:358–361.
81. Page SL, Shaffer LG. Nonhomologous Robertsonian translocations form predominantly during female meiosis. *Nat Genet* 1997;15, 231–232.
82. Wu Y-Q, Heilstedt HA, Bedell JA, May KM, Starkey DE, McPherson JD, Shapira SK, Shaffer LG. Molecular refinement of the 1p36 deletion syndrome reveals size diversity and a preponderance of maternally derived deletions. *Hum Mol Genet* 1999; 8:312–321.
83. Zhang Y, Rowley JD. Chromatin structural elements and chromosomal translocations in leukemia. *DNA Repair (Amst)* 2006;5:1282–1297.
84. Gu W, Zhang F, Lupski JR. Mechanisms for human genomic rearrangements. *Pathogenetics* 2008;1(4):1–17.
85. Deininger PL, Batzer MA. Alu repeats and human disease. *Mol Genet Metab* 1999;67:183–193.
86. Shaw CJ, Lupski JR. Non-recurrent 17p11.2 deletions are generated by homologous and nonhomologous mechanisms. *Hum Genet* 2005;116: 1–7.
87. Stankiewicz P, Lupski JR. Genome architecture, rearrangements and genomic disorders. *Trends Genet* 2002;18(2):74–82.
88. Giglio S, Calvari V, Gregato G, Gimelli G, Camanini C, Giorda R, Ragusa A, Guerrieri S, Selicorni A, Stumm M, Tonnies H, Ventura M, Zollino M, Neri G, Barber J, Wieczorek D, Rocchi M, Zuffardi O. Heterozygous submicroscopic inversions involving olfactory receptor-gene clusters mediate the recurrent t(4;8)(p16;p23) translocation. *Am J Hum Genet* 2002; 71:276–285.
89. Stankiewicz P, Shaw CJ, Dapper JD, Wakui K, Shaffer LG, Withers M, Elizondo L, Park S-S, Lupski JR. Genome architecture catalyzes nonrecurrent chromosomal rearrangements. *Am J Hum Genet* 2003;72:1101–1116.
90. Kurahashi H, Bolor H, Kato T, Kogo H, Tsutsumi M, Inagaki H, Ohye T. Recent advance in our understanding of the molecular nature of chromosome abnormalities. *J Hum Genet* 2009;54:253–260.
91. Oldenburg J, El-Maari O. New insight into the molecular basis of hemophilia A. *Int J Hematol* 2006;83:96–102.
92. Shaw CJ, Lupski JR. Implications of human genome architecture for rearrangement-based disorders: the genomic basis for disease. *Hum Mol Genet* 2004;13(1):R57–R64.
93. Lupski JR. Genome structural variation and sporadic disease traits. *Nat Genet* 2006;38(9):974–976.
94. Giglio S, Broman KW, Matsumoto N, Calvari V, Gimelli G, Neumann T, Ohashi H, Vouillaire L, Larizza D, Giorda R, Weber JL, Ledbetter DH, Zuffardi O. Olfactory receptor-gene clusters, genomic-inversion polymorphisms, and common chromosome rearrangements. *Am J Hum Genet* 2001;68:874–883.
95. Shimokawa O, Kurosawa K, Ida T, Harada N, Kondoh T, Miyake N, Yoshiura K, Kishino T, Ohta T, Niikawa N, Matsumoto N. Molecular characterization of inv dup del(8p): analysis of five cases. *Am J Med Genet* 2004;128A(2):133–137.
96. Kurahashi H, Shaikh T, Takata M, Toda T, Emanuel BS. The constitutional t(17;22): another translocation mediated by palindromic AT-rich repeats. *Am J Hum Genet* 2003; 72:733–738.
97. Kurahashi H, Inagaki H, Ohye T, Kogo H, Kato T, Emanuel BS. Palindrome-mediated chromosomal translocations in humans. *DNA Repair* 2006;5:1136–1145.
98. Lee JA, Carvalho CMB, Lupski JR. A DNA replication mechanism for generating nonrecurrent rearrangements associated with genomic disorders. *Cell* 2007;131:1235–1247.

99. Zhang F, Khajavi M, Connolly AM, Towne CF, Batish SD, Lupski JR. The DNA replication FoSTeS/MMBIR mechanism can generate genomic, genic and exonic complex rearrangements in humans. *Nat Genet* 2009;41(7):849–853.
100. Kloosterman WP, Cuppen E. Chromothripsis in congenital disorders and cancer: similarities and differences. *Curr Opin Cell Biol* 2013;25:341–348.
101. Maher CA, Wilson RK. Chromothripsis and human disease: piecing together the shattering process. *Cell* 2012;148:29–32.
102. Kloosterman WP, Tavakoli-Yaraki M, van Roosmalen MJ, van Binsbergen E, Renkens I, Duran K, Ballarati L, Vergult S, Giardino D, Hansson K, Ruivenkamp CA, Jager M, van Haeringen A, Ippel EF, Haaf T, Passarge E, Hochstenbach R, Menten B, Larizza L, Guryev V, Poot M, Cuppen E. Constitutional chromothripsis rearrangements involve clustered double stranded DNA breaks and nonhomologous repair mechanisms. *Cell Rep* 2012;1(6):648–655.
103. Liu P, Erez A, Sreenath Nagamani SC, Dhar SU, Kolodziejska KE et al. Chromosome catastrophes involving replication mechanisms generating complex genomic rearrangements. *Cell* 2011;146:889–903.
104. Gardner RJM, Sutherland GR. Variant chromosomes and abnormalities of no phenotypic consequence. In: Motulsky AG, Bobrow M, Harper PS, Scriver C, Epstein CJ, Hall J, eds. *Chromosome Abnormalities and Genetic Counseling*. New York: Oxford University Press, 2004;243,244.
105. Schwartz S, Kumar A, Becker LA, Crowe CA, Haren JM, Tsuchiya K, Wandstrat AE, Wolff DJ. Molecular and cytogenetic analysis of de novo “terminal” deletions: implications for mechanism of formation. *Am J Hum Genet Suppl* 1997;61: A7.
106. Helstedt HA, Ballif BC, Howard LA, Lewis RA, Stal S, Kashork CD, Bacino CA, Shapira SK, Shaffer LG. Physical map of 1p36, placement of breakpoints in monosomy 1p36 and clinical characterization of the syndrome. *Am J Hum Genet* 2003;72:1200–1212.
107. Yatsenko SA, Brundage EK, Roney EK, Cheung SW, Chinault AC, Lupski JR. Molecular mechanisms for subtelomeric rearrangements associated with the 9q34.3 microdeletion syndrome. *Hum Mol Genet* 2009;18(11):1924–1936.
108. Wilkie AOM, Lamb J, Harris PC, Finney RD, Higgs DR. A truncated human chromosome 16 associated with alpha thalassaemia is stabilized by addition of telomeric repeat (TTAGGG)_n. *Nature* 1990;346:868–871.
109. Flint J, Craddock CF, Villegas A, Bentley DP, Williams HJ, Galenello R, Cao A, Wood WG, Ayyub H, Higgs DR. Healing of broken human chromosomes by the addition of telomeric repeats. *Am J Hum Genet* 1994;55(3):505–512.
110. Varley H, Di S, Scherer SW, Royle NJ. Characterization of terminal deletions at 7q32 and 22q13.3 healed by de novo telomere addition. *Am J Hum Genet* 2000;67(3):610–622.
111. Ballif BC, Kashork CD, Shaffer LG. FISHing for mechanisms of cytogenetically defined terminal deletions using chromosome-specific subtelomeric probes. *Eur J Hum Genet* 2000;8:764–770.
112. Ballif BC, Wakui K, Gajecka M, Shaffer LG. Translocation breakpoint mapping and sequence analysis in three monosomy 1p36 subjects with der(1)t(1;1)(p36;q44) suggest mechanisms for telomere capture in stabilizing de novo terminal rearrangements. *Hum Genet* 2004;114:198–206.
113. Meltzer PS, Guan X-Y, Trent JM. Telomere capture stabilizes chromosome breakage. *Nat Genet* 1993;4(3):252–255.
114. Gardner RJM, Sutherland GR. Inversions. In: Motulsky AG, Bobrow M, Harper PS, Scriver C, Epstein CJ, Hall J, eds. *Chromosome Abnormalities and Genetic Counseling*. New York: Oxford University Press, 2004;144.
115. Smith ACM, Spuhler K, Williams TM, McConnell T, Sujansky E, Robinson A. Genetic risk for recombinant 8 syndrome and the transmission rate of balanced inversion 8 in the Hispanic population of the southwestern United States. *Am J Hum Genet* 1987;41:1083–1103.
116. Madan K, Pieters MHEC, Kuyt LP, van Asperen CJ, de Pater JM, Hamers AJH, Gerssen-Schoorl KBJ, Hustinx TWJ, Breed ASPM, Van Hemel JO, Smeets DFCM. Paracentric inversion inv(11)(q21q23) in The Netherlands. *Hum Genet* 1990;85:15–20.
117. Chodirker BN, Greenberg CR, Pabello PD, Chudley AE. Paracentric inversion 11q in Canadian Hutterites. *Hum Genet* 1992;89:450–452.
118. Lupski JR. Genome structural variation and sporadic disease traits. *Nat Genet* 2006;38(9):974–976.
119. Bunge S, Rathmann M, Steglich C, Bondeson M-L, Tylki-Szymanska A, Popowska E, Gal A. Homologous nonallelic recombination between the iduronate-sulfatase gene and pseudogene cause various intragenic deletions and inversions in patients with mucopolysaccharidosis type II. *Eur J Hum Genet* 1998;6:492–500.

120. Madan K, Nieuwint AWM. Reproductive risks for paracentric inversion heterozygotes: inversion or insertion? That is the question. *Am J Med Genet* 2002;107:340–343.
121. Saffery R, Irvine DV, Griffiths B, Kalitsis P, Wordeman I, Choo KH. Human centromeres and neocentromeres show identical distribution patterns of >20 functionally important kinetochore-associated proteins. *Hum Mol Gene* 2000;9:175–185.
122. Marshall OJ, Chueh AC, Wong LH, Choo KH. Neocentromeres: new insights into centromere structure, disease development, and karyotype evolution. *Am J Hum Genet* 2008;82:261–282.
123. Earle E, Shaffer LG, Klitsis P, McQuillan C, Dale S, Choo KHA. Identification of DNA sequences flanking the breakpoint of human t(14q21q) Robertsonian translocations *Am J Hum Genet* 1992;50:717–724.
124. Gravholt CH, Friedrich U, Caprani M, Jorgensen AL. Breakpoints in Robertsonian translocations are localized to satellite III DNA by fluorescence in situ hybridization. *Genomics* 1992;14:924–930.
125. Wolff DJ, Schwartz S. Characterization of Robertsonian translocations by using fluorescence in situ hybridizations. *Am J Med Genet* 1992;50:174–178.
126. Wolff DJ, Miller AP, VanDyke DL, Schwartz S, Willard HF. Molecular definition of breakpoints associated with human Xq isochromosomes: implication for mechanisms of formation. *Am J Hum Genet* 1996;58:154–160.
127. Lemyre E, Der Kaloustian VM, Duncan AMV. Stable non-Robertsonian dicentric chromosomes: four new cases and review. *J Med Genet* 2001;38:76–79.
128. Battaglia A. The inv dup(15) or idic(15) syndrome (tetrasomy 15). *Orphanet J Rare Dis* 2008;3:30.
129. Sullivan BA, Schwartz S. Identification of centromeric antigens in dicentric Robertsonian translocations: CENP-C and CENP-E are necessary components of functional centromeres. *Hum Mol Genet* 1995;4(12):2189–2197.
130. Sullivan BA, Willard HF. Functional status of centromeres in dicentric X chromosomes: evidence for the distance-dependence of centromere/kinetochore assembly and correlation with malsegregation in anaphase. *Am J Hum Genet Suppl* 1996;59(4):A14.
131. Schwartz S, Depinet TW. Studies of “acentric” and “dicentric” marker chromosomes: implications for definition of the functional centromere. *Am J Hum Genet Suppl* 1996;59(4):A14.
132. Shaffer LG, McCaskill C, Haller V, Brown JA, Jackson-Cook CK. Further characterization of 19 cases of rea(21q21q) and delineation as isochromosomes or Robertsonian translocations in Down syndrome. *Am J Med Genet* 1993;47:1218–1222.
133. Shaffer LG, McCaskill C, Han J-Y, Choo KHA, Cutillo DM, Donnenfeld AE, Weiss L, Van Dyke DL. Molecular characterization of de novo secondary trisomy 13. *Am J Hum Genet* 1994;55:968–974.
134. Fioretos T, Strombeck B, Sandberg T, Johansson B, Billstrom R, Borg A, Nilsson PG, Van Den Berghe H, Hagemeijer A, Mitelman F, Hoglund M. Isochromosome 17q in blast crisis of chronic myeloid leukemia and other hematologic malignancies is the result of clustered breakpoints in 17p11 and is not associated with coding TP53 mutations. *Blood* 1999;94:225–232.
135. Darlington CD. Misdivision and the genetics of the centromere. *J Genet* 1939;37(2):341–364.
136. Kaiser-Rogers K, Rao K. Structural Chromosome Rearrangements. In: Gersen SL, Keagle MB, eds. *The Principles of Clinical Cytogenetics*, 2nd ed. New Jersey: Humana Press, 2005;165–206.
137. Barbouti A, Stankiewicz P, Nusbaum C, Cuomo C, Cook A, Hoglund M, Johansson B, Hagemeijer A, Park SS, Sung-Sup P, Mitelman F, Lupski JR, Fioretos T. The breakpoint region of the most common isochromosome, i(17q), in human neoplasia is characterized by a complex genomic architecture with large, palindromic, low copy repeats. *Am J Hum Genet* 2004;74:1–10.
138. Chen H, Young R, Mu X, Nandi K, Miao S, Prouty L, Ursin S, Gonzalez J, Yanamandra K. Uniparental isodisomy resulting from 46,XX,i(1p),i(1q) in a woman with short stature, ptosis, micro/retrognathia, myopathy, deafness and sterility. *Am J Med Genet* 1999;82(3):215–218.
139. Bjorck EJ, Anderlid BM, Blennow E. Maternal isodisomy of chromosome 9 with no impact on the phenotype in a woman with two isochromosomes: i(9p) and i(9q). *Am J Med Genet* 1999;87(1):49–52.
140. Albrecht B, Mergenthaler S, Eggermann K, Zerres K, Passarge E, Eggermann T. Uniparental isodisomy for paternal 2p and maternal 2q in a phenotypically normal female with two isochromosomes, i(2p) and i(2q). *J Med Genet* 2001;38(2):214.

141. Kovaleva NV. Nonmosaic balanced homologous translocations of major clinical significance: some may be mosaic. *Am J Med Genet* 2007;143A:2843–2850.
142. Baumer A, Basaran S, Taralczak M, Cefle K, Ozturk S, Palanduz S, Schinzel A. Initial maternal meiotic I error leading to the formation of a maternal i(2q) and a paternal i(2p) in a healthy male. *Cytogenet Genome Res* 2007;118(1):38–41.
143. Callen DF, Mulley JC, Baker EG, Sutherland GR. Determining the origin of human X isochromosomes by use of DNA sequence polymorphisms and detection of an apparent i(Xq) with Xp sequences. *Hum Genet* 1987;77:236–240.
144. Harbison M, Hassold T, Kobryn C, Jacobs PA. Molecular studies of the parental origin and nature of human X isochromosomes. *Cytogenet Cell Genet* 1988;47:217–222.
145. Antonarakis SE, Adelsberger PA, Petersen MB, Binkert F, Schinzel AA. Analysis of DNA polymorphisms suggests that most de novo dup(21q) chromosomes in patients with Down syndrome are isochromosomes and not translocations. *Am J Hum Genet* 1990;47:968–972.
146. Lorda-Sanchez I, Binkert F, Maechler M, Schinzel A. A molecular study of X isochromosomes: parental origin, centromeric structure, and mechanisms of formation. *Am J Hum Genet* 1991;49:1034–1040.
147. Robinson WP, Bernasconi F, Basaran S, Yuksel-Apak M, Neri G, Serville F, Balicek P, Haluza R, Farah LMS, Luleci G, Schinzel AA. A somatic origin of homologous Robertsonian translocations and isochromosomes. *Am J Hum Genet* 1994;54:290–302.
148. Bugge M, deLozier-Blanchet C, Bak M, Brandt CA, Hertz JM, Nielsen JB, Duprez L, Petersen MB. Trisomy 13 due to rea(13q;13q) is caused by i(13) and not rob(13;13)(q10;q10) in the majority of cases. *Am J Med Genet* 2005;132A:310–313.
149. Riegel M, Wisser J, Baumer A, Schinzel A. Postzygotic isochromosome formation as a cause for false-negative results from chorionic villus chromosome examinations. *Prenat Diagn* 2006;26:221–225.
150. De Ravel TJL, Keymolen K, van Assche E, Wittevronghel I, Moerman P, Sadde I, Matthijs, JSG, Fryns JB, Vermeesch JR. Post-zygotic origin of isochromosome 12p. *Prenat Diagn* 2004;24:984–988.
151. Wyandt HE. Ring autosomes: identification, familial transmission, causes of phenotypic effects and in vitro mosaicism. In: Daniel A, ed. *The Cytogenetics of Mammalian Autosomal Rearrangements*. New York:Alan R. Liss, Inc., 1988; 667–696.
152. Kosztolanyi G, Mehes K, Hook EB. Inherited ring chromosomes: an analysis of published cases. *Hum Genet* 1991;87:320–324.
153. Friedman JM, Harrod MJ, Howard-Peebles PN. Complementary duplication and deletion or 17(pcen-p11.2): A family with a supernumerary chromosome comprised of an interstitially deleted segment. *Am J Med Genet* 1992;44:37–40.
154. Quack B, Van Roy N, Verschraegen-Spaë MR, Klein F. Interstitial deletion and ring chromosome derived from 19q. Proximal 19q trisomy phenotype. *Ann Genet* 1992;35: 241–244.
155. Baldwin EL, May LF, Justice AN, Martin CL, Ledbetter DH. Mechanisms and consequences of small supernumerary marker chromosomes: from Barbara McClintock to modern genetic-counseling issues. *Am J Hum Genet* 2008;82:398–410.
156. Stetten G, Tuck-Muller CM, Blakemore KJ, Wong C, Kazazian HH Jr, Antonarakis SE. Evidence for involvement of a Robertsonian translocation 13 chromosome in formation of a ring chromosome 13. *Mol Biol Med* 1990;7(6):479–484.
157. McGinniss MJ, Kazazian HH Jr, Stetten G, Petersen MB, Boman H, Engel, E, Greenberg F, Hertz JM, Johnson A, Laca Z, Mikkelsen M, Patile SR, Schinzel AA, Tranebjærg L, Antonarakis SE. Mechanisms of ring chromosome formation in 11 cases of human ring chromosome 21. *Am J Hum Genet* 1992;50(1):15–28.
158. Muroya K, Yamamoto K, Fukushima Y, Ogata T. Ring chromosome 21 in a boy and a derivative chromosome 21 in the mother: implication for ring chromosome formation. *Am J Med Genet* 2002;110:332–337.
159. Petersen MB, Bartsch O, Adelsberger PA, Mikkelsen M, Schwinger E, Antonarakis SE. Uniparental isodisomy due to duplication of chromosome 21 occurring in somatic cells monosomic for chromosome 21. *Genomics* 1992;13:269–274.
160. Temple IK, James RS, Crolla JA, Sitch FL, Jacobs PA, Howell WM, Betts P, Baum JD, Shield JPH. An imprinted gene(s) for diabetes? *Nat Genet* 1995;3:110–112.
161. Crolla JA. FISH and molecular studies of autosomal supernumerary marker chromosomes excluding those derived from chromosome 15: review of the literature. *Am J Med Genet* 1997;75:367–381.

162. Miyoshi O, Kondoh T, Taneda H, Otsuka K, Matsumoto T, Niikawa N. 47,XX,UPD(7)mat,+r(7)pat/46,XX,UPD(7)mat mosaicism in a girl with Silver-Russell syndrome (SRS): possible exclusion of the putative SRS gene from a 7p13-q11 region. *J Med Genet* 1999;36(4):326–329.
163. Rothlisberger B, Zerova T, Kotzot D, Buzhievskaya TI, Balmer D, Schinzel A. Supernumerary marker chromosome (1) of paternal origin and maternal uniparental disomy 1 in a developmentally delayed child. *J Med Genet* 2001;38:885–888.
164. Werner M, Ben-Neriah Z, Silverstein S, Lerer I, Dagan Y, Abeliovich D. A patient with Prader-Willi syndrome and a supernumerary marker chromosome r(15)(q11.1–13p11.1)pat and maternal heterodisomy. *Am J Med Genet* 2004;129A(2):176–179.
165. Combi R, Sala E, Villa N, Crosti F, Beccaria L, Cogliardi A, Tenchini ML, Dalprà L. Maternal heterodisomy/isodisomy and paternal supernumerary ring of chromosome 7 in a child with Silver-Russell syndrome. *Clin Dysmorphol* 2008;17(1):35–39.
166. Cote GB, Katsantoni A, Deligeorgis D. The cytogenetic and clinical implications of a ring chromosome 2. *Ann Genet* 1981;24:231–235.
167. Shaffer LG, Slovak ML, Campbell LJ. *An International System for Human Cytogenetic Nomenclature (2009)*. Basel: S.Karger, 2009.
168. Liehr T, Weise A. Frequency of small supernumerary marker chromosomes in prenatal, newborn, developmentally retarded and infertility diagnostics. *International J Mol Med* 2007;19:719–731.
169. Crolla JA, Youings SA, Ennis S, Jacobs PA. Supernumerary marker chromosomes in man: parental origin, mosaicism and maternal age revisited. *Eur J Hum Genet* 2005;13:154–160.
170. Dalprà L, Giardino D, Finelli P, Corti C, Valtora C, Guerneri S, Ilardi, P, Fortuna R, Covello D, Nocera G, Amico FP, Martinoli E, Sala E, Villa N, Crosti F, Chiodo F, di Cantogno LV, Savin E, Croci G, Franchi F, Venti G, Donti E, Migliori V, Pettinari A, Bonifacio S, Centrone C, Torricelli F, Rossi S, Simi P, Granata P, Casalone R, Lenzini E, Artifoni L, Pecile V, Barlati S, Bellotti D, Caufin D, Police A, Cavani S, Piombo G, Pierluigi M, Larizza L. Cytogenetic and molecular evaluation of 241 small supernumerary marker chromosomes: Cooperative study of 19 Italian laboratories. *Genet Med* 2005;7(9):620–625.
171. Liehr T, Mrasek K, Weise A, Dufke A, Rodriguez L, Martinez Guardia N, Sanchis A, Vermeesch JR, Ramel C, Polityko A, Haas OA, Anderson J, Claussen U, von Eggeling F, Starke H. Small supernumerary marker chromosomes—progress towards a genotype-phenotype correlation. *Cytogenet Genome Res* 2006;112:23–34.
172. Stankiewicz P, Bocian E, Jakubow-Durska K, Obersztyn E, Lato E, Starke H, Mroczek K, Mazurczak T. Identification of supernumerary marker chromosomes derived from chromosomes 5, 6, 19 and 20 using FISH. *J Med Genet* 2000;37:114–120.
173. Anderlid BM, Sahlen S, Schoumans J, Holmberg E, Ahsgren I, Mortier G, Speleman F, Blennow E. Detailed characterization of 12 supernumerary ring chromosomes using micro-FISH and search for uniparental disomy. *Am J Med Genet* 2001;99:223–233.
174. Daniel A, Malafiej P. A series of supernumerary small ring marker autosomes identified by FISH with chromosome probe arrays and literature review excluding chromosome 15. *Am J Med Genet* 2003;117A:212–222.
175. Youings S, Ellis K, Ennis S, Barber J, Jacobs P. A study of reciprocal translocations and inversion detected by light microscopy with special reference to origin, segregation, and recurrent abnormalities. *Am J Med Genet* 2004;126A:46–60.
176. Van Dyke DL, Weiss L, Roberson JR, Babu VR. The frequency and mutation rate of balanced autosomal rearrangements in man estimated from prenatal genetic studies for advanced maternal age. *Am J Hum Genet* 1983;35:301–308.
177. Warburton D. De novo balanced chromosome rearrangements and extra marker chromosomes identified at prenatal diagnosis: clinical significance and distribution of breakpoints. *Am J Hum Genet* 1991;49:995–1013.
178. Kurahashi H, Inagaki H, Ohye T, Kogo H, Kato T, Emanuel BS. Palindrome-mediated chromosomal translocations in humans. *DNA Repair* 2006;5:1136–1145.
179. Kehrer-Sawatzki H, Haussler J, Krone W, Bode H, Jenne DE, Mehnert KU, Tummers U, Assum G. The second case of a t(17;22) in a family with neurofibromatosis type I: sequence analysis of the breakpoint regions. *Hum Genet* 1997;99:237–247.
180. Kurahashi H, Shaikh T, Takata M, Toda T, Emanuel BS. The constitutional t(17;22): another translocation mediated by palindromic AT-rich repeats. *Am J Hum Genet* 2003;72:733–738.
181. Wieczorek D, Krause M, Majewski F, Albrecht B, Meinecke P, Riess O, Gillessen-Kaesbach G. Unexpected high frequency of de novo unbalanced translocations in patients with Wolf-Hirschhorn syndrome (WHS). *J Med Genet* 2000;37:798–804.

182. Tonnies H, Stumm M, Neumann L, Völleth M, Grumpelt U, Musebeck J, Annuss G, Neitzel H. Two further cases of WHS with unbalanced de novo translocation t(4;8) characterized by CGH and FISH. *J Med Genet* 2001;38(6):e21:1–4.
183. Zollino M, Lecce R, Seliconi A, Murdolo M, Mancuso I, Marangi G, Zampino G, Garavelli L, Ferrarini A, Rocchi M, Opitz JM, Neri G. A double cryptic chromosome imbalance is an important factor to explain phenotypic variability in Wolf-Hirschhorn syndrome. *Eur J Hum Genet* 2004;12:797–804.
184. Chen C-P, Lin S-P, Chern S-R, Lee C-C, Chen L-F, Chen Y-J, Wang W. Molecular cytogenetic analysis of de novo parital monosomy 4p (4p16.2 → pter) and partial trisomy 8p (9p23.2 → pter). *Genet Counsel* 2006;17(1):81–85.
185. Hamerton JL, Canning N, Ray M, Smith S. A cytogenetic survey of 14,069 newborn infants: I. incidence of chromosomal abnormalities. *Clin Genet* 1975;8:223–243.
186. Jacobs PA. Mutation rates of structural chromosome rearrangements in man. *Am J Hum Genet* 1981;33:44–54.
187. Nielsen J, Wohlert M. Chromosome abnormalities found among 34,910 newborn children: results from a 13-year incidence study in Arhus, Denmark. *Hum Genet* 1991;87:81–83.
188. Schmutz SM, Pinno E. Morphology alone does not make an isochromosome. *Hum Genet* 1986;72:253–255.
189. Gardner RJM, Sutherland GR. Robertsonian translocations. In: Motulsky AG, Bobrow M, Harper PS, Scriver C, Epstein CJ, Hall J, eds. *Chromosome Abnormalities and Genetic Counseling*. New York: Oxford University Press, 2004;122–137.
190. Gardner RJM, Sutherland GR. Insertions. In: Motulsky AG, Bobrow M, Harper PS, Scriver C, Epstein CJ, Hall J, eds. *Chromosome Abnormalities and Genetic Counseling*. New York: Oxford University Press, 2004;163–177.
191. Pai GS, Thomas GH, Mahoney W, Migeon BR. Complex chromosome rearrangements; Report of a new case and literature review. *Clin Genet* 1980;18:436–444.
192. Chiang C, Jacobsen JC, Ernst C, Hanscom C, Heilbut A, Blumenthal I, Mills RE, Kirby A, Lindgren AM, Rudiger SR, McLaughlan CJ, Bawden CS, Reid SJ, Faull RL, Snell RG, Hall IM, Shen Y, Ohsumi TK, Borowsky ML, Daly MJ, Lee C, Morton CC, MacDonald ME, Gusella JF, Talkowski ME. Complex reorganization and predominant non-homologous repair following chromosomal breakage in karyotypically balanced germline rearrangements and transgenic integration. *Nat Genet* 2012;44(4):390–7.
193. Astbury C, Christ LA, Aughton DJ, Cassidy SB, Fujimoto A, Pletcher BA, Shafer IA, Schwartz S. Delineation of complex chromosomal rearrangements: evidence for increased complexity. *Hum Genet* 2004;114:448–457.
194. Gribble SM, Prigmore E, Burford DC, Porter KM, Ng BL, Douglas EJ, Fiegler H, Carr P, Kalaitzopoulos D, Clegg S, Sandstrom R, Temple IK, Youings SA, Thomas NS, Dennis NR, Jacobs PA, Crolla JA, Carter NP. The complex nature of constitutional de novo apparently balanced translocations in patients presenting with abnormal phenotypes. *J Med Genet* 2005;42(1):8–16.
195. De Gregori M, Ciccone R, Magini P, Pramparo T, Gimelli S, Messa J, Novara F, Vetro A, Rossi E, Maraschio P, Bonaglia MC, Anihini C, Ferrero GB, Silengo M, Fazzi E, Zatteralle A, Fischetto R, Previderé C, Belli S, Turci B, Calabrese G, Bernardi R, Meneghelli E, Riegel M, Rocchi M, Guerneri S, Lalatta F, Zelante L, Romano C, Fichera M, Mattina T, Arrigo G, Zollino M, Giglio S, Lonardo F, Bonfante A, Ferlini A, Cifuentes F, Van Esch H, Backx L, Schinzel A, Vermeesch JR, Zuffardi O. Cryptic deletions are a common finding in “balanced” reciprocal and complex chromosome rearrangements: a study of 59 patients. *J Med Genet* 2007;44:750–762.
196. Baptista J, Mercer C, Prigmore E, Gribble SM, Carter NP, Maloney V, Thomas NS, Jacobs PA, Crolla JA. Breakpoint mapping and array CGH in translocations: comparison of a phenotypically normal and an abnormal cohort. *Am J Hum Genet* 2008;82:927–936.
197. Olson SB, Magenis RE. Preferential paternal origin of de novo structural chromosome rearrangements. In: Daniel A, ed. *The Cytogenetics of Mammalian Autosomal Rearrangements*. New York: Alan R. Liss Inc. 1988;583–589.
198. Gorski JL, Kistenmacher ML, Punnett HH, Zackai EH, Emanuel BS. Reproductive risks for carriers of complex chromosome rearrangements: analysis of 25 families. *Am J Med Genet* 1988;29:247–261.
199. Daniel A, Hook EB, Wulf G. Risks of unbalanced progeny at amniocentesis to carriers of chromosome rearrangements: data from United States and Canadian laboratories. *Am J Med Genet* 1989;31:14–53.
200. Batista DAS, Pai GS, Stetten G. Molecular analysis of a complex chromosomal rearrangement and a review of familial cases. *Am J Med Genet* 1994;53:255–263.

CHAPTER 10

Genomic imprinting

R. Ellen Magenis

*(deceased) formerly, Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA

10.1 Introduction[†]

Genomic imprinting is a process leading to differential expression of genetic material (genes/alleles) dependent on the parent (male or female) of origin. The term “imprinting” was first used in reference to chromosomes by Crouse in 1960 [1] in her description of the selective elimination of paternal translocation products in the fly *Sciara*. However, it originally was used by Lorenz in 1935 [2] to describe the behavior of newly hatched goslings. At hatching, a gosling is said to be “imprinted” upon the first object it sees, as though the object were its mother, thus the term implies a change that is imposed by an external event.

Experimental evidence for the existence of imprinting comes from pronuclear transplantation performed in mice [3–6]. These experiments involved constructing zygotes in which both haploid nuclear gene sets are derived from either the father or mother (called uniparental disomy, or UPD). Those with paternally derived chromosomes had poor development of embryonic structures and relatively normal development of membranes. Those with maternally derived chromosomes had good embryonic development but poor development of membranes and placentas. Both conditions were lethal.

Naturally occurring human analogies include conceptuses with *triploidy*, the *hydatidiform mole* and the *teratoma*. Human triploids with two paternal and one maternal complement have a large cystic placenta with partial molar changes (Figure 10.1a), while two maternal haploid complements and one paternal complement have a small underdeveloped placenta (Figure 10.1b) and are found primarily in study of early spontaneous abortuses. The hydatidiform mole has two paternal complements with no maternal copy and no fetus (Figure 10.2a,b). The *ovarian teratoma*, an embryonal tumor occurring in children and adults, with only two maternal haploid complements and no paternal contribution (Figure 10.3) may have differentiated ectodermal tissues, such as hair and teeth and are said to be *parthenogenetic* in origin [7].

Imprinted genes, which are subject to differential expression, comprise only a small proportion of the total numbers of genes in mammals. Thus far, about 83 imprinted genes have been identified in placental mammals [8]; computer-based analysis of the mouse genome predicts that up to 600 [9] of the approximate 30,000 protein-coding genes could be imprinted or exhibit imprinting [10]. The number in the human has not yet been determined, though 55 imprinted genes have been characterized and localized [9] (Table 10.1).

* Editors' note: Dr. Magenis, world-renowned for her intuitiveness and accomplishments, passed away on February 4, 2014, at the age of 88, following years of health issues after a chronic neurodegenerative disease. Dr. Magenis had prepared this chapter prior to her illness; therefore, the chapter may not contain the most current discoveries, but it is, nonetheless, filled with this Genetic Giant's lifelong work, passion, and brilliance. We only wish she could be here to read it herself.

† Note: Italicized words are defined in the glossary at the end of this chapter.

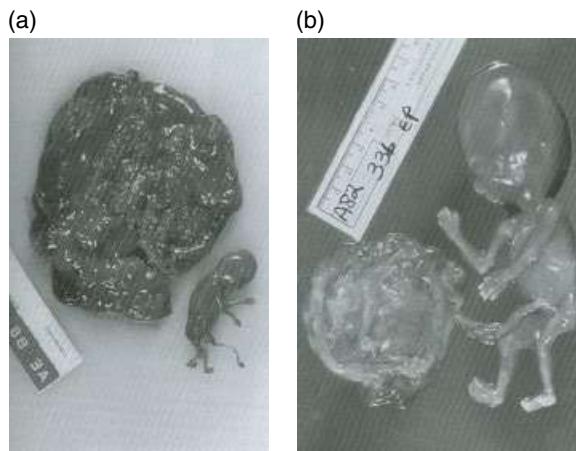


Figure 10.1 Triploidy. (a) Human triploid with 2 paternal and 1 maternal complement. Note large cystic placenta and under developed fetus. (b) Human triploid with 2 maternal and 1 paternal complement. Note underdeveloped placenta and relatively large fetus. Gorlin 2001. Reproduced with permission of Wiley.

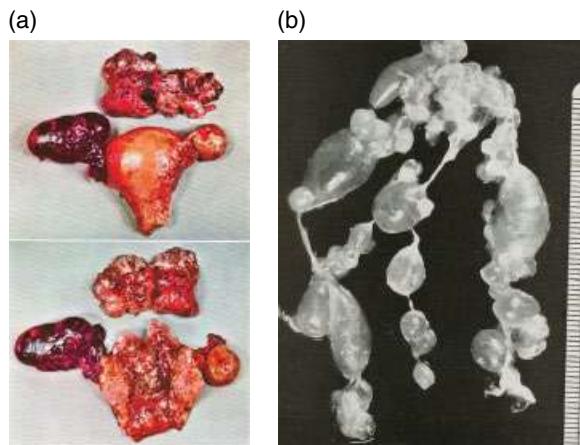


Figure 10.2 Hydatidiform mole. (a) (Top) Uterus with tubes containing a hydatidiform mole. (Bottom) Uterus open showing remains of the mole. (b) Hydatidiform mole, vesicles isolated by submerging in water. De Lee, JB, Greenhill, JP. Principles and Practice of Obstetrics. Philadelphia and London. W.B. Saunders Company. 1948. See insert for color representation of this figure.

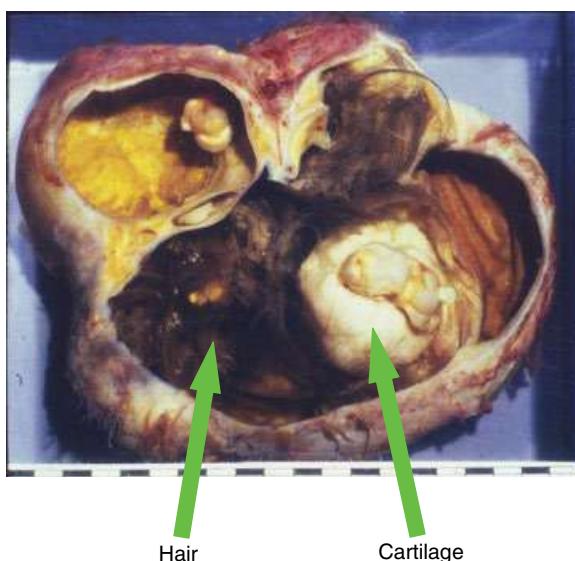


Figure 10.3 Teratoma. Ovarian teratoma with two maternal haploid complements, no paternal contribution. Note hair and cartilage. <http://medwebstudents2.wordpress.com>. Used under CC-BY-SA 4.0 <https://creativecommons.org/licenses/by/4.0/>. See insert for color representation of this figure.

Table 10.1 List of currently characterized and localized human imprinted genes

Gene	Aliases	Location	Status	Expressed allele
<i>TP73</i>	P73, Trp73	(1p36.3)	Imprinted	Maternal
<i>DIRAS3</i>	ARHI, NOEY2	(1p31)	Imprinted	Paternal
<i>PLAGL1</i>	ZAC, LOT1, ZAC1, MGC12627, 5MGC126276, DKFZp781P1017	(6q24-q25)	Imprinted	Paternal
<i>HYMAI</i>		(6q24)	Imprinted	Paternal
<i>MESTIT1</i>	PEG1-AS, paternally expressed gene, antisense transcript	(7q32)	Imprinted	Paternal
<i>COPG2IT1</i>	CIT1	(7q32)	Imprinted	Paternal
<i>GRB10</i>	RSS, IRBP, MEG1, GRB-IR, KIAA0207	(7p12-p11.2)	Imprinted	Isoform Dependent
<i>SGCE</i>	ESG, DYT11	(7q21-q22)	Imprinted	Paternal
<i>PEG10</i>	Edr, HB-1, MEF3L, RGAG3, KIAA1051	(7q21)	Imprinted	Paternal
<i>PPP1R9A</i>	NRB1, NRBI, FLJ20068, KIAA1222, Neurabin-I	(7q21.3)	Imprinted	Maternal
<i>DLX5</i>		(7q22)	Imprinted	Maternal
<i>CPA4</i>	CPA3	(7q32)	Imprinted	Maternal
<i>MEST</i>	PEG1, MGC8703, MGC111102, DKFZp686L18234	(7q32)	Imprinted	Paternal
<i>KCNQ10T1</i>	KCNQ10T1, KvLQT1-AS, long QT intronic transcript 1	(11p15)	Imprinted	Paternal
<i>H19</i>	ASM, BWS, ASM1, MGC4485, PRO2605, D11S813E	(11p15.5)	Imprinted	Maternal
<i>IGF2</i>	INSIGF, pp9974, C11orf43, FLJ22066, FLJ44734	(11p15.5)	Imprinted	Paternal
<i>IGF2AS</i>	PEG8	(11p15.5)	Imprinted	Paternal
<i>INS</i>	INS2	(11p15.5)	Imprinted	Paternal
<i>KCNQ1</i>	LQT, RWS, WRS, LQT1, SQT2, ATFB1, JLNS1 KCNA8, KCNA9, Kv1.9, Kv7.1, KVLT1, FLJ26167	(11p15.5)	Imprinted	Maternal
<i>KCNQ1DN</i>	BWRT, HSA404617	(11p15.4)	Imprinted	Maternal
<i>CDKN1C</i>	BWS, WBS, p57, BWCR, KIP2	(11p15.5)	Imprinted	Maternal
<i>SLC22A18</i>	BWSCR1A, SLC22A1L, p45-BWR1A, DKFZp667A184	(11p15.5)	Imprinted	Maternal
<i>PHLDA2</i>	IPL, BRW1C, BWR1C, HLDA2, TSSC3	(11p15.5)	Imprinted	Maternal
<i>OSBPL5</i>	ORP5, OBPH1, FLJ42929	(11p15.4)	Imprinted	Maternal
<i>WT1-Alt trans</i>	WT1, GUD, WAGR, WT33, WIT-2	(11p13)	Imprinted	Paternal
<i>DLK1</i>	FA1, ZOG, pG2, PREF1, Pref-1	(14q32)	Imprinted	Paternal
<i>MEG3</i>	GTL2, FP504, prebp1, PRO0518, PRO2160, FLJ31163, LJ42589	(14q32)	Imprinted	Maternal
<i>SNORD109A</i>	HBII-438A, HBII-438A C/D box snoRNA	(15q11.2)	Imprinted	Paternal
<i>SNORD115@</i>	HBII-52	(15q11.2)	Imprinted	Paternal
<i>PWCR1</i>	PET1, noncoding RNA in the Prader-Willi critical region	(15q11.2)	Imprinted	Paternal
<i>SNRPN</i>	SMN, SM-D, RT-LI, HCERN3, SNRNP-N, SNURF-SNRPN	(15q11.2)	Imprinted	Paternal

(Continued)

Table 10.1 (Continued)

Gene	Aliases	Location	Status	Expressed allele
<i>MKRN3</i>	D15S9, RNF63, Zfp127, ZNF127, MGC88288	(15q11-q13)	Imprinted	Paternal
<i>MAGEL2</i>	nM15, NDNL1	(15q11-q12)	Imprinted	Paternal
<i>NDN</i>	HsT16328	(15q11.2-q12)	Imprinted	Paternal
<i>SNURF</i>		(15q12)	Imprinted	Paternal
<i>SNORD107</i>	HBII-436, HBII-436 C/D box snoRNA	(15q11.2)	Imprinted	Paternal
<i>SNORD64</i>	HBII-13, HBII-13 snoRNA	(15q12)	Imprinted	Paternal
<i>SNORD108</i>	HBII-437, HBII-437 C/D box snoRNA	(15q11.2)	Imprinted	Paternal
<i>SNORD109B</i>	HBII-438B, HBII-438B C/D box snoRNA	(15q11.2)	Imprinted	Paternal
<i>UBE3A</i>	AS, ANCR, E6-AP, HPVE6A, EPVE6AP, FLJ26981	(15q11-q13)	Imprinted	Maternal
<i>ATP10A</i>	ATPVA, ATPVC, ATP10C, KIAA0566	(15q11.2)	Imprinted	Maternal
<i>TCEB3C</i>	HsT829, TCEB3L2, MGC119353	(18q21.1)	Imprinted	Maternal
<i>ZIM2</i>	ZNF656	(19q13.4)	Imprinted	Paternal
<i>PEG3</i>	PW1, ZSCAN24, KIAA0287, DKFZp781A095	(19q13.4)	Imprinted	Paternal
<i>ZNF264</i>	Zfp264	(19q13.4)	Imprinted	Maternal
<i>GNAS-AS1</i>	SANG, NESPAS	(20q13.32)	Imprinted	Paternal
<i>NNAT</i>	Peg5, MGC1439	(20q11.2-q12)	Imprinted	Paternal
<i>L3MBTL</i>	L3MBTL1, KIAA0681, H-L(3)MBT, dJ138B7.3, DKFZp586P1522	(20q13.12)	Imprinted	Paternal
<i>GNAS</i>	XL, AHO, GSA, GSP, POH, XL2, GPSA, NESP, SCG6, GNAS1, PHP1A, PHP1B, GNASXL, NESP55, C20orf45, MGC33735, XLalphas, dJ309F20.1.1, dJ806M20.3.3	(20q13.3)	Imprinted	Maternal

Although few in number, imprinted genes are seldom found as isolated genes; they are usually found in clusters, which may reflect coordinated regulation of these genes in chromosome domains (Table 10.1). These genes are unusually rich in *CpG islands*. Eighty-eight percent of imprinted genes in the mouse occur within or near *CpG islands*, as compared with the 47% of nonimprinted genes. The *CpG islands* are regions of DNA with many cytosine–guanine base pairs. These islands may show complete methylation or none at all. Some exhibit differentially methylated regions (DMR), which may play a central role in imprinting. Most imprinted genes show differences in DNA methylation between the parental alleles. Direct base-pair repeats are commonly found near or within *CpG islands*; the role of these repeats is unclear [11].

The methylation process is catalyzed by the enzyme DNA methyltransferase which connects a methyl group (CH_3) to a cytosine in DNA. This change slightly alters the shape of the double helix, preventing binding of many types of proteins after the methyl groups have been added. The methylation state can be maintained even when copied. Thus, the pattern can be retained through development.

However, the imprinting process is highly complex, and involves more than the methylation process. *Histone proteins* are involved; indeed, DNA methylation may be connected mechanically to histone modification and may recruit other proteins that bind the DNA, which in turn attract other enzymes that remove acetyl groups from histones. This complex of proteins condenses chromatin and limits transcription [8]. Methylation of H3 9Lys is a feature of condensed regions of chromatin including heterochromatin. Methylation of 79Lys in H3 may be necessary for formation of repeats at telomeres [12]. Methylation of both DNA and histones is a feature of inactive chromatin [12]. The imprinting process must be reversible from generation to generation. It must involve imprint establishment during gamete formation – maintenance through embryogenesis into somatic tissues, but erasure and resetting in the germline. Stable and differential modifications are required. This process is illustrated in Figure 10.4 [8].

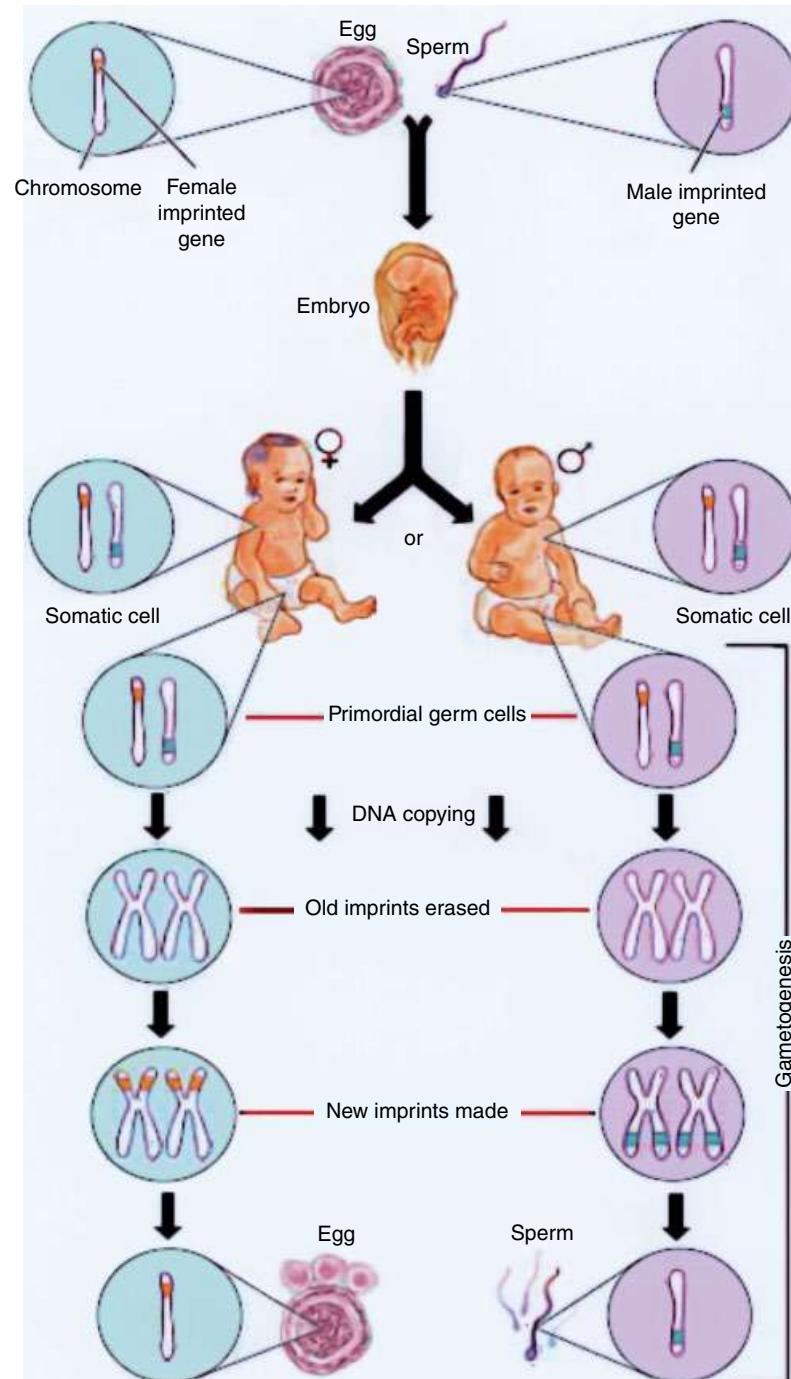


Figure 10.4 Germline imprint resetting process. The sex-specific imprints on DNA from the sperm and egg persist in somatic cells throughout the child's body. Parent specific imprint is reset in each new generation to ensure appropriate gene activity in the germline. In primordial germ cells, DNA copying is followed by the erasing of old imprints and the re-establishment of newly uniform imprints that reflect the offspring's own sex. From: Jirtle R, Weidman J. Imprinted and More Equal. *Am Sci.* Volume 35. March/April 2007. Reproduced with permission of American Scientist and with the kind permission of Randy Jirtle. See insert for color representation of this figure.

Imprinted genes appear to be preferentially involved in growth regulation and in behavior. There is also evidence of involvement in the aging process and in malignancies. This regulation is part of the transcriptional process involving checks and balances between domains in differentially imprinted clusters of genes. Figure 10.5 is a diagram of normal regulation of the *IGF2-H19* and *KCNQ1* domains and epigenetic changes resulting in disorders of growth, notably Beckwith–Wiedemann, an overgrowth syndrome exhibiting macroglossia, omphalocele, visceromegaly (Figure 10.6), and Silver–Russell syndrome (Figure 10.7), with short stature (prenatal onset), body asymmetry, triangular facies and often café-au-lait pigmentation [13].

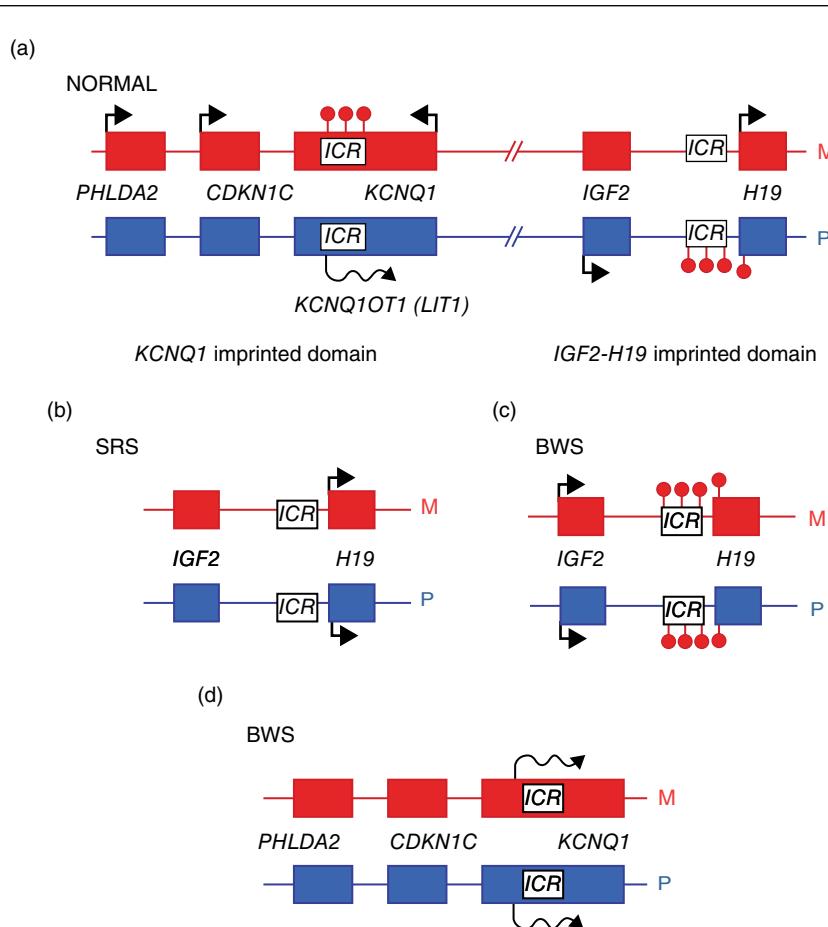


Figure 10.5 *IGF2* normal maternal/paternal gene regulation of *IGF2*. Epigenetic deregulation at the imprinted *IGF2-H19* and *KCNQ1* domains on chromosome 11p15. (a) Epigenetic organization in normal individuals. The *H19* ICR (open box) is methylated (lollipops) exclusively on the paternal allele (P). This conveys paternal expression of the *IGF2* growth factor gene, and maternal expression of the *H19* gene. The ICR regulating the flanking *KCNQ1* domain is methylated only on the maternal allele (M). On the unmethylated paternal allele, this region produces a noncoding RNA (*KCNQ1OT1*). The paternal silencing encompasses the negative growth regulator *CDKN1C* and *PHLDA2*, whose placental expression is aberrant in intrauterine growth restriction. (b) In a third of Silver-Russell syndrome (SRS) patients, there is a loss of methylation at the *H19* ICR. This causes a strong reduction in *IGF2* expression, and biallelic expression of *H19*. (c) The opposite epigenetic change is observed in ten percent of patients with Beckwith-Wiedemann syndrome (BWS). Here biallelic methylation at the *H19* ICR (and *H19*) causes biallelic *IGF2* expression, and loss of *H19* expression. (d) In about half the BWS patients, there is loss of methylation at the KvDMR1 ICR. This correlates with biallelic expression of the noncoding *KCNQ1OT1* transcript, and a strong reduction in the expression of genes along the domain, including *CDKN1C*. BioEssays; 28 (5), 2006, 453–459. Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. See insert for color representation of this figure.

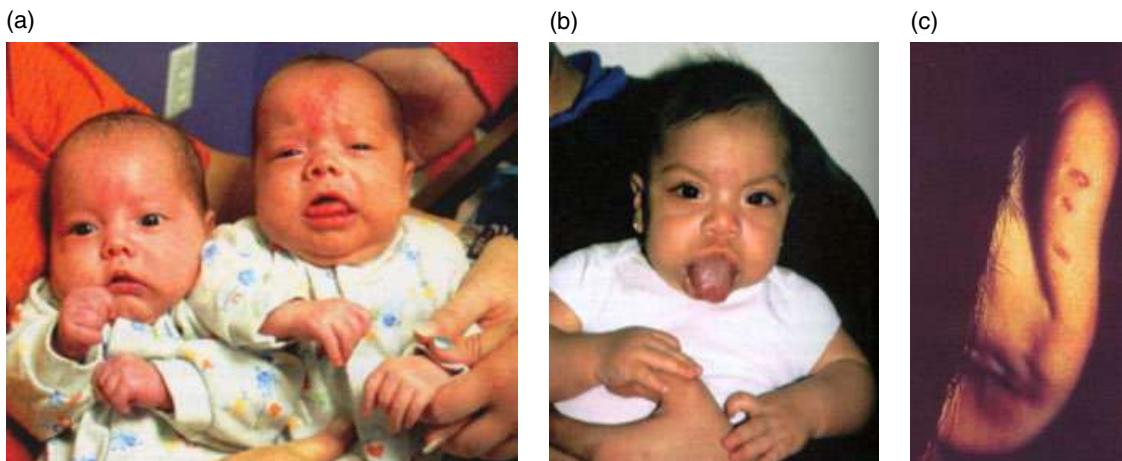


Figure 10.6 Patients with Beckwith–Wiedemann syndrome. (a) Newborn monozygotic twins discordant for Beckwith–Wiedemann syndrome. Reproduced with the kind permission of Dr. Cynthia Curry, Professor of Pediatrics, UCSF. (b) Child showing facial features of Beckwith–Wiedemann syndrome. Partial glossectomy was performed at 18 months. Reproduced with the kind permission of Lynne Bird. (c) Indentations on posterior helix of child with BWS. From Jones KL, Beckwith–Wiedeman Syndrome, in *Smith’s Recognizable Patterns of Human Malformation*, pages 174–177. Reproduced with permission of Elsevier. See insert for color representation of this figure.



Figure 10.7 Patients with Silver–Russell syndrome. (a) Female newborn infant. Small size, triangular face. Reproduced with the kind permission of Rady Children’s Hospital, San Diego. Diagnostic studies show maternal uniparental disomy of chromosome 7 consistent with SRS. (b) Same female at 20 months of age. Reproduced with the kind permission of Rady Children’s Hospital, San Diego. (c) A 3½ year old boy, diagnosed with SRS (left) with his 2-year old unaffected sister. Note comparable sizes. Reproduced with the kind permission of Lynne Bird. See insert for color representation of this figure.

10.2 Human genomic disease and imprinting

The important role of imprinting in human genomic disease is rapidly becoming known. Imprinting provides explanation for the unusual inheritance patterns found in some genetic disorders, such as:

- multiple congenital anomaly syndromes
- growth disorders
- certain behavioral conditions
- cancers.

10.2.1 Chromosomal syndromes

The Prader–Willi and Angelman deletion syndromes are the most thoroughly studied and best known conditions resulting from genomic imprinting. The clinical features (Figures 10.8 and 10.9) of the two syndromes are distinct (Table 10.2), even though both conditions involve the same chromosomal deletion region on the proximal long arm of chromosome 15 (Figure 10.10).

About 70% [14] of cases with Prader–Willi syndrome (PWS) and Angelman syndrome (AS) are caused by deletions of chromosomes 15q11.2 (Figure 10.11). The deletions in PWS are paternal in origin [15], while the deletions are of maternal

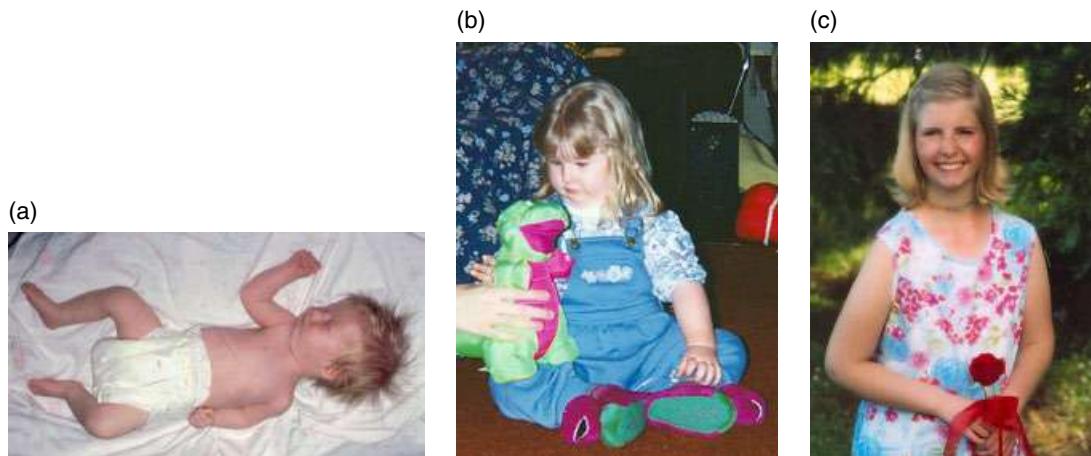


Figure 10.8 Female with Prader–Willi syndrome. (a) In infancy. (b) Age 3 years, showing mild/moderate obesity. (c) Age 14 years, post-treatment with growth hormone and diet control. Reproduced with kind permission of Rachel Williams. See insert for color representation of this figure.



Figure 10.9 Female with Angelman syndrome. (a) At 10 months of age. Note alert expression. (b,c) At 4–5 years of age. Note limb posturing. (d) At 25 years of age. Reproduced with the kind permission of Oregon Health and Science University Hospitals and Clinics. See insert for color representation of this figure.

Table 10.2 Clinical features seen in Prader–Willi and Angelman syndromes

Prader–Willi syndrome	Angelman syndrome
Decreased fetal movement	Feeding problems
Feeding problems in early infancy	Delayed motor development
Hypotonia	Lack of speech
Genital hypoplasia/cryptorchidism	Severe intellectual disabilities
Delayed motor development	Sleep difficulties
Hyperphagia/obesity	Seizures
Speech delay/articulation difficulties	Ataxic gait
Mild intellectual disabilities	Limb posturing
Behavioral difficulties/ obsessive/ tantrums	Brisk reflexes
Sleep problems	Tongue protrusion
Mild short stature	Drooling
Hypogonadism	Prognathism
Small hands and feet	Wide spaced teeth
Narrow frontal diameter	Wide mouth
Almond-shaped palpebral fissures	Unusual laughter
Thin/ down-turned upper lip	

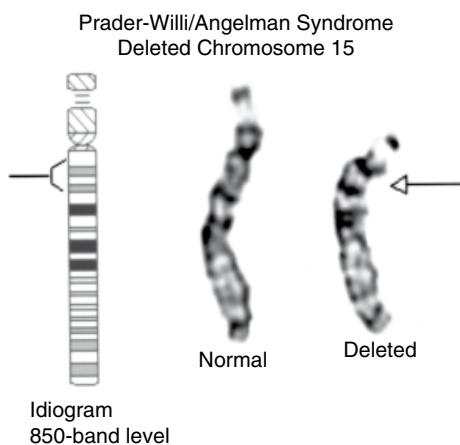


Figure 10.10 Deleted chromosome 15 of Prader–Willi/Angelman syndrome. Both PWS and AS are caused by the absence of genetic material at the same q12 region of chromosome 15. Reproduced with the kind permission of Oregon Health and Science University Hospitals and Clinics.

origin in AS [16]. Approximately 25% of additional cases of PWS are caused by the presence of uniparental disomy [14] with no deletion detectable, but both chromosome 15 gene sets are of maternal origin (Figure 10.12). There are usually no paternally derived genes found. Only a few cases of AS have been found with paternal uniparental disomy. These phenomena are caused by the differential silencing of the maternal and paternal imprinted genes (Figure 10.12).

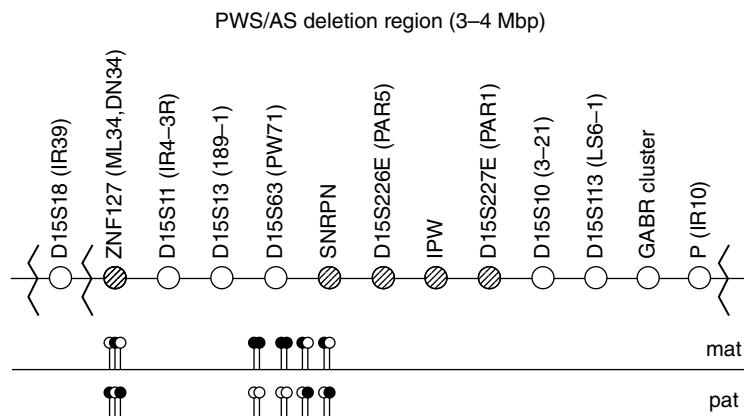


Figure 10.11 Locus order in 15q11-13. The common deletion region in PWS and AS extends from D15S18 to the pigmentation gene P and comprises 3–4 Mb (not drawn to scale). Open circle, anonymous marker or nonimprinted gene; hatched circle, imprinted gene (paternally expressed); *MKRN3*, makorin ring finger protein 3 (formerly *ZNF127*, zinc finger protein 127); *SNRPN*, small nuclear ribonucleoprotein N; GABR cluster, GABA_A receptor gene cluster (*GABRB3*, *GABRA5*, *GABRG3*); P, pigment gene; wavy line, common deletion breakpoint; filled lollipop, methylated CpG; open lollipop, unmethylated CpG; mat, maternal chromosome; pat, paternal chromosome. Reproduced with the kind permission of Oregon Health and Science University Hospitals and Clinics.

UPD causes clinical disease because of lack of **functional gene products**, either paternal as in PWS, or maternal as in AS. When there are only maternally imprinted genes present, as in maternal UPD, these methylated genes are not expressed, there is lack of paternal gene function, and PWS occurs. In paternal uniparental disomy, there are no maternally derived expressed genes present, leading to AS [17] (Figure 10.13).

In PWS an additional approximate 1% of cases have an imprinting center defect; and <1% are the result of an apparently balanced translocation [14]. Mutations of the imprinted *UBE3A* gene, ubiquitin protein ligase E3A (localized to 15q11.3-q13), have been detected in some cases of AS with no gene loss. No cases with single gene mutation have been found in PWS [18].

10.2.2 Whole chromosome uniparental disomies

Whole chromosome uniparental disomies have been detected for all twenty-four chromosomes at low incidence, with the exception of chromosomes 3, 18 and 19 [19]. An important cause of many uniparental disomies is, first, a gain of a chromosome by nondisjunction, such that a gamete will contain 24 chromosomes. This would result in 47 chromosomes after fertilization, which in many cases is lethal. However, if one of the trisomic chromosomes is lost as a result of anaphase lag or nondisjunction (*trisomy rescue*), the embryo could end up either chromosomally and phenotypically normal – or it could have paternal or maternal uniparental disomy, depending on the parent of origin. In a reverse manner but with a similar outcome, if the zygote were to lose one of its two homologues, and the remaining single chromosome compensates for the loss by duplicating, called *monosomy rescue*, the cell may appear normal, but the genetic outcome again is UPD. Some of these abnormal disomic cases show highly abnormal outcomes, including congenital abnormalities, possibly caused by *dose effect*.

UPD abnormalities may not always result from the presence of differentially methylated imprinted genes. They can also result from “unmasking” adverse gene mutations that are inherited from the parent contributing the disomy. Families with Robertsonian translocations, particularly between chromosomes 14 and 15, have shown an increased risk not just for uniparental disomy due to *meiotic malsegregation*, but also for adverse outcomes due to the expression of deleterious imprinted genes that have been unmasked on these chromosomes [20]. A further causative mechanism for UPD expression is the formation of *isochromosomes* together with the presence of a normal homologue.

The phenotypic effects of whole chromosome uniparental disomy are highly variable, dependent on the chromosome involved and the parent of origin. To date, 55 documented imprinted genes have been localized to 16 different chromosomes, with clusters of these genes on chromosomes 7, 11, 15, and 20 (Table 10.1). For example, Wang [19], in his literature review, described nine cases of **paternal** UPD 14 [21–23]. These patients had similar clinical features, which included *polyhydramnios*, low birth weight, hirsute forehead, *blepharophimosis*, *protruding philtrum*, small ears, abnormal ribs and joint contractures, suggesting some form of imprinting effect. In his review of twenty published cases of **maternal** UPD 14 [24–28], he also

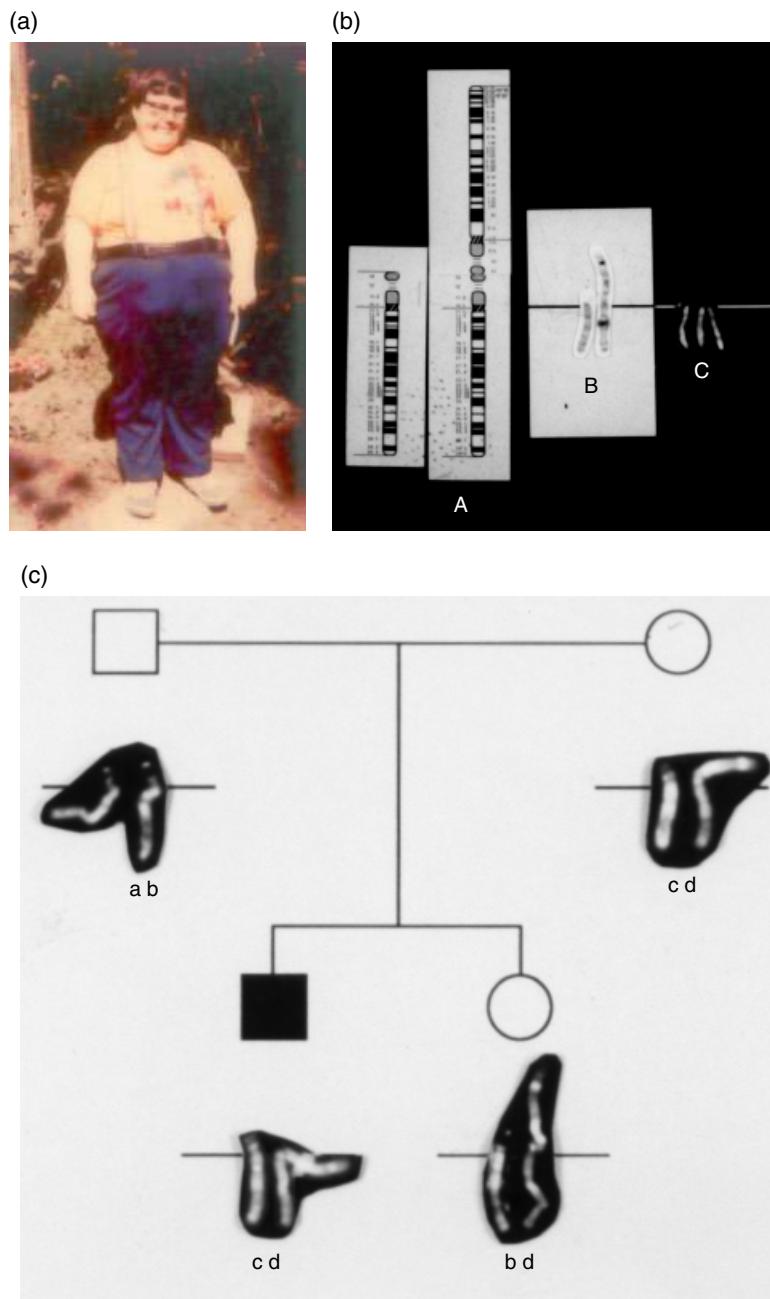


Figure 10.12 Male with Prader–Willi syndrome due to uniparental disomy. (a) Twenty-four-year-old male with Prader–Willi syndrome. Note massive obesity. (b) Chromosome studies show a **14/15** Robertsonian translocation along with a normal **15** (normal **14** not shown). No deletion by G-banding or Q-banding is evident, as depicted in ISCN ideogram. (c) Q-banded chromosomes of parents and female sibling show that mother and sibling carry the translocation. The proband's normal **15**, by comparison of the marker short arm, stalk and satellite regions, is inherited from his mother as well as the **t(14/15)**. Thus, he has maternal UPD as a cause of his PWS features. His sibling received a normal **15** from her father—and has a normal phenotype. For quinacrine staining methods and identification of short arms, stalks and satellite marker regions shown in (c), see Magenis RE, Overton KM, Chamberlin J, Brady T, Lovrien E. Parental origin of the extra chromosome in Down's syndrome. *Hum Genet* 1977; 37: 7–16. Surani (1997). By permission of Oxford University Press. See insert for color representation of this figure.

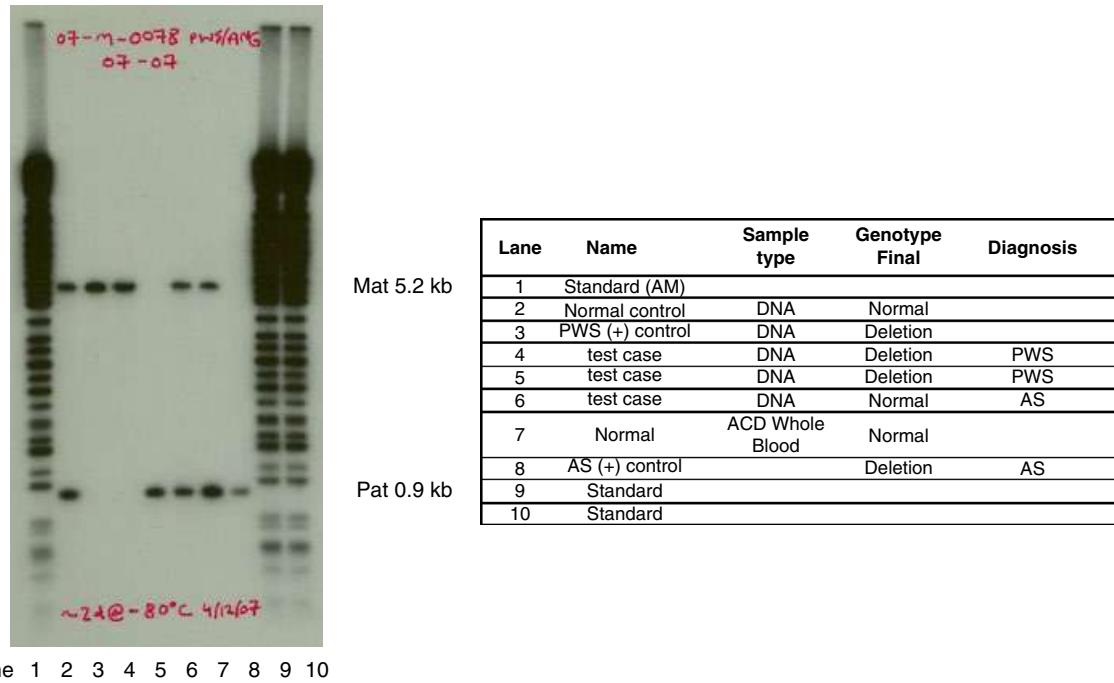


Figure 10.13 PWS/AS methylation assay. The DNA test for Prader–Willi and Angelman syndromes is based on an assessment of the imprinting status of the 15q11–q13 region. This is done by determining the methylation status of the small nuclear ribonucleoprotein-associated polypeptide N (*SNRNP*) gene. The methylation pattern of the *SNRNP* gene is assessed by Southern blot analysis of genomic DNA using the restriction enzymes Xba 1 and Not 1. Not 1 is a methylation sensitive enzyme. The probe pSNP-1, which is specific for the 5'-end of the *SNRNP* gene, detects a 5.2 kb Xba 1 fragment that contains Not 1 sites. When the *SNRNP* gene is methylated, the Xba 1 fragment is not digested by Not 1 (maternal allele). When the *SNRNP* gene is hypomethylated, the Xba 1 fragment is cut by Not 1 (paternal allele); the probe pSNP-1 detects a 0.9 kb fragment.

noted a distinct phenotype, including moderate motor and/or developmental delay, hypotonia, short stature, precocious puberty, and minor dysmorphic features – different imprinting defects from those found with **paternal UPD 14**.

The phenotype also may vary widely when a recessive gene has been unmasked. Wang [19] found five cases of **paternal UPD 1** [29–33]. One case was a 7-year-old male with *pycnodysostoses*, resulting from a homozygous mutation of the *cathepsin K* gene inherited from his father. The boy was otherwise normal. Three other cases of UPD 1 had differing features – one with congenital insensitivity to pain with *anhidrosis*, the second with *Herlitz junctional epidermolysis bullosa* and the third with *Leber congenital amaurosis* [30–32]. None had overt dysmorphisms. These clinical findings were thus presumed as the result of mutated recessive genes from their fathers. A fifth case was a 43-year-old female with short stature, ptosis, micrognathia, scoliosis, hearing loss and myopathy due to *isochromosomes* of the short arm and the long arm of chromosome 1. It was not clear whether the features were due entirely to imprinting or homozygosity for an unknown recessive allele.

Wang [19] also summarized three reported cases of **maternal UPD 1**. One case had *junctional epidermolysis bullosa*, recessive type, from mutation of the *LAMB3* gene inherited from the mother. This patient had no dysmorphic features or malformations. Another case had *Chediak–Higashi syndrome* due to homozygosity for a mutation of the *LYST* gene inherited from the mother. Still another patient, who had insulin-dependent diabetes mellitus and **maternal UPD** of chromosome 1, was discovered through a family linkage study; this patient was otherwise assessed as normal. These findings suggest that neither **maternal** nor **paternal UPD** for chromosome 1 has imprinting effects [34–36].

10.2.3 Partial (segmental) uniparental disomies

With the use of genome-wide single nucleotide polymorphism analysis (SNP), partial (segmental) uniparental disomy affecting only portions of involved chromosomes has been detected in acute myeloid leukemias. About 20% (12 cases) of the 64 acute myeloid leukemias studied by Raghavan et al. [37] showed large regions of homozygosity that were not visible at the chromosome

level. These 12 cases showed homozygosity involving ten different chromosome regions. Remission samples on five of the affected patients were normal. The amount of DNA by SNP signal value and fluorescence in situ hybridization was concordant with the presence of *two copies* of each gene in the homozygous regions. The authors concluded that these regions of homozygosity were caused by *somatic recombination events*.

Further studies of subjects with acute myeloid leukemia were performed to detect the presence of homozygous mutations in genes known to be “mutational targets” in these leukemias. In 7 of 13 cases with detected uniparental disomy, they found concurrent homozygous mutations at four loci (*WT1*, *FLT3*, *CEBPA*, and *RUNX1*). This suggests that the mutation occurs first, followed by mitotic recombination [38]. These data indicate a new etiological type of leukemia.

10.3 Germ cell tumors – UPD and imprinting

Because the imprint reset occurs in the germ cell, the imprint role in germ cell tumors (GCTs) has been investigated. GCTs are uncommon in childhood, but are found at every age. These tumors are considered to be of germ cell origin, the primordial germ cell, though the tumors may be of diverse histologic types. It is in the germ cell where the imprint switch takes place, with loss of parental imprinting occurring before entry into the gonadal ridge, followed by appropriate resetting according to the sex of the embryo. The precise process and timing of the tumorigenic processes giving rise to the various tumor types is not clear.

One large cytogenetic GCT study of 81 pediatric tumors procured through Children’s Oncology Group protocols showed differences in chromosome results between tumors by **sex**, **age**, **tumor location**, and **histology**. For example, 16 of 17 benign ovarian tumors from older girls with normal G-band karyotypes were homozygous for Q-band heteromorphisms in all variable regions, suggestive of a meiosis II error and uniparental disomy for all 23 chromosome pairs. Twenty-three malignant endodermal sinus tumors in infants and young children showed varied abnormal karyotypes [39]. This study also showed that loss of 1p was associated with a malignant phenotype. Amplification of 12p, commonly found in adult testicular tumors, was uncommon in pediatric GCTs. The relation of these tumors to specific imprinted genes is not clear, although an imprinted gene is known to be located in the deleted chromosome 1p region (Table 10.1).

10.3.1 Common chromosome abnormalities

A follow-up study was performed on these pediatric GCTs using interphase fluorescence in situ hybridization [40]. Fifty-three pediatric GCTs were analyzed for loss of 1p36 and 12p amplification. Probes targeted the centromere and short arm of chromosomes 1 and 12. Twelve tumors that showed loss of 1p36 also had malignant histology. Five of 18 tumors from males had amplification of 12p, but only one of 35 tumors from female patients showed amplifications; however, five female patients had numerical abnormalities of chromosome 12. This male/female ratio is not yet explained, but the data provided further evidence for different causative mechanisms between males and females.

10.3.2 Imprinting status

Since the cell of origin in GCTs is the germ cell, where the imprint switch occurs in embryonic development, it was important to determine the imprint status for documentation of any chromosome errors, particularly related to sex of origin. To study this requires comparison of gene expression in the tumors to expression in the normal tissues from the same individual. In addition, a developmental stage comparison of the tumor to normal tissue is required.

Bussey et al. [41] used the methylation patterns of the imprinted gene *SNRPN* in GCTs as a reflection of primordial germ cell development. They compared the pattern to that seen in normal tissues from the same patients with tumors, and in non-germ-cell tumors. Of 84 GCTs from children and adults, 51 demonstrated a nonsomatic methylation pattern after dual digestion with enzymes XbaI and Not I, and Southern blot analysis. Only two of 18 control samples (16 non-GCT and two normal ovaries) showed a nonsomatic pattern. In both cases, the result was due to copy number differences.

This finding suggests that the abnormal methylation pattern seen in GCTs may help identify the developmental stage of the progenitor cell, based on the tumor’s location as it relates to known germ cell migration patterns. Based on data from studies on ovarian teratomas [42] and human oocytes [43], it has been further postulated that if tumors located in the sacrum had developed from a primary germ cell that had not yet reached the gonadal ridge for imprint erasing, they would have a somatic methylation pattern. Ten of eleven such tumors in the data of Bussey et al. showed a somatic pattern [41].

Uniparental disomy, imprinting and parent of origin differences are all important and subtle causes of genetic imbalance, leading to genetic syndromes and cancer. Additional such causes and effects are sure to be discovered along with the emergence, clarification and refinement of new molecular diagnostic technologies [17,44,45].

Glossary

Acetyl groups: Acetyl is the radical of acetic acid. It contains a methyl group single-bonded to a carbonyl. The carbon of the carbonyl has a bond available to the remainder of the molecule. The organic group of acetic acid ($\text{CH}_3\text{CO}-$) is a chemical group that is added and removed from histones. Acetylation of histones allows transcription to occur, and deacetylation inhibits transcription.

Anhidrosis: Lack of sweating.

Blepharophimosis: Dysplasia of eyelids with short palpebral fissures, lateral displacement of inner canthi, ptosis of eyelids.

Candiak–Higashi syndrome: A rare, inherited autosomal recessive disease of the immune and nervous systems characterized by pale-colored hair, eyes, and skin. Mutations have been found in the LYST gene. The primary defect in this disease is in special granules present in skin pigment cells and certain white blood cells.

CpG (Cytosine-phosphodiester-Guanine) islands: In molecular genetics, a short region of DNA rich in CpG sites, often located near the promoters of genes. Methylation of the CpG sites influences the activity of nearby genes and is critical to the regulation of gene expression.

DNA methylation: Process by which methyl groups are added to certain nucleotides in genomic DNA. This affects gene expression, as methylated DNAs are not easily transcribed. The degree of methylation is passed on to daughter strands at mitosis by maintenance DNA methylases. Accordingly, DNA methylation is thought to play an important developmental role in sequentially restricting the transcribable genes available to distinct cell lineages.

Endodermal sinus tumors: A malignant germ cell tumor of children that represents a proliferation of both yolk sac endoderm and extraembryonic mesenchyme. It is characterized by a labyrinthine glandular pattern with a papillary projection into a sinus-like space; frequently there are hyaline bodies and Schiller-Duval bodies. It produces α -fetoprotein and most often occurs in the testes, but is also seen in the ovaries and some extragonadal sites.

Epidermolysis bullosa: A group of rare inherited disorders in which blistering of the skin occurs in response to skin trauma. Large fluid-filled blisters can occur in response to injury, skin rubbing, chafing or even increases in room temperature.

Epigenetic: Incompletely understood mechanism by which there is differential expression of imprinted genes, usually according to parent of origin, without changes in the genomic sequence.

Gamete: A reproductive cell (ovum or sperm) with the haploid chromosome number.

Gametic complementation: For gametic complementation to occur, there is aneuploid or nondisjunction gamete formation in both parents – followed by chance fertilization of two aneuploid but complementing gametes. These events may lead to euploid offspring with UPD.

Gene dosage effect: Phenomenon whereby an increase in gene number can result in higher levels of the gene product if the gene is not subject to autogenous regulation.

Genomic imprinting: The differential silencing or expression of genetic material depending on the parent of origin and the methylation state of the gene(s).

Gonadal ridge: An elevation of thickened mesothelium and underlying mesenchyme on the ventromedial border of the embryonic mesonephros in which the primordial germ cells become embedded and establish it as the primordium of the testis or the ovary.

Heterochromatin: Heterochromatin is contained in regions of the genome which are highly condensed, are not transcribed, and are late-replicating. Heterochromatin is divided into two types: constitutive and facultative.

Constitutive heterochromatin: consists of simple repeats of nitrogenous bases that are generally located around the centromeres of all chromosomes and at the distal end of the Y chromosome. Chromosomes 1, 9, 16 and Y have variably sized constitutive heterochromatic regions. There are no transcribed genes located in constitutive heterochromatin, which explains the lack of copy number variant effect on the phenotype. The heterochromatic regions of these chromosomes stain differentially with various special staining techniques, revealing that the DNA structure of these regions is not the same as the structure of the euchromatic regions on the same chromosomes. The only established function of constitutive heterochromatin is the regulation of crossing over—the exchange of genes from one sister chromatid to the other during cell division.

Facultative heterochromatin: One X chromosome in female cells is randomly inactivated. The inactivated X is condensed during interphase and replicates late during the synthesis stage of the cell cycle. It appears in interphase as a condensed, dark region which is termed facultative heterochromatin.

Histone proteins: Proteins associated with DNA in the chromosomes that are rich in basic amino acids (lysine or arginine) and virtually unchanged throughout eukaryote evolution.

Hydatidiform mole: An abnormality of the placenta which causes it to grow to resemble a hydatid cyst or bunch of grapes, associated with very abnormal fetal development. In 90% of *complete moles*, the karyotype is 46,XX, representing duplication

of the chromosomes of the sperm with no maternal contribution. In 10% the karyotype is 46, XY, indicating dispermy. A *partial mole* is triploid, usually with an extra paternal chromosome set.

Imprinting center (imprinting control regions): ICRs are specialized sequence elements marked by allele-specific epigenetic modifications active on one of the two parental copies only. They regulate gene expression in an allele specific manner.

Isochromosomes: An isochromosome contains two copies of the same chromosome arm joined through an apparently single centromere such that the arms form mirror images of one another. Individuals with 46 chromosomes, 1 of which is an isochromosome, are monosomic for the genes within the lost arm and trisomic for all genes present on the isochromosome. Tetrasomy for the involved chromosome segment is present when an isochromosome is present as an extra (supernumerary) chromosome. Many theories have been proposed to explain the mechanism of isochromosome formation. The most commonly held is that isochromosome formation is the result of centromere misdivision. Instead of splitting longitudinally to separate the two sister chromatids, the centromere was hypothesized to undergo a transverse split that separated the two arms from one another. Recent molecular studies, however, suggest that the breakage and reunion events required to form some isochromosomes might occur predominantly within the area adjacent to the centromere, rather than within the centromere itself. It is clear that multiple mechanisms of isochromosome formation are likely to exist. Precisely which mechanism is found to predominate could largely depend on the chromosomal origin of the isochromosome, whether the chromosome is present in a disomic karyotype or represents an extra or supernumerary chromosome, and whether formation occurs during meiosis or mitosis. Clearly, additional studies are needed to establish a more complete understanding of isochromosome formation.

Leber congenital amaurosis: A characteristic and rare type of blindness transmitted as an autosomal recessive trait, occurring at or shortly after birth and associated with an atypical form of diffuse pigmentation and commonly with optic atrophy and attenuation of the retinal vessels.

Meiotic malsegregation: Nondisjunction during gamete genesis can give rise to chromosomally unbalanced offspring, such as trisomies or monosomies. Malsegregation, particularly when balanced chromosome rearrangements are present, may also result in unbalanced offspring of many types dependent on the breakpoints of the translocations.

Methylation: 1. In chemistry, the addition of a methyl group to a molecule. 2. On a protein level, the addition of a methyl group or groups to the amino acid arginine or lysine in a protein. 3. On a DNA level, the addition of a methyl group to a cytosine residue to convert it to 5-methylcytosine. Methylation of DNA occurs at CpG sites, where cytosine (C) lies next to guanine (G). The CpG sites are in regions near the promoters of a genes. These regions are known as CpG islands. The state of methylation of CpG islands is critical to both gene activity and gene expression.

Micrognathia: Abnormally small mandible.

Monosity rescue: Nondisjunction in meiosis in a parent leading to a gamete that is nullisomic for the chromosome involved. Following fertilization with a normal haploid gamete, the conceptus is monosomic. Mitotic rescue may result if nonjunction, duplication or isochromosome formation of the single homologue present occurs.

Not 1: A methylation reactive restriction enzyme that does not cut at methylated sites.

Parthenogenetic: Refers to an embryo or tumor produced from a female gamete without the participation of a male gamete.

Philtrum: The area from below the nose to the upper lip.

Polyhydramnios: Excessive amniotic fluid with pregnancy.

Post-zygotic: Occurring after the completion of fertilization and formation of the zygote.

Pycnodysostosis: An autosomal recessive inherited disorder due to mutation in the cathepsin K gene, which is involved with bone resorption. Features include small stature sclerosis of bone, persistence of anterior fontanelle, lack of frontal sinus, facial hypoplasia, dysplasia of fingers.

Segmental isodisomy: Occurs due to regional mitotic recombination changing the involved DNA segments from a heterodisomic state to an isodisomic state.

Segmental (partial) UPD: Segmental UPD (uniparental disomy): Regions of acquired homozygosity, the result of mitotic recombination, found in about 20% of acute myeloid leukemias.

SNP: Single nucleotide polymorphism. A polymorphism in DNA sequence consisting of variation in a single base.

Somatic mitotic recombination events: An error occurring post fertilization in the zygote. This may result in a balanced (normal) state, maternal isodisomy or paternal isodisomy.

Southern blot: A technique, devised by the British biochemist Ed Southern, for preparation of a filter to which DNA has been transferred, following restriction enzyme digestion and gel electrophoresis to separate the DNA molecules by size. Specific DNA molecules can then be detected on the filter by their hybridization to labeled probes.

Teratoma: A type of germ cell tumor that may contain several different types of tissue and sometimes mature elements such as hair, muscle, and bone. Teratomas occur most often in the ovary, testis, and in the sacrococcygeal region (near the tailbone) in children. A teratoma may be benign or malignant.

Trifunctional protein deficiency: A recessive hereditary disorder of fatty acid oxidation causing lethargy, hypoglycemia, hypotonia and feeding problems in infancy.

Triploidy: 69, XXX/69, XXY or 69, XYY: Three haploid chromosome sets are present per cell. Fetal loss is frequent. Survivors may show body asymmetry, syndactyly (fused digits) of third and fourth fingers, club foot, abnormal male genitalia, kidney abnormalities; heart defects, dysplastic (deformed) ears and cleft lip and/or palate. Iris/choroid colobomas are common. The most frequent cause is fertilization of the egg by two sperm (dispermy).

Trisomy rescue: Nondisjunction in meiosis in a parent leading to disomy in a gamete. Following fertilization, trisomy is present in the conceptus; loss of a homologue (rescue) may result in uniparental disomy or normal disomy.

UBE3A gene: Ubiquitin protein ligase E3A. This gene provides instruction for making the enzyme, ubiquitin protein ligase E3A. Maternal copy (only one) is active in the brain.

Uniparental disomy: The presence in the karyotype of two copies of a specific chromosome, both inherited from one parent, with usually no representative of that chromosome from the other parent. If both homologues from the parent are present, the situation is termed *heterodisomy*; if one parental homologue is present in duplicate, the situation is termed *isodisomy*.

Unmasking of a recessive allele: Uniparental disomy from a parent carrying a deleterious recessive allele, with both copies of the disomic chromosome (isodisomy) containing that allele, presenting with full features of the disorder.

WT1: Wilms tumor suppressor gene, location 11p13 – zinc finger transcription factor – may exhibit biallelic inactivation in Wilms tumors (<15%), with 11p13 deletion in some cases.

Xba 1: A restriction enzyme which cuts DNA at a specific sequence of nucleotides.

Zygote: A fertilized ovum.

References

1. Crouse HV. The controlling element in sex chromosome behaviour in *Sciara*. *Genetics* 1960;45:1429–1443.
2. Lorenz K. Imprinting. *J Orn* 1935;83:137–213,289–413.
3. Hoppe PC, Illmensee K. Microsurgically produced homozygous-diploid uniparental mice. *Proc Natl Acad Sci USA* 1977;74:5657–5661.
4. McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* 1984;37:179–183.
5. Surani MAH, Barton SC, Norris PML. Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome. *Cell* 1986;45:127–136.
6. Surani M. Evidences and consequences of differences between maternal and paternal genomes during embryogenesis of the mouse. In Rossant J, Pederson RA, eds. *Experimental Approaches to Mammalian Embryonic Development* 1986; New York: Cambridge University Press, 401–435.
7. Linder D, McCaw BK, Hecht F. Parthenogenic origin of benign ovarian teratomas. *N Engl J Med* 1975;292:63–66.
8. Jirtle R, Weidman J. Imprinted and More Equal. *Am Sci* March/April 2007(35).
9. Jirtle R. Geneimprint – Service of Jirtle Laboratory, Duke University Copyright 2006. <http://www.geneimprint.com>
10. NIH News Advisory. The Mouse Genome and the Measure of Man. National Human Genome Research Institute. National Institute of Health 2002 Release.
11. Reik R, Walter J. Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2001; 2(1):21–32.
12. Lewin B. *Genes IX* 2007; London: Jones & Bartlett, Chapters 30–31.
13. Delaval K, Wagschal A, Feil R. Epigenetic deregulation of imprinting in congenital diseases of aberrant growth. *BioEssays* 2006;28(5):453–459.
14. Buiting K, Horsthemke B. Molecular genetic findings in Prader-Willi syndrome. In Butler M, Lee P, Whitman B, eds. *Management of Prader-Willi Syndrome* 2006. USA: Springer.
15. Butler G, Palmer PG. Parental origin of chromosome 15 deletion in Prader-Willi syndrome. *Lancet* 1983;1:1285–1286.
16. Horsthemke B. Imprinting in the Prader-Willi/Angelman syndrome region on human chromosome 15. In Reik W, Surani A, eds. *Genomic Imprinting* 1997. New York: Oxford. By permission of Oxford University Press.
17. Shaffer L. Uniparental disomy: mechanisms and clinical consequences. *Fetal Maternal Med Rev* 2003;14:155–175.
18. Engel E, Antonarakis S. *Genomic Imprinting and Uniparental Disomy in Medicine: Clinical and Molecular Aspects* 2002; New York: Wiley-Liss Inc.

19. Wang J-C. Genomic Imprinting and Uniparental Disomy. In Gersen S, Keagle M, eds. *The Principles of Clinical Cytogenetics*, 2nd ed. 2004; Totowa, NJ: Humana Press.
20. Berend SA, Horwitz J, McCaskill C, Shaffer L. Identification of uniparental disomy following prenatal detection of Robertsonian translocations and isochromosomes. *Am J Hum Genet* 2000;66:1787–1793.
21. Wang J-CC, Passage MB, Yen PH, Shapiro LJ, Mohandas TK. Uniparental heterodisomy for chromosome 14 in a phenotypically abnormal familial balanced 13/14 Robertsonian translocation carrier. *Am J Hum Genet* 1991;48:1069–1074.
22. Papenhausen PR, Mueller OT, Johnson VP, Sutcliffe M, Diamond TM, Kousseff BG. Uniparental isodisomy of chromosome 14 in two cases: an abnormal child and a normal adult. *Am J Med Genet* 1995;59:271–275.
23. Kurosawa K, Sasaki H, Sato Y, Yamanaka M, Shimizu M, Ito Y, Okuyama T, Matsuo M, Imaizumi K, Kuroki Y, Nishimura G. Paternal UPD14 is responsible for a distinctive malformation complex. *Am J Med Genet* 2002;110:268–272.
24. Hordijk R, Wierenga H, Scheffer H, Leege B, Hofstra RMW, Stolte-Dijkstra I. Maternal uniparental disomy for chromosome 14 in a boy with a normal karyotype. *J Med Genet* 1999;36:782–785.
25. Sanlaville D, Aubry MC, Dumez T, Nolen MC, Amiel J, Pinson MP, Lyonnet S, Munnich A, Vekemans M, Morichon-Delvallez N. Maternal uniparental heterodisomy of chromosome 14: chromosomal mechanism and clinical follow up. *J Med Genet* 2000;37:525–528.
26. Temple IK, Cockwell A, Hassold T, Pettay D, Jacobs P. Maternal uniparental disomy for chromosome 14. *J Med Genet* 1991;28:511–514.
27. Berends MJW, Hordijk R, Scheffer H, Oosterwijk JC, Halley DJ, Sorgedrager N. Two cases of maternal uniparental disomy 14 with a phenotype overlapping with the Prader-Willi phenotype. *Am J Med Genet* 1999;84:76–79.
28. Towner DR, Shaffer LG, Yang SP, Walgenbach DD. Confined placental mosaicism for trisomy 14 and maternal uniparental disomy in association with elevated second trimester maternal serum human chorionic gonadotrophin and third trimester fetal growth restriction. *Prenat Diagn* 2001;21:395–398.
29. Gelb BD, Willner JP, Dunn TM, Kardon NB, Verloes A, Poncin J, Desnick RJ. Paternal uniparental disomy for chromosome 1 revealed by molecular analysis of a patient with pycnodynatosostosis. *Am J Hum Genet* 1998;62:848–854.
30. Miura, Y, Hiura, M, Torigoe, K, Numata O, Kuwahara A, Matsunaga M, Hasegawa S, Boku N, Ino H, Mardy S, Endo F, Matsuda I, Indo Y. Complete paternal uniparental isodisomy for chromosome 1 revealed by mutation analyses of the TRKA (NTRK1) gene encoding a receptor tyrosine kinase for nerve growth factor in a patient with congenital insensitivity to pain with anhidrosis. *Hum Genet* 2000;107:205–209.
31. Takizawa Y, Pulkkinen L, Chao SC, Nakajima H, Nakano Y, Shimizu H, Uitto J. Mutation report: complete paternal uniparental isodisomy of chromosome 1: a novel mechanism for Herlitz junctional epidermolysis bullosa. *J Invest Dermatol* 2000;115:307–311.
32. Thompson DA, McHenry CL, Li Y, Richards JE, Othman MI, Schwinger E, Vollrath D, Jacobson SG, Gal A. Retinal dystrophy due to paternal isodisomy for chromosome 1 or chromosome 2, with homoallelism for mutations in RPE65 or MERTK, respectively. *Am J Hum Genet* 2002;70:224–229.
33. Chen H, Young R, Mu X, Nandi K, Miao S, Prouty L, Ursin S, Gonzalez J, Yanamandra K. Uniparental isodisomy resulting from 46,XX,i(1p)i(1q) in a woman with short stature, ptosis, micro/retrognathia, myopathy, deafness, and sterility. *Am J Med Genet* 1999;82:215–218.
34. Pulkkinen L, Bullrich F, Czarnecki P, Weiss L, Uitto J. Maternal uniparental disomy of chromosome 1 with reduction to homozygosity of the LAMB3 locus in a patient with Herlitz junctional epidermolysis bullosa. *Am J Hum Genet* 1997;61:611–619.
35. Dufourcq-Lagelouse R, Lambert N, Duval M, Viot G, Vilmer E, Fischer A, Prieur M, de Saint Basile G. Chediak-Higashi syndrome associated with maternal uniparental isodisomy of chromosome 1. *Eur J Hum Genet* 1999;63:633–637.
36. Field LL, Tobias R, Robinson WP, Paisey R, Bain S. Maternal uniparental disomy of chromosome 1 with no apparent phenotypic effects. *Am. J. Hum. Genet* 1998;63:1216–1220.
37. Raghavan M, Lillington D, Skoulakis S, Debernardi S, Chaplin T, Foot N, Lister TA, Young B. Genome-wide single nucleotide polymorphism analysis reveals frequent partial uniparental disomy due to somatic recombination in acute myeloid leukemias. *Cancer Research* 2005;65:375–378.

38. Fitzgibbon J, Smith L-L, Raghavan M, Smith M, Debernardi S, Skoulakis S, Lillington D, Lister TA, Young B. Association between acquired uniparental disomy and homozygous gene mutation in acute myeloid leukemias. *Cancer Research* 2005;65:9152–9154.
39. Bussey K, Lawce H, Olson S, Arthur D, Kalousek D, Kralio M, Giller R, Heifetz S, Womer R, Magenis RE. Chromosome abnormalities of eighty-one pediatric germ cell tumors: sex-, age-, site-, and histopathology-related differences – a Children's Cancer Group Study. *Genes Chromosomes Cancer* 1999;25:134–146.
40. Bussey K, Lawce H, Himoe E, Shu XO, Suijkerbuijk R, Olson S, Magenis RE. Chromosome 1 and chromosome 12 abnormalities in pediatric germ cell tumors by interphase FISH. *Cancer Genet Cytogenet* 2001;125:112–118.
41. Bussey K, Lawce H, Himoe E, Shu XO, Heerema N, Perlman E, Olson S, Magenis RE. SNRPN methylation patterns in germ cell tumors as a reflection of primordial germ cell development. *Genes Chromosomes Cancer* 2001;32:342–352.
42. Miura K, Obama M, Yun K, Masuzaki H, Ikeda Y, Yoshimura S, Akashi T, Niikawa N, Ishimaru T, Jinno Y. Methylation imprinting of *H19* and *SNRPN* genes in human benign ovarian teratomas. *Am J Hum Genet* 1999;65:1359–1367.
43. El-Maarri O, Buiting K, Peery EG, Kroisel PM, Balaban B, Wagner K, Urman B, Heyd J, Lich C, Brannan CI, Walter J, Horstemke B. Maternal methylation imprints on human chromosome 15 are established during or after fertilization. *Nat Genet* 2001;27:341–344.
44. Shaffer L, Bejjani BA, Torchia B, Kirkpatrick S, Coppinger J, Ballif BC. The identification of microdeletion syndromes and other chromosome abnormalities: cytogenetic methods of the past, new technologies for the future. *Am J Med Genet Part* 2007;145C:335–345.
45. Shaffer LG, Bejjani BA. Medical applications of array CGH and the transformation of clinical cytogenetics. *Cytogenet Genome Res* 2006;115:303–309.

CHAPTER 11

Cytogenetic analysis of hematologic malignant diseases

Nyla A. Heerema

Department of Pathology, The Ohio State University, Columbus, OH, USA

This chapter describes cytogenetic rearrangements with ISCN (International System for Human Cytogenomic Nomenclature) terminology. The reader is referred to Chapter 8 for an understanding of ISCN nomenclature.

11.1 Introduction

Hematologic malignant diseases include leukemias and lymphomas. Leukemias are malignancies of the blood and bone marrow and originate in the bone marrow. Lymphomas are malignancies of the lymphatic system, originating in lymph nodes (see the Glossary at the end of this chapter for additional definitions). The two can be very similar and have some overlap. For those that are similar, presentation in the bone marrow generally indicates leukemia, while presentation in the lymphatic system generally indicates lymphoma. These diseases also include some “ premalignant” conditions, which have some features of malignancies, but are not frankly malignant.

The cytogenetics of hematologic malignancies has contributed immensely to their diagnosis, staging, prognosis, treatment, treatment response evaluation and understanding their biology. Cytogenetic abnormalities define the particular genetic subtypes of leukemias. The World Health Organization (WHO) Classification of Tumours incorporates cytogenetic aberrations, as well as morphologic, immunophenotypic and clinical features, in the classification of leukemias and lymphomas [1]. Cytogenetic studies are recommended at diagnosis and at regular intervals thereafter [1]. The cytogenetic/genetic aberrations may be identified by conventional banded metaphase analysis, fluorescence *in situ* hybridization (FISH) or other molecular techniques, such as reverse transcriptase polymerase chain reaction (RT-PCR), array-based comparative genomic hybridization (aCGH) (see Chapter 18, section 18.2.2, aCGH for characterization of acquired genetic changes) and single nucleotide polymorphism (SNP) arrays to detect copy number aberrations.

Many recurring cytogenetic abnormalities occur in the leukemias and lymphomas, and many clonal abnormalities are unique to a particular patient. Abnormalities in hematologic malignancies are considered clonal when a structural abnormality(s) or gain of a chromosome(s) is seen in at least two cells, or loss of a chromosome(s) is seen in at least three cells. The abnormalities can occur in any form and combination. Numerical abnormalities include both gains and losses. There may be gain or loss of a single chromosome or of many, including ploidy abnormalities. Structural abnormalities may be balanced or unbalanced. Balanced abnormalities are reciprocal translocations, inversions and insertions. Unbalanced aberrations include derivative chromosomes, isochromosomes, deletions, duplications, marker chromosomes, double minutes, and homogeneously staining regions (rare). However, some abnormalities are more common than others, and some occur with other particular abnormalities more often than by chance (Table 11.1).

Table 11.1 Common recurring aberrations – (bold ISCN indicates that an example is in Figure 11.2)

a. AML/MDS						
Cytogenetic aberration	Frequency	Genes/probes	Prognosis	Cytogenetic variants	Secondary aberrations/ comments	References
t(8;21)(q22;q22.3)	5–10% of adult, 20% of pediatric AML	<i>RUNX1T1(ETO)-RUNX1</i>	Good	insertions	-X or Y, del(9)(q22)	[4,5]
inv(16)(p13.1q22)/ t(16;16)(p13.1q22)	10–12%	<i>MYH11-CBF</i>	Good	t(1;16)(p31-32;q22), t(3;16)(q21;q22), t(5;16)(q33;q22)*	+8, +22, del(7q), +21	[4,5]
t(15;17)(q24;q21)	100% of APL/30–40% of AML	<i>PML-RARA</i>	Good	t(11;17)(q23;q21), ins(15;17), ins(17;15) (see 11.2 Myeloid leukemias)	+8, ider(17q), del(9q)	[28]
t(11;v)(q23;v)	5–6% overall, >80% of infants	<i>KMT2A-v</i>	Poor	<i>KMT2A</i> has many different partners. Metaphase analysis is required to determine the partner chromosome, as prognosis differs		
t(1;11)(q21;q23)	3% of pediatric	<i>KMT2A-MLLT11(AF1q)</i>	Excellent			
t(9;11)(p22;q23)	2–5%	<i>KMT2A-MLLT3(AF9)</i>	Intermediate			
t(6;11)(q27;q23)		<i>KMT2A-MLLT4(AF6)</i>	Poor	Sometimes T-ALL	+8, +3, +19, +21	[32–34]
t(10;11)(p12;q23)		<i>KMT2A-MLLT10(AF10)</i>	Poor	ins(10;11)(p12;q23q13)	Also in T-ALL	[1]
t(11;19)(q23;p13.1)		<i>KMT2A-ELL</i>	Poor	Also occurs in ALL		
t(11;19)(q23;p13.3)		<i>KMT2A-MLLT1(ENL)</i>	Poor			
t(6;9)(p23;q34)	1%	<i>DEK-NUP214(CAN)</i>	Poor			
inv(3)(q21q26)/ t(3;3)(q21;q26)	2% of AML	<i>RPN1-MECOM(EV1)</i>	Poor			
t(3;v)(q26;v)		<i>MECOM-v</i>	MECOM has many different partners. Prognosis varies dependent on the partner gene.			
t(8;16)(p11;p13.3)	rare	<i>KAT6A(MYST3)-CREBPP(CBP)</i>	Poor	Complex	Frequently therapy-related	[245]

del(20q)	2-5% AML, 6% PV, 1% MDS	20q12 probe	Poor except sole in MPS	[11,43]
+8	10-15% AML, 15-20% MDS	Intermediate (most studies)	Can be primary or secondary, the latter with many different primary	[6,8]
-7/7q-	Adults 30% RAEB/ RAEB-T, 20% CMML, 5% RA with abnormal karyotype; >50% of T-AML	CEN7/7q probes	Both poor adults, -7 poor pediatric	-5/5q-, often complex, more common in elderly [3,5]
-5/5q- excluding 5q- syndrome	10% of AML	5p/5q probes	Poor	-7/7q-, often complex, more common in elderly [3,5]
t(2;11)(p21;q23)		KMT2A	Poor	del(5) (q13q33), therapy-related [246]
t(11;16)(q23;p13.3)		KMT2A-CREBBP(CBP)	Poor	Therapy-related [247]
del(9q)		KMT2A	Good	Often is secondary [248]
amp(KMT2A)	rare	KMT2A	Poor	[32]
t(1;22)(p13;q13)	25-30% of pediatric acute megablastic leukemia	RBM15(OTT)-MLKL(MAL)	Poor	Megakaryocytic leukemia <3 years old [249]
t(7;12)(q36;p13)		MNX1(HLXB9)-ETV6	Poor	+19, +8; found in infants [250]
der(1;7)(q10;p10)	0.5-2% de novo	CEN7/CEN1 probes	Poor, but better than other -7/7q-	+8, +21, +9; frequently T-MDS/AML [251,252]
t(4;v)(q12;v)		FIP1L1-PDGFR _A CHIC2 probes	t(1;4)(q44;q12) and t(4;10)(q12;p11) sometimes result in this cryptic 4q deletion	Produces a chimeric oncogene. CEL and T-cell ALL with eosinophilia respond to imatinib. [78]
Loss Y	up to 90% in elderly males		Controversial whether sole -Y is age-related or malignancy-related	[253,254]

(Continued)

Table 11.1 (Continued)

b. ALL					
Cytogenetic aberration	Frequency	Genes/probes	Prognosis/pediatric	Cytogenetic variants	Secondary aberrations
Hyperdiploidy, mn >50	25%–30% pediatric ALL	Good			[86,88–90]
Hyperdiploidy, mn >46 <51		Intermediate			Usually have structural abnormalities, -50% recurrent [86,88–90]
Pseudodiploidy		Intermediate			[86,88–90]
Hypodiploidy, mn 44–45		Intermediate			Usually have structural abnormalities [94]
Hypodiploidy, mn <44		Poor		Few structural abnormalities, frequent doubling in mn <40	[93]
B-lineage ALL					
t(9;22)(q34;q11.2)	5% pediatric; 25% adult	<i>BCR-ABL1</i>	Good with a tyrosine kinase inhibitor	5% complex hyperdiploidy, del(7), del(9p)	Frequency increases with age [130–132]
t(12;21)(p13;q22.3)	25% of pediatric; 5% of adult ALL	<i>ETV6-RUNX1</i>	Good	Many, del(12p), del(6q), del(11q) common	Cytogenetically cryptic. Many other <i>ETV6</i> rearrangements; may differ in clinical characteristics and prognosis [105,107,112]
t(4;11)(q21;q23)	11q23: 5% of ALL, 80% of infants	<i>KMT2A-MLLT2(AF4)</i>	Poor	Insertions, 3-way translocations i(7q)	Frequent in infants. Outcome may differ with age [35,102]
Other 11q23		<i>KMT2A-other</i>	Poor/Intermediate		Frequent in infants. Outcome may differ with age and partner [243]
t(1;11)(p32;q23)		<i>KMT2A-EPS15(AF1P)</i>			
t(6;11)(q27;q23)		<i>KMT2A-MLLT4</i>			
t(9;11)(p22;q23)		<i>KMT2A-MLLT3(AF9)</i>	Better than other 11q23		[33,34]
t(11;19)(q23;p13.3)		<i>KMT2A-MLLT1(ENL)</i>	Good, T-ALL & <10years		Also in T-ALL [33,255]

t(1;19)(q23;p13.3)	4–5% ALL, 30% pre-B ALL	<i>PBX1-TCF3(E2A)</i>	Not prognostic	der(19)t(1;19) (q23;p13.3); t(17;19)(q22;p13.3)	[123,132,137,138]
del(6q)	5–15% B-lineage, 10–20% T-ALL	<i>MYB</i> some cases	Not prognostic	Frequent in <i>ETV6-RUNX1</i> cases	[256]
t(6;14)(p22;q32)	Rare	<i>IGH-ID4</i>	Fair	del(9p), i(9q)	[257]
del(7p)	4%	<i>PAX5</i> and <i>CDKN2A</i>	Poor		[258]
del(9p)	30%	<i>ETV6</i>	Not prognostic	Often cryptic Other genes may also be involved	[139,259,260] [261]
del(12) (p11.2~p13)		<i>PAX5</i>		t(9;22) (q34;q11.2)	[262]
dic(7;9)(p11.2;p13)		<i>PAX5-ETV6</i>	Good	t(12;21) (p13;q22)	Not all have <i>PAX5-ETV6</i> rearrangement [143,145,263,264]
dic(9;12) (p13;p11.2)	4%	<i>PAX5</i>	Intermediate	+X, +21	Sometimes with <i>ETV6-RUNX1</i> fusion genes [144,146,147]
dic(9;20) (p13;q11.2)		<i>IGH-CEBPD</i>			
t(8;14)(q11.2;q32.3)		<i>IGH-ID3</i>	Poor	+X, i(7q), del(12p), +19	Frequent in Down syndrome [265]
t(5;14)(q31;q32.3)	Rare	<i>RUNX1</i>	Poor	Associated with hypereosinophilia [266]	
der(21)/add(21) iAMP21	2%		der(21) appears in many guises	Amplification of <i>RUNX1</i> or near gene. Designated iAMP21.	[154,267]
t(X or Y;14) (pter;q32.3, del(X) (p22.3p22.3) or del(Y)(p11.3p11.3)	1% with t 4–7% with del	<i>IGH-CRLF2 t P2RY3-CRLF2 del</i>	Poor in non-Down syndrome	+X, del(9p) in Down syndrome Mutations of <i>JAK1</i> , <i>JAK2</i> , <i>JAK3</i> and <i>IKZF1</i> . Deletion in 55% of Down syndrome (DS) ALL	[149–151]
T-lineage ALL					
del(9)(p21)	65% T-ALL	<i>CDKN2A(p16)</i>	Poor in T-ALL	Especially frequent in T-ALL, often cryptic. Breakpoints differ.	[142,268]
inv(7)(p15q34)	3–5% TAL	<i>TRB-HOXA</i>		t(7;7)(p15;q34)	[269]
t(1;14)(p32;q11.2)	10% translocation 30% deletion pediatric T-ALL	<i>TAL1(SCL)-TRD(TCRD)</i>		del(1)(p32p32) cryptic; t(1;7)(p32;q34)	Cryptic deletion resulting in <i>TAL1-SCL</i> fusion common [270]

(Continued)

Table 11.1 (Continued)

			b. ALL				
Cytogenetic aberration	Frequency	Genes/probes	Prognosis/pediatric	Cytogenetic variants	Secondary aberrations	Comments	Reference
t(6;7)(q23.3;q34)	5% T-ALL	TRB-MYB				Seen in very young patients; can be cryptic	[271]
t(6;11)(q27;q23)	rare	MLLT4-KMT2A	Poor			See AML	[1]
14q11.2 rearrangement	3-4%	TRD(TCRD)	Not prognostic	7p15, 7q34			
t(8;14) (q24.2;q11.2)		TRD-MYC	Poor				[272]
t(10;14)(q24;q11.2)	10% pediatric, 30% adult	TRD(TCRD)-TLX1(HOX11)	Favorable	t(7;10)(q34q24)			[273]
t(11;14)(p13;q11.2)	5-10% pediatric, 2% adult T-ALL	TRD(TCRD)-LMO2		t(7;11)(q34;p13)	+17		[274]
t(11;14)(p15;q11.2)	Rare	TRD-LMO1		t(7;11)(q34;p15)			[274,275]
t(5;14)(q35;q32)	20% pediatric; 13% adult	TLX3(HOX11L2)-BCL11B	Poor			Cryptic	[276-279]
ins(10;11) (p13;q23;q13)		KMT2A-MLLT10(AF10)	Poor			May be cryptic	[280]
t(10;11)(p12;q14)	5% T-ALL	MLLT10(AF10)- PICALM(CALM)	Poor			Also in AML	[280,281]
amp(NUP214/ ABL1)	<6% T-ALL	NUP214-ABL1	Poor			Cytogenetically cryptic, FISH required, rearrangement on an episome	[164,165,282]
amp(MYB)	8% T-ALL	MYB				Cryptic, frequently also have NOTCH1 mutation	[271]

c. Chronic leukemias						
Disease	Cytogenetic Aberration	Frequency	Genes/probes	Prognosis	Variants	Secondary Abnormalities
CML	t(9;22)(q34;q11.2)	100% of CML	BCR-ABL1	Good with imatinib mesylate	Myeloid: +der(22) t(9;22), t(17)(q10) , +8, +19.	Complex; cryptic in 5%
Polycythemia vera	del(20)(q12)	20q			Lymphoid: del(7q)	[54,55]
Chronic idiopathic myelofibrosis, essential thrombocythemia	del(13)(q12q22)				Some with +8, +9, del(13q), del(9p), del(1)(p11)	[68,69]
Primary myelofibrosis	der(6)t(1;6) (q21 - q23;p21.3)				Some with +8, 20q-, -77q-, 11q-	[283,284]
Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB, or FGFR1	t(4;v)(q12;v)		PDGFRA		del(20q), abnormal 1q	[1]
Chronic eosinophilic leukemia (CEL)						[1]
CMMI						[61-63,82]
JMML	-7	+8, -7/7q-, abn(12p)	30%-40%	CEN7	Poor	[61-64]
MDS	+8, -7/7q-, -5/5q-, 20q- , complex			CSF1R	Generally poor Good	[66] [285]
MDS – pediatric	-7, complex with a structural abnormality				Poor	[46,47] [64,286]

(Continued)

Table 11.1 (Continued)

Disease	Cytogenetic Aberration	c. Chronic leukemias						References
		Frequency	Genes/probes	Prognosis	Variants	Secondary Abnormalities	Comments	
CLL	11q-	20%	ATM	Poor				[171,172,179–181]
	13q-	50%	<i>miR15, miR16</i> D13S319	Intermediate				[171,172,179–181]
	+12	20%	CEN112 (<i>MDM2</i>)	Good				[171,172,179–181]
	17p-	10%	<i>TP53</i>	Poor				[171,172,179–181]
	6q-	5%	<i>MYB</i> or 6q21 probe	Intermediate				[287,288]
	t(14;19)(q32.3;q13.2)		<i>IGH-BCL3</i>					[289]
	Complex			Poor				[290,291]
T-PLL	inv(14)(q11.2;q32)	70% T-PLL	<i>TRA-TCL1A</i>	Poor	<i>t(14;14) (q11.2;q32)</i>	<i>i(8q), +8</i>	T-cell mitogen required	[167,168]
MM (MGUS)	+3, +5, +7, +9, +11, +15, +19, +21		Localized to specific chromosomes	Good				[187,193]
	t(11;14)(q13;q32.3)	30%	<i>IGH-CCND1</i>	Good				[193]
	t(4;14)(p16;q32.3)	25%	<i>IGH-WHSC1(MMS67)</i> and <i>FGFR3</i>	Poor				[193]
	t(8;14)(q24.2;q32.3)	5%	<i>IGH-MYC</i>	Not prognostic				[193]
	t(14;16)(q32.3;q23)	1%	<i>IGH-MAF</i>	Poor				[193]
	t(14;20) (q32.3;q11.2–13.1)		<i>IGH-MAFB</i>	Poor				[193]
	other 14q32.3		<i>IGH</i>					[193]
	-13, del(13)(q14)	48% by FISH	D13S319/ <i>RB1</i>	Poor, usually with other poor prognostic factors				[193]
	17p-	11% by FISH	<i>TP53</i>	Poor				[193]

d. Lymphomas						
Type	Cytogenetic Aberration	Frequency	Genes/probes	Prognosis	Cytogenetic Variants	Secondary Aberrations/Comments
Mantle cell	t(11;14)(q13;q32.3)	100% (by FISH)	<i>IGH-CCND1</i>	Poor	t(2;11)(p11.2;q13), t(11;22)(q13;q11.2)	+3,+7,del(9p),+18 [292,293]
Follicular/diffuse large B-cell	t(14;18)(q32.3;q21.3)	80% of FL, 20%-30% DLBCL,10-20% MALT	<i>IGH-BCL2</i>	FL, not prognostic: DLBCL poor	t(2;18)(p11.2;q21), t(18;22)(q21;q11.2)	[294-296]
Diffuse large B-cell	t(3;14)(q27;q32.3)	Associated with DLBCL, 5%-10% of FL	<i>IGH-BCL6</i>	Unknown	Many partners of 3q27	[297,298]
Marginal zone B-cell (MZBCL)	t(11;18)(q21;q21)	25-50% MALT	<i>BIRC3/API2)-MALT1</i>	Indolent		[299-301]
	t(3;14)(p14.1;q32.3)	10% MALT	<i>IGH-FOXP1</i>	Indolent		[302]
	t(1;14)(p21;q32.3)	5-10% MALT	<i>IGH-BCL10</i>	May be unresponsive to antibiotics	Also in FL and DLBCL	[303]
	del(7)(q21 - q32)	40% MZBCL				[304]
Burkitt	t(8;14)(q24.2;q32.3)	100% BL (including variants), also in mature B-cell ALL and BLL	<i>IGH-MYC,IGK-MYC,IGL-MYC</i>	Good	t(2;8)(p11.2;q24.2), t(8;22)(q24.2;q11.2)	Abnormal 1q, abnormal 13q [208,305,306]
Lymphoblastic	t(9;14)(p13;q32.3)	rare	<i>IGH-PAX5</i>	Adverse	t(2;9)(p11.2;p13), t(9;22)(p13;q11.2)	Also present in other B-cell malignancies [307,308]
Anaplastic large cell MALT	t(2;5)(p23;q35) t(3;14)(p14.1;q32.3)	100% ALCL	<i>NPM1-ALK</i>	Good	Many	[226-234] [302]
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL (double/triple hit lymphoma)	t(8;v)(q24.2;v) with t(14;v)(q32.3;v) or t(2;v) (p12;v) or t(22;v)(q11;v)	100%	<i>MYC-IGH-IGK-GL</i>	Indolent Adverse	Many	[1]

Approximately 60,140 individuals were predicted to be diagnosed with different leukemias in 2016, and approximately 24,400 individuals with leukemia were predicted to die in 2016 (Table 11.2) [2]. The incidences of all leukemias except acute lymphoblastic leukemia (ALL) increase with age.

The leukemias can be differentiated by their clinical presentation, their cell type/pathology and their genetic aberrations. Historically, the cell types defined the leukemias, and they correlated with various normal cells of the hematopoietic system (Figure 11.1). Leukemias can occur in most of the different cell types and at different stages of cell maturation. It is not known whether genetic aberrations associated with different cell types occur in those particular cell types only or whether they occur in a more primitive cell, which differentiates to the specific cell type associated with the genetic abnormality.

Leukemias also can be either chronic or acute. Chronic leukemias generally have an indolent course and are not always treated. In cells of the myeloid lineage, they include chronic myelogenous leukemia, chronic neutrophilic leukemia and chronic eosinophilic leukemia (not otherwise specified). In the lymphoid lineage, they are comprised primarily of mature B-cell neoplasms, such as chronic lymphocytic leukemia. Acute leukemias have a much more aggressive course and are rapidly fatal if not treated. Recurring cytogenetic aberrations have been described for many of these disorders, although not for all.

Diagnosis of leukemia usually requires a bone marrow aspirate and biopsy. The various leukemias have different morphologic and cytochemical features, and many have distinctive immunophenotypes [1]. Leukemias are distinguished from preleukemias by the presence of ≥20% blasts in the bone marrow. General clinical presenting characteristics of leukemia are shown in Table 11.3.

11.2 Myeloid leukemias

11.2.1 Acute myeloid leukemia (AML)

The acute myeloid leukemias (AML) are characterized by a predominance of immature forms of myeloid lineage cells in the bone marrow, with a concomitant loss of normal hematopoiesis. AML is more common in adults than in children, with a median age of 67 years at diagnosis. It accounts for 70–80% of adult acute leukemias, but only 15% of childhood acute leukemias. AML frequency increases with age, markedly so with advanced patient age (Table 11.2) [3]. Although infrequent in children, AML is the most common congenital leukemia.

Table 11.2 Incidences of Leukemia

Disease ¹	Estimate for 2016 [2]		Statistics from 2009–2013 [241]				2006–2012 [2]
	No. diagnosed	No. of deaths	Median age at diagnosis (years)	% under 20 years	% over 65 years	Median age of death (years)	5-year survival (%)
AML	19,950	10,430	67	5.3	56	72	26 (66.5 for <15yr)
CML	8,220	1,070	64	2.2	49	76	63.2 ²
CLL	18,960	4,660	71	0.1	67.2	80	84.8
ALL	6,590	1,430	15	57.2	11.4	54	70.1 (92.2% for >1<5yr)
MM	30,330	12,650	69	0	62.2	75	49
NHL	72,580	20,150	66	1.6	55.2	76	72
HD	8,500	1,120	39	12.6	17.8	65	88
Leukemia ³	60,140	24,400	66	9	53.7	75	62

¹ AML: acute myeloid leukemia; CML: chronic myelogenous leukemia; CLL: chronic lymphocytic leukemia; ALL: acute lymphoblastic leukemia; MM: multiple myeloma; NHL: non-Hodgkin lymphoma; HD: Hodgkin disease (lymphoma)

² Since advent of imatinib mesylate and other tyrosine kinase inhibitors, this may change

³ Excludes MM and lymphomas

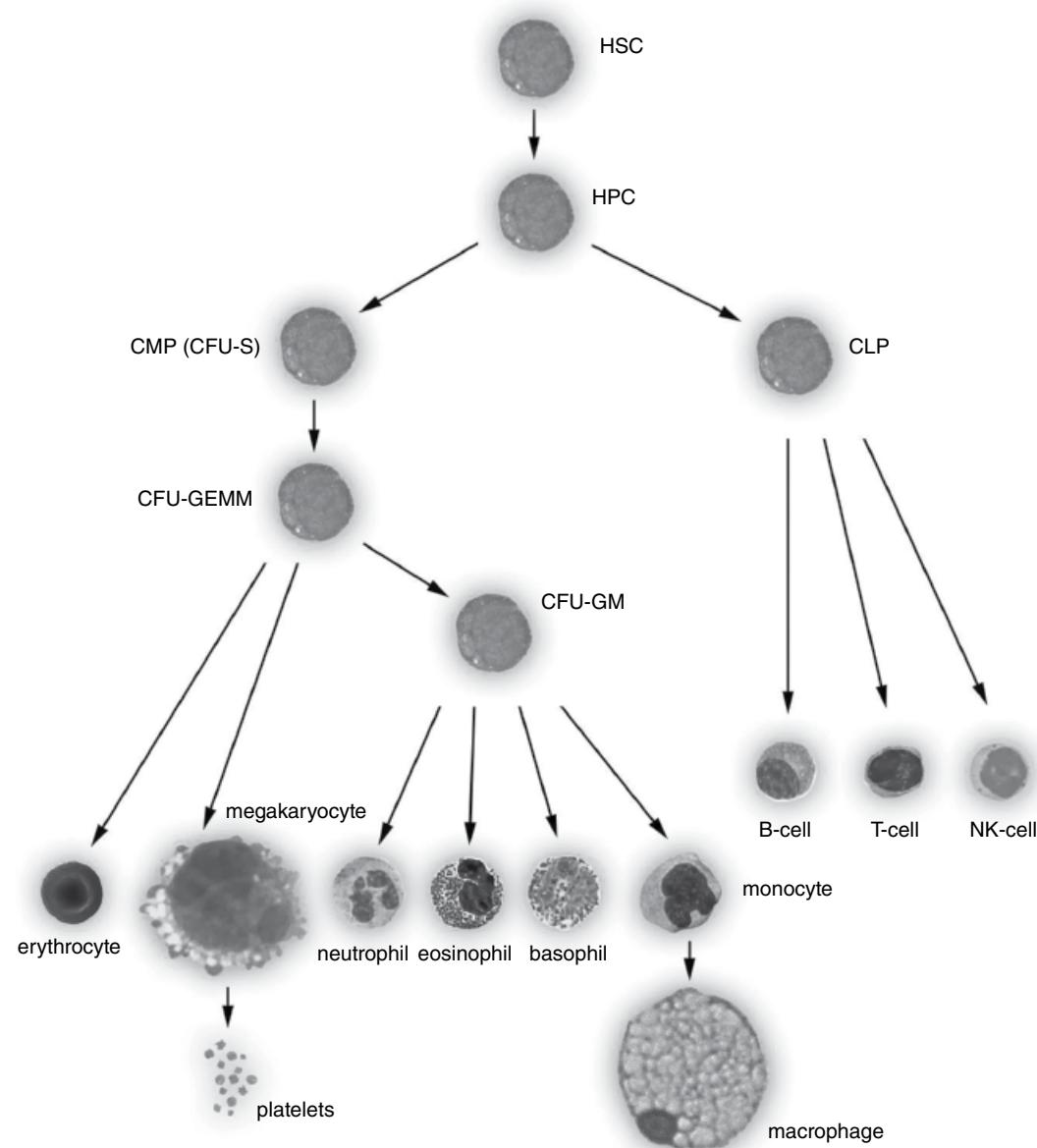


Figure 11.1 The hematopoietic system. The hematopoietic system as developed from a hematopoietic stem cell. The myeloid system and lymphoid systems separate early, and the various myeloid and lymphoid cell types develop from the myeloid and lymphoid (respectively) stem cells. As the cells mature and differentiate, they lose their ability to divide. Abbreviations: HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell; CMP (CFU-S), common myeloid progenitors (colony forming unit-spleen); CFU-GEMM, colony forming unit that generates myeloid cells; CFU-GM, colony forming unit granulocyte-monocyte, the precursor for monoblasts and myeloblasts; CLP, common lymphoid progenitor; NK cell, natural killer cell. Reproduced with the kind permission of William Lensch. See insert for color representation of this figure.

Patients with AML present with symptoms of bone marrow failure, basically cytopenia, increased fatigue, bleeding, and infections. Their white blood cell (WBC) counts are frequently elevated, and there is an increased frequency of blasts. They may also experience weight loss, bone pain, and symptoms secondary to extramedullary leukemic infiltrates, particularly in the liver, lymph nodes, mouth, and skin (Table 11.3). The proportion of blasts in the marrow varies considerably, and lower frequencies are often present in acute promyelocytic leukemia (APL), acute erythroid leukemia and other AML with substantial maturation [1].

Table 11.3 General presenting characteristics of leukemia

Elevated white blood cell (WBC) count
Fatigue
Malaise (vague feeling of bodily discomfort)
Abnormal bleeding
Excessive bruising
Paleness
Weakness
Reduced exercise tolerance
Weight loss
Bone or joint pain
Infection and fever
Abdominal pain or “fullness”
Enlarged spleen, lymph nodes, and liver

11.2.2 Common recurring cytogenetic abnormalities in AML

A significant number of AML subtypes have recurrent cytogenetic abnormalities, and some are defined by their cytogenetic/genetic aberrations (Table 11.1a). Figure 11.2 demonstrates some of the cytogenetic aberrations in these leukemias. Many of the genes involved in these aberrations have been described, and some of the aberrations can also be diagnosed with FISH, using probes such as those listed in Table 11.1a. AML with t(8;21)(q22;q22) and AML with inv(16)(p13.1q22)/t(16;16)(p13.1;q22) are classified as “core binding factor” leukemias [4,5]. (Note: acute lymphoblastic leukemia (ALL) with a t(12;21) also is a “core binding factor” leukemia; see section on ALL.) Core binding factor leukemias have a relatively good prognosis. Similarly, t(15;17)(q24;q21), which is diagnostic for acute promyelocytic leukemia (APL), has a good prognosis. Poor cytogenetic prognostic indicators are monosomy 5 and 7, 5q deletions, t(3;3)(q21;q26)/inv(3)(q21q26), t(6;9)(p23;q34), 11q23 abnormalities (see 11.2.2, 11q23 Abnormalities in AML), abnormal 17p, complex (≥ 3 or ≥ 4 unrelated abnormalities), and monosomal karyotypes [1, 5–11]. Interestingly, the incidence of de novo AML with monosomy 5 or monosomy 7 increases with age, but the incidence of AML with balanced translocations does not [12].

t(8;21)(q22;q22) AML

The t(8;21)(q22;q22) AML accounts for 5–12% of all AML. It is more common in younger patients and rare in elderly AML [3,13]. Morphologically, t(8;21) leukemia cells generally show maturation, with large blasts having abundant basophilic cytoplasm, and granulocytic involvement is generally obvious [14]. Granulocytic sarcoma also is present occasionally [15]. The t(8;21) results in fusion of the *RUNX1T1* (formerly called *ETO*, eight-twenty-one) gene on chromosome 8 and the *RUNX1* (formerly called *AML1*) gene on chromosome 21, and codes for a chimeric protein. Variant forms of t(8;21) occur in 3% of cases and include both insertions and three-way translocations [16]. Because of this, FISH for a cryptic rearrangement is important when an AML specimen shows a rearrangement involving the 8q breakpoint.

inv(16)(p13.1q22)/t(16;16)(p13.1;q22) AML

An inversion (inv) within one chromosome 16 or a translocation (t) involving both chromosomes 16 (i.e., inv(16)(p13.1q22) and t(16;16)(p13.1;q22)) is found in 6–12% of pediatric and 3–4% of adult AML [17,18]. Morphologically, these leukemias have monocytic and granulocytic differentiation. AML with one of these rearrangements is sometimes referred to as AML M4Eo (acute myelomonocytic leukemia with abnormal eosinophils), as these leukemias have atypical eosinophils and/or eosinophilia. The eosinophils are present in all stages of maturation, and eosinophilic granules generally are present. Patients with inv(16)/t(16;16) also may have granulocytic sarcomas. The genes rearranged in these aberrations are *MYH11* (16p13.1) and *CBFB* (16q22) and result in a chimeric protein [19]. An inv(16)/t(16;16) is occasionally present in blast crisis of chronic myelogenous leukemia (CML).

Both *RUNX1* and *CBFB* are normally part of a protein complex that acts as a “master switch” in normal myeloid regulation [20]. The fusion proteins derived from t(8;21) and inv(16)/t(16;16) AML result in loss of myeloid regulation.

t(15;17)(q24;q21) APL

A t(15;17)(q24;q21) leukemia, also known as acute promyelocytic leukemia (APL), is a third AML with a relatively good prognosis. APL accounts for 10–15% of pediatric AML and 4–10% of adult AML, and is more common in older children and young adults [21]. Morphologically, abnormal promyelocytes predominate, and there is an error in maturation. Typical APL is hypergranular with large azurophilic granules in the cytoplasm, although a microgranular type also occurs, usually with a very high WBC count. These leukemias have a propensity for life-threatening disseminated intravascular coagulation (DIC) and fibrinolysis, and rapid diagnosis and treatment with anti-coagulants are often necessary [22]. Consequently, the cytogenetic laboratory must treat samples from these patients as “STAT,” and FISH should be done on a STAT basis to accurately diagnose these patients.

In APL, a t(15;17)(q24;q21) is diagnostic. It results in a chimeric oncogene, *PML-RARA*. The normal locations of these genes are 15q24 for *PML* and 17q21 for *RARA*. The significant partner is the der(15), resulting in expression of *PML-RARA*. Three-way and complex abnormalities resulting in *PML-RARA* fusion occur in 10% of cases. These include cryptic insertions of part of *RARA* into *PML* and cryptic insertions of *PML* into *RARA* [23]. Most of these insertions, as well as complex rearrangements, can be detected by FISH, although not all.

Treatment includes all-trans retinoic acid (ATRA). ATRA therapy is based on the block of maturation resulting from the chimeric oncogene *PML-RARA* and overcomes this block by degrading the PML–RARA complex [24–27]. ATRA is not curative. Essentially all patients go into remission; but without additional therapy, all relapse [28]. The additional therapy usually includes an anthracycline or arsenic trioxide (As_2O_3), which has high efficacy in this disease [28]. Another reason for STAT analysis of samples from patients with a differential diagnosis that includes APL is that therapy for APL differs from other AML, and correct treatment requires an accurate diagnosis. With current treatment, APL is the most curable subtype of AML [28].

There are several other rearrangements of *RARA* that result in a clinical picture of APL. These include t(5;17)(q35;q21) (*NPM1-RARA*), t(11;17)(q13;q21) (*NUMA-RARA*), rearrangements with 17q11.2 (*STAT5B-RARA*) and t(11;17)(q23;q21) [*ZBTB16* (formerly *PZLF*)-*RARA*]. Cases with t(11;17)(q23;q21) and *STAT5B* rearrangements do not respond to ATRA; hence, it is imperative that these aberrations are identified rapidly. The other aberrations apparently do respond to ATRA [29]. FISH using fusion probes for *PML* and *RARA* will show an extra (a split) *RARA* signal for any of the variant translocations, but not fusion of the two probes. A break-apart probe for *RARA* will not distinguish between the primary and variant translocations. Metaphase analysis is important to verify these variant translocations.

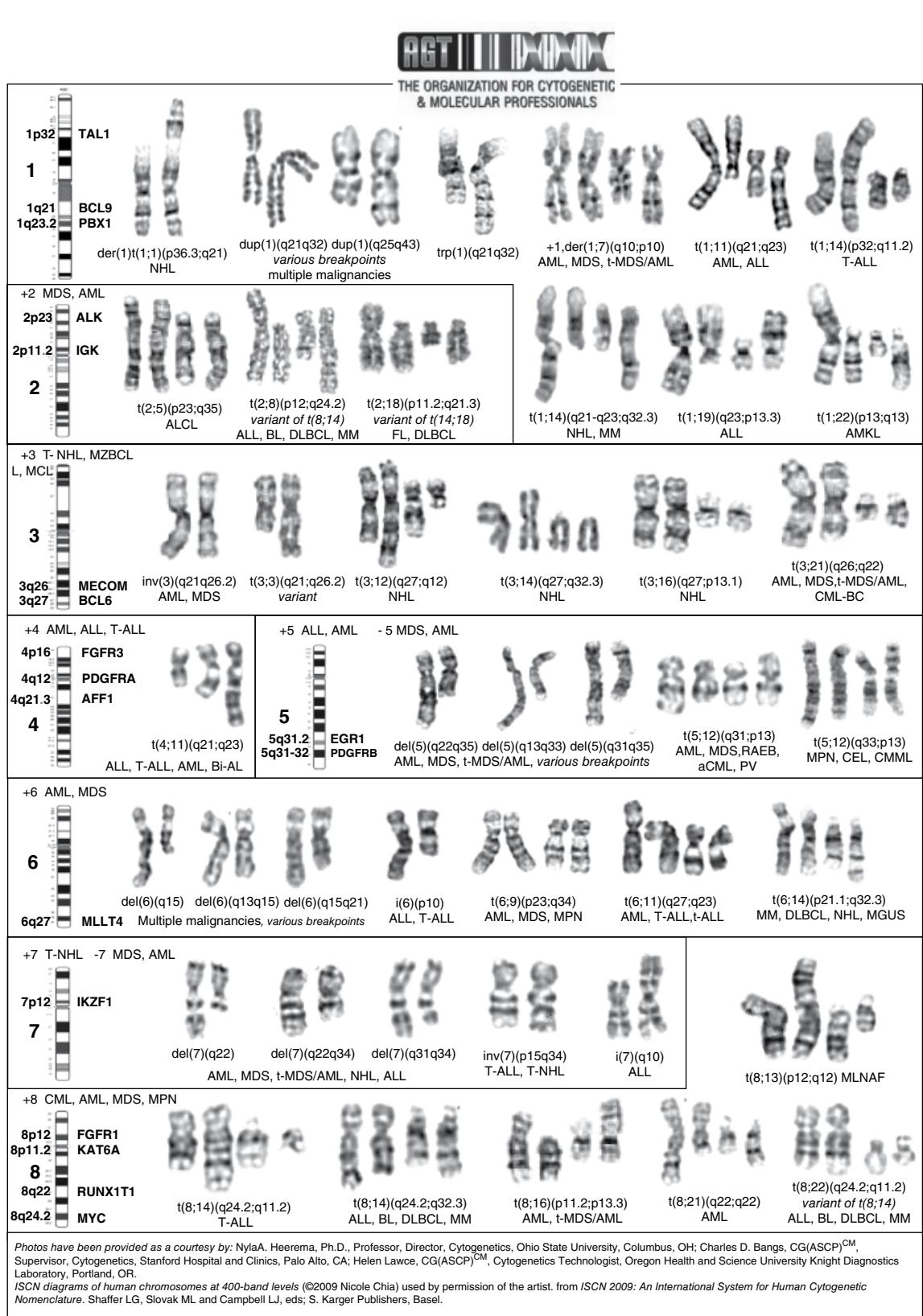
11q23 Abnormalities in AML

Chromosome band 11q23 is the location of the *KMT2A* gene (formerly *MLL*), and most, but not all, AML aberrations with an 11q23 breakpoint have a *KMT2A* rearrangement. Since not all 11q23 breakpoints result in an *KMT2A* rearrangement, FISH is important to confirm *KMT2A* involvement. Most AML blasts with *KMT2A* rearrangements are characterized morphologically as monocytes or myelomonocytes.

KMT2A is a gene with ubiquitous rearrangements and has been reported in rearrangements with 120 different partners [30]. This gene regulates *HOX* gene expression, and the fusion proteins encoded by *KMT2A* translocations may deregulate *HOX* gene expression [31,32]. *KMT2A* rearrangements are usually [1], but not always [6] associated with a poor outcome. In particular, t(9;11)(p22;q23), the most common *KMT2A* translocation in AML, has been reported to have a better outcome compared with other *KMT2A* rearrangements [33,34]. One notable *KMT2A* rearrangement, the ins(10;11)(p12;q23q13), can be difficult to recognize and benefits from *KMT2A* FISH detection.

KMT2A rearrangements occur in both AML and ALL and are particularly common in infants with AML or ALL. Infants with *KMT2A* rearrangements respond particularly poorly to therapy, and have an even worse outcome than infants with other cytogenetic aberrations [35]. *KMT2A* rearrangements also occur in acute leukemias of older children and adults. The translocation partners in ALL are generally different from those found in AML, although some partners, such as 9p22, occur in both AML and ALL.

KMT2A rearrangements in both AML and ALL occur in de novo cases and in therapy-related disease, sometimes called secondary disease [36,37] (see 11.2.4, Therapy-related MDS and AML). When the disease is therapy-related, the patient typically has been treated with an epipodophyllotoxin such as etoposide, teniposide, amsacrine and mitoxantrone, or an anthracycline such as doxorubicin (Adriamycin, Doxil, Myocet) and epirubicin. These drugs inhibit topoisomerase (Topo) II.

**Figure 11.2** Hematological rearrangements (G-banding). In order to improve legibility, gene names have not been italicized.

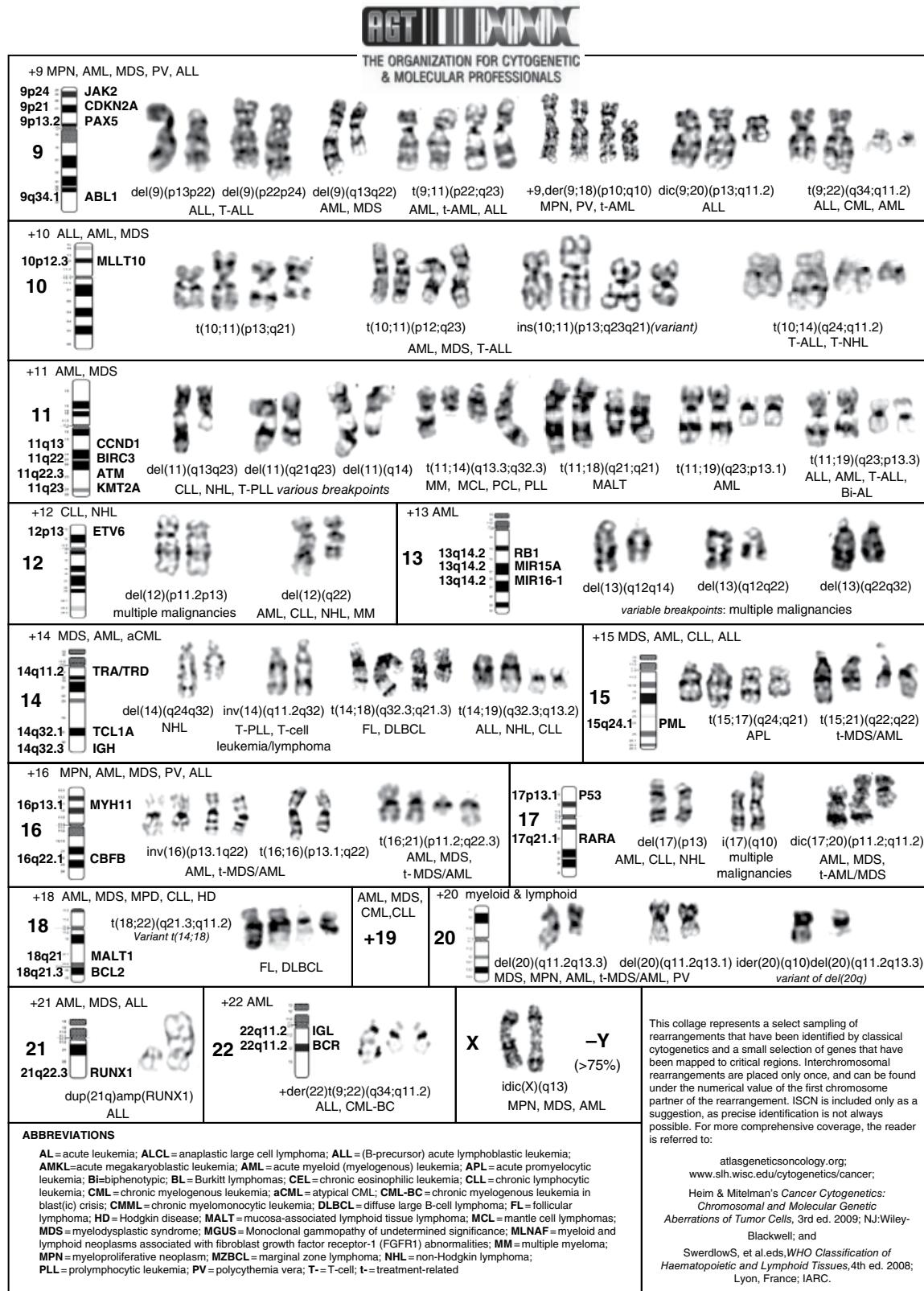


Figure 11.2 (Continued)

Topo II functions in DNA replication to relax the twisting of the double helix. The DNA breaks to permit unwinding, and Topo II holds the ends together promoting reuniting of the ends broken to relax the DNA. Topo II inhibitors prevent the reuniting of the DNA [38]. (For an excellent review of *KMT2A*, see Muntean and Hess [39].)

11.2.3 Other abnormalities recognized by the WHO [1]

There are several additional cytogenetic rearrangements used for diagnostic classification by the WHO. These include t(6;9) (p23;q34), inv(3)(q21q26.2/t(3;3)(q21;q26.2) and t(1;22)(p13;q13) [1] (see Table 11.1a). In addition, AML cases with normal karyotypes frequently have point mutations with prognostic implications. Several of these are also used for patient stratification [1].

Philadelphia chromosome (Ph) positive AML

Philadelphia chromosome (Ph) positive AML occurs in only about 1% of AML. This is the typical t(9;22)(q34;q11.2), in which the der(22) is called a Philadelphia chromosome. Cells of Ph AML are immature with little if any differentiation. These patients have a poor prognosis. Currently, Ph AML patients may be treated with imatinib mesylate (Gleevec®; Novartis), or another second-generation tyrosine kinase inhibitor (TKI) which has improved outcome (see 11.4.1, Chronic myelogenous leukemia).

11.2.4 Therapy-related MDS and AML (t-MDS and t-AML)

There are two types of therapy-related MDS (myelodysplastic syndrome) and AML, sometimes called secondary MDS and AML [40]. These diseases generally are caused by treatment that the patient received for another malignancy or other disease. The “primary” (first) malignancy can be any type; however, the most common is Hodgkin lymphoma [41]. The risk factors are the type and amount of therapy received, including the schedule of administration. Over 90% of these patients have cytogenetic abnormalities in their bone marrow. The two types of drugs resulting in therapy-related AML or MDS are:

1. *Alkylating agent/Ionizing radiation therapy-related:* Some of the commonly used drugs in this category are busulfan, chlorambucil (CAB), cyclophosphamide (CP, or its metabolite), dacarbazine, mechlorethamine, melphalan, mitomycin-C (MMC), nitrosoureas, and thiotepa. The alkylating agent kills cells by transferring an alkyl group to DNA causing inhibition of both replication and transcription. The latency period from initial treatment to development of secondary disease is approximately 4–6 years. MDS frequently precedes development of this t-AML. These diseases typically have unbalanced karyotypes, frequently with deletions of 5q and 7q and monosomy 5 and 7. Cytogenetic evolution often heralds transformation from MDS to overt AML. The patients generally have a poor response to treatment and poor survival [40].
2. *Topoisomerase II inhibitor therapy-related (epipodophyllotoxins and related agents):* These t-AML have a short latency period of only 1–3 years. There is no, or a very brief, MDS phase. At presentation there is a striking predominance of blasts, usually monoblasts. The karyotypes generally have balanced translocations, frequently involving *KMT2A* (see 11.2.2, 11q23 Abnormalities in AML above). Rearrangements of *RUNX1*, as well as t(15;17) and inv(16)/t(16;16) also occur. Response to treatment is generally good; but the relapse rate is high, especially of the *KMT2A* rearranged patients [37].

11.3 Myelodysplastic syndromes

Myelodysplastic syndromes (MDS) are clonal stem cell disorders characterized by single or multilineage dysplasia, cytopenia, ineffective hematopoiesis, intact maturation with a variable increase in blasts, and an increased risk for development of AML. By definition, there are less than 20% blasts. MDS are more common in elderly patients, particularly those over the age of 70, and only 8% of MDS occur in patients <40 years of age [42]. MDS can be either de novo or therapy-related.

Abnormal chromosomes are found in 30–60% of MDS [43]. MDS with abnormal cytogenetics are more likely to progress to overt AML and have a worse prognosis than MDS with normal cytogenetics. MDS cytogenetic abnormalities are predominantly unbalanced losses, especially losses of 5q and 7q. Patients with isolated loss of the Y chromosome, del(11q), del(12p), or isolated del(5q) (see 5q- syndrome later) have a relatively good prognosis, while patients with complex karyotypes with more than three unrelated abnormalities [44] or with monosomy 7 [1] have a poor outcome. Deletion of 20q has been classified as both a poor prognostic feature and as having a relatively good prognosis [11,43]. In elderly patients, the chromosomes of MDS are similar to those in AML, which might suggest a similar etiology [45].

5q- syndrome

5q- syndrome (i.e., deletion of all or part of the long (q) arm of chromosome 5) accounts for about 7% of MDS. It is more prevalent in elderly females. Cytogenetically, the deletion of 5q is the sole abnormality, generally with a proximal breakpoint in q13 - 15 and a distal breakpoint in q33 - 35 [46,47]. The commonly deleted region has been identified as 5q33.1, but the gene(s) lost in these patients is currently unknown, and more than one gene, including microRNAs (miRs), are likely important [48,49]. The mechanism of action may be haploinsufficiency, as mutations of genes in the region have not been found on the normal homologue. The 5q- syndrome is unique, as it rarely transforms to AML. Therefore, unlike del(5q) and monosomy 5 when associated with additional cytogenetic abnormalities, isolated del(5q) has a good prognosis. These patients, as well as some patients with del(5q) and a single additional abnormality, generally respond to lenalidomide; therefore, it is important to identify them [50].

Pediatric MDS

Pediatric MDS is rare, occurring in only 0.4/100,000 children; it accounts for <5% of hematologic malignancies in children [51]. In children there is a high association of MDS and myeloproliferative neoplasms (MPN) with genetic disorders, such as neurofibromatosis type I, Fanconi anemia, congenital neutropenia, and Shwachman–Diamond syndrome. Isolated monosomy 7 is particularly common, especially in patients <5 years [52]. Older children (>5 years) are more likely to develop “adult-type” MDS.

11.4 Myeloproliferative neoplasms

The incidence of MPN is 1/100,000 individuals, but is uncommon in children. The chronic MPN are briefly described.

11.4.1 Chronic myelogenous leukemia

Chronic myelogenous leukemia (CML) comprises ~15% of all adult leukemias. Although rare, it does occur in children; 2–5% of childhood leukemias are CML. There is a slight predominance of males [2]. Presenting symptoms are often nonspecific, such as fatigue, malaise, headache, and weight loss, or symptoms related to pronounced splenomegaly. It is a striking granulocytic hyperplasia leading to a markedly elevated WBC count, usually with <5% blasts and a predominance of mature neutrophils. Because it is a stem cell disease, thrombocytosis, basophilia, and eosinophilia are also frequently present [53].

This neoplasm is well known in the cytogenetics laboratory. Diagnosis requires the presence of a *BCR-ABL1* rearrangement, cytogenetically seen as a Philadelphia chromosome (Ph) in most cases. The Ph is the der(22) which results from a t(9;22)(q34;q11.2) in 95% of cases, and the remaining cases have either a complex or a cryptic rearrangement resulting in a *BCR-ABL1* chimeric gene. The *BCR-ABL1* rearrangement can be detected by RT-PCR or by FISH, although generally cytogenetic evidence of a t(9;22)(q34;q11.2) in a patient with the clinical picture of CML confirms the disease. FISH is recommended, however, in order to establish the baseline pattern of FISH signals for follow-up of the disease. This is particularly important since the advent of imatinib mesylate (Gleevec®; Novartis) and other TKI therapies, which require monitoring of response to treatment by RT-PCR or by cytogenetics and FISH. Imatinib mesylate, and the second generation newer tyrosine kinase inhibitors such as dasatinib, are a new type of drug specifically targeting protein tyrosine kinases, including the protein encoded by the chimeric *BCR-ABL1* gene. This treatment results in complete cytogenetic response in 87% of patients [54], but a persistent, complete response (continued absence of disease by RT-PCR) in only about 4% of patients [55]. Most patients with CML continue imatinib therapy indefinitely [54].

Cryptic deletions of distal *BCR* and proximal *ABL1*, which can be detected by FISH, have been reported; but with imatinib mesylate treatment their clinical significance appears to be eliminated [56]. After treatment with imatinib mesylate, clonal cytogenetic abnormalities may appear in Ph negative cells, frequently trisomy 8. The clinical significance of these abnormalities is not clear at this time [57]. When CML progresses, the disease enters an accelerated phase or blast crisis. Secondary abnormalities are present in about 70% of patients in blast crisis. The most common secondary cytogenetic abnormalities are a second copy of the Ph chromosome, trisomy 8, i(17q), and trisomy 19 [58–60], although many other, frequently unique, abnormalities also are observed.

11.4.2 Chronic myelomonocytic leukemia

Chronic myelomonocytic leukemia (CMML) (MDS/MPN by WHO) may present with leukocytosis or leukopenia, hepatosplenomegaly and other extramedullary disease. There are <20% blasts in the marrow, and the marrow is hypercellular. CML (a *BCR-ABL1* rearrangement) must be excluded by cytogenetic or molecular studies. Only 20–40% of CMML have

cytogenetic abnormalities. Monosomy 7 is the most common abnormality, and del(7q), +8, del(11q), abnormal(12p), and del(20q) also recur [1,61–64]. These abnormalities are not specific and are seen in other myelodysplastic and myeloproliferative disorders. Abnormalities of chromosome 7, trisomy 8, and complex karyotypes are associated with a shorter survival [61,65]. Juvenile myelomonocytic leukemia (JMML) is chronic myelomonocytic leukemia in young children, usually diagnosed before the age of 4 years. There is an association with neurofibromatosis type 1 and with Noonan syndrome [66]. Cytogenetically, monosomy 7 or other abnormalities of chromosome 7 are frequent [66].

11.4.3 Polycythemia vera

Polycythemia vera (PV) is an increase in red blood cells. It is usually indolent, with a median survival exceeding 10–15 years. The median age at diagnosis is 60–65 years. Nearly all patients with PV have a *JAK2* mutation [67]. As with other MPN, a Philadelphia chromosome must be ruled out, as the Ph is indicative of CML. Only about 20% of PV have cytogenetic abnormalities at diagnosis, and patients with abnormal cytogenetics are more likely to progress to acute leukemia [68,69].

11.4.4 Essential thrombocythemia

Essential thrombocythemia (ET) is characterized by pronounced megakaryocytic hyperplasia in the bone marrow and peripheral blood thrombocytosis. It occurs primarily in the elderly (50–60 years) and has a median survival of 10–15 years [70]. Fifty percent of these patients also have a *JAK2* mutation [67]. Cytogenetic abnormalities occur at presentation in only ~5% and are much more prevalent in patients who go on to develop acute leukemia, a phenomenon almost always linked to prior therapy with radioactive phosphorous or an alkylating agent [71,72].

11.4.5 Primary myelofibrosis

Primary myelofibrosis (PMF) is a clonal myeloproliferative disorder in which bone marrow myelofibrosis occurs secondary to nonclonal fibroblast proliferation. Extramedullary hematopoiesis causes massive hepatosplenomegaly. The median survival is 3–5 years, although some patients live >15 years from presentation. As in ET, 50% of these patients have *JAK2* mutations [67]. Cytogenetic abnormalities are found in 30–50% of cases, with a favorable outcome for patients with chromosome 1 abnormalities, +9, 13q– or 20q–, and a worse outcome for patients with sole +8, -5/5q–, -7/7q–, i(17q), inv(3), 12p–, 11q23 breakpoint or a complex karyotype [73].

11.4.6 Chronic neutrophilic leukemia

Chronic neutrophilic leukemia (CNL) is a very rare disorder generally affecting older adults. Normal chromosomes are found in 90% of cases. A 20q– has been reported in some cases [74].

11.4.7 Chronic eosinophilic leukemia

Chronic eosinophilic leukemia (CEL) is difficult to distinguish from reactive eosinophilia. If clonal cytogenetic aberrations are present, the condition is considered neoplastic. It is more frequent in young to middle-age adults. Recurring cytogenetic abnormalities are *PGFRB* rearrangements (see later) and +8 [75–77]. CEL is different from inv(16)/t(16;16) AML (AML M4Eo) and from hypereosinophilic syndrome, which is not neoplastic.

11.4.8 Other myeloid and lymphoid disorders

Myeloid and lymphoid disorders with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* or *FGFR1* are a new classification established by the WHO in the 4th edition [1]. It includes three rare diseases, myeloid and lymphoid neoplasias with (1) *PDGFRA* and (2) *FGFR1* rearrangements, and (3) myeloid neoplasms with *PDGFRB* rearrangements. A cryptic rearrangement of *FIP1L1-PDGFR*A (both at 4q12) results in deletion of *CHIC2* and can be detected by FISH [78]. Patients with loss of *CHIC2* respond to imatinib mesylate; therefore, identification of this abnormality is very important [79,80]. Occasionally, a translocation of *PDGFRA* is detected. Clinically, these patients present with CEL [81]. *FGFR1* is located at 8p12 and rearranges with many partners. Most patients with an *FGFR1* aberration are relatively young. Clinically, they can present with CEL, AML, or T-cell lymphoblastic lymphoma (T-LBL). *PDGFRB* is located at 5q33 and rearranges with many partners, although a t(5;12)(q33;p13), which results in a chimeric *ETV6-PDGFRB* gene, is the most common

rearrangement. It is important to identify the t(5;12) or another rearrangement of *PDGFRB* in these patients, as they respond to imatinib mesylate [82]. Patients with a *PDGFRB* rearrangement most often present as CMML, but also with CEL or MPN with eosinophilia [1].

11.5 B- and T-cell lymphoid neoplasms

11.5.1 Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most common childhood leukemia (Table 11.2). The incidence is 3/100,000 children/year, and >60% of cases are in children <15 years of age. The peak age at onset is 4 years. There is a slight male predominance. Patients present with fever, bleeding, splenomegaly and/or hepatosplenomegaly, and bone marrow failure. Less than 2% have a preleukemic state, which consists of a hypocellular episode preceding overt leukemia. ALL is a clonal B- or T-cell neoplasm, characterized by loss of normal hematopoietic elements and predominance of immature B- or T-cells that are capable of minimal, if any, maturation. The cell-surface antigen profile is similar to that of normal B- or T-cell precursors. B-lineage ALL accounts for 75–90% of cases. The WBC count is highly variable, from <4,000/ μ L to >1,000,000/ μ L, and there are usually >90% blasts. The WBC counts tend to be higher in T-cell ALL with most >50,000/ μ L, but are highest in infants, with some >200,000/ μ L.

Eighty to ninety percent of pediatric ALL cases have visible metaphase cytogenetic aberrations [83–85]. The aberrations are both numerical and structural (Table 11.1b). The cases, as well as prognoses, are classified by ploidy and by specific chromosome abnormalities.

High hyperdiploidy (>50 chromosomes)

High hyperdiploidy (>50 chromosomes) (see Figure 11.3) occurs in about 25% of pediatric cases and typically is associated with a good outcome [86–90]. However, the good outcome associated with high hyperdiploidy may be attributed to the presence of specific extra chromosomes. The Children's Oncology Group (COG) found that extra copies of 4, 10 and 17 predicted a good outcome [91], whereas the Medical Research Council (MRC) of the United Kingdom found that extra copies of chromosome 18 were associated with an especially good outcome [92].

Low hyperdiploidy (47–50 chromosomes)

Low hyperdiploidy (47–50 chromosomes) is associated with an intermediate prognosis [86,88–90]. However, these patients frequently have recurring structural abnormalities; and then their prognosis is best predicted by the structural abnormality.

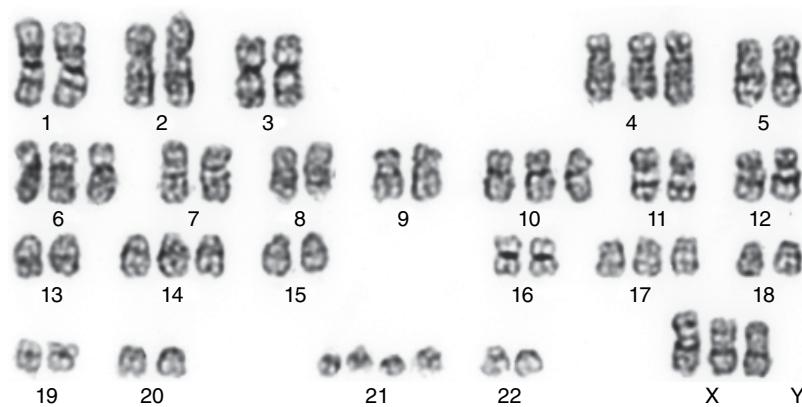


Figure 11.3 ALL high hyperdiploid. Karyogram of a pediatric acute lymphoblastic case with high hyperdiploidy. The ISCN karyotype is 54,XX,+X,+4,+6,+10,+14,+17,+21,+21. Reproduced with the kind permission of N. Heerema, Department of Pathology, The Ohio State University, Columbus, OH.

Pseudodiploidy

Prognosis for patients with *pseudodiploidy* is associated with the structural abnormality present when the structural abnormality has prognostic significance [86,88–90]. Otherwise, these patients are considered intermediate risk.

Hypodiploidy

Hypodiploidy with 44 or 45 chromosomes has an intermediate prognosis, while **hypodiploidy with ≤ 43 chromosomes** has a very poor outcome [93,94]. Cases with hypodiploidy with <40 chromosomes may have a doubling of the hypodiploid clone, with few or no remaining hypodiploid cells, masking the case as hyperdiploid. The cytogeneticist must be able to recognize the pattern of extra chromosomes with two and four copies of homologues suggestive of doubling of a hypodiploid clone, as opposed to three copies of homologues, the pattern for most chromosomes in hyperdiploidy. These patients have a poor prognosis, identical to that of severe hypodiploid cases without a doubling of the abnormal clone [93] (see Figure 11.4).

In addition to numerical aberrations, there are multiple recurring structural cytogenetic abnormalities in ALL, and many are listed in Table 11.1b. Interestingly, many of the cytogenetic abnormalities in ALL have been shown to be prenatal in origin [95–101]. These abnormalities can be found in newborn blood spots of both children who later develop ALL and some who do not, indicating that the cytogenetic abnormality is insufficient for leukemogenesis (at least one additional mutation is required), and the development of leukemia after the generation of the recurring aberration is not inevitable.

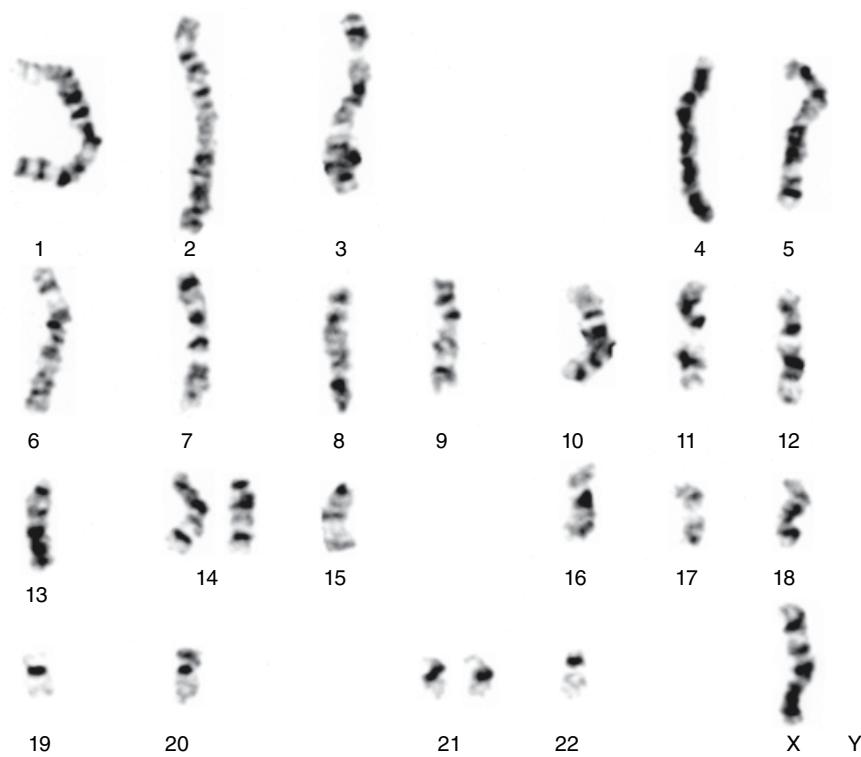


Figure 11.4 Near-haploid ALL. Karyogram of a pediatric acute lymphoblastic leukemia case with near-haploid, showing two copies (a diploid number) of only two chromosomes: chromosome 14 and chromosome 21. The ISCN karyotype is written 25,X,+14,+21. Reproduced with the kind permission of N. Heerema, Department of Pathology, The Ohio State University, Columbus, OH.

Rearrangements of KMT2A

Rearrangements of *KMT2A*, with an 11q23 breakpoint, occur in 4% of children (unpublished COG data) and 3–7% of adults [21] with ALL. They generally have a poor outcome, although infants less than 12 months and children ten years of age and older usually do worse [102], and there is considerable clinical heterogeneity in children with *KMT2A*-rearranged ALL [103]. Some 11q23 partner chromosomes are unique to ALL (e.g., 4q21), some do not occur in ALL and are unique to AML (e.g., 6q27), whereas some occur in both ALL and AML (e.g., 9p22) (see 11.2.2, 11q23 abnormalities in AML).

t(12;21)(p13;q22)

A t(12;21)(p13;q22) is the most frequent translocation in pediatric ALL, occurring in 20–25% of cases [104–106]. It is found in children ages 1–10 years, but is more common in children ages 3–6 years [107,108]. It is rare in adults and has been reported in only a few, usually young adults [109–111]. This aberration is cytogenetically cryptic, and FISH or other molecular techniques are required to determine its presence. It signifies an excellent prognosis [105,107,112]. Immunophenotypically, the ALL cells are B-precursor, and are CD9 and CD20 negative and CD10 positive, with frequent myeloid antigen co-expression [113–115]. The translocation results in rearrangement of the *ETV6* and *RUNX1* genes (formerly *TEL* and *AML1*). The aberration occurs prenatally [116] and has been shown to be an initiating event, with an additional mutation(s) required for leukemogenesis [117]. Deletion of the *ETV6* gene on the chromosome 12 homologue not involved in the t(12;21) is a frequent secondary abnormality and is thought to be one of the foremost events associated with progression [118–120]. Between 80% and 90% of these cases have other cytogenetically detectable aberrations [109,121].

Philadelphia chromosome (Ph), t(9;22)(q34;q11.2)

A Philadelphia chromosome (Ph), t(9;22)(q34;q11.2), occurs in 2–5% of pediatric and 20–40% of adult ALL [122–125]. The frequency of Ph ALL increases with advancing age [124]. Nearly all cases are B-lineage ALL, although a few T-lineage cases occur. Patients usually have a high WBC count. Cytogenetically, the Philadelphia chromosome is indistinguishable from the Ph present in CML. However, molecularly, most pediatric cases have a rearrangement of the e1a2 exons of *BCR* and *ABL1*, while up to half of adults have the b2a2 rearrangement typical in CML, and the rest have the e1a2 fusion gene [126]. The e1a2 rearrangement results in a 190 kD protein, while the b2a2 rearrangement results in a 210 kD protein [126]. These differences can be distinguished by some FISH probes. There are no known differences in clinical features or outcome with the two different rearrangements. Secondary abnormalities are present at diagnosis in about half of Ph ALL, with a second Philadelphia chromosome, trisomy 21, 9p abnormalities, hyperdiploidy, monosomy 7 and loss of 7q common secondary abnormalities [127,128]. Ph ALL traditionally has a very poor outcome [129,130]. However, early results indicate that treatment with tyrosine kinase inhibitors, such as imatinib mesylate, will improve outcome for these patients [131].

t(1;19)(q23;p13.3)

The t(1;19)(q23;p13.3) of ALL occurs both as a balanced translocation and an unbalanced der(19)t(1;19). It is infrequent and found in 5% or less of both pediatric and adult ALL [123,132]. The immunophenotype is always pre-B, which is a more mature cell than the more common B-precursor cell in most ALL [133]. The translocation results in a chimeric oncogene, *TCF3(E2A)-PBX1*, which is a potent transcriptional activator [134–136]. Although initially associated with a poor outcome, current intensive treatments result in intermediate outcomes [123,132,137]. A der(19)t(1;19)(q23;p13.3) that does not involve *TCF3* or *PBX1* also occurs in hyperdiploid ALL. This rearrangement cannot be distinguished cytogenetically from a *TCF3-PBX1* rearrangement, and FISH or other molecular methods are required to distinguish the two rearrangements, as some hyperdiploid cases do have a *TCF3-PBX1* rearrangement [138].

del(9p)

A del(9p) is also recurrent in ALL. This deletion involves the *CDKN2A/CDKN2B* locus on 9p21. The deletion is frequently cryptic and often can be detected by FISH with a probe for the 9p21 region. It is seen in both B-cell precursor ALL and T-ALL. The prognostic significance of del(9p) is not known, as reports have shown both no prognostic significance [139,140] and an unfavorable outcome [141,142] for these patients.

Dicentric chromosomes

Dicentric (dic) chromosomes are common in ALL, and most often involve chromosome 9. Although loss of 9p is variously associated with a poor outcome [139–142], some reports suggest a good outcome for some of the dicentric cases, particularly dic(9;12) [143]. The dic(9;20) may be difficult to detect, and monosomy 20 in an ALL patient often heralds the dic(9;20). However, some cases with a dic(9;20) also have two normal chromosomes 20. FISH may be required to confirm its presence [144–147]. Many, although not all, dic(9;v) have a breakpoint in *PAX5* and result in a fusion gene with *PAX5* [148].

CRLF2 abnormalities

Two recurring cryptic rearrangements in ALL involve the *CRLF2* gene which is located in the pseudoautosomal regions of the X and Y chromosomes [149–151]. Both a translocation with the *IGH* locus and an interstitial deletion resulting in a *P2RY8-CRLF2* fusion occur, and both result in deregulation of *CRLF2*. The rearrangement with *IGH* is less frequent, but can be detected with metaphase FISH using an *IGH* probe. *CRLF2* rearrangements occur in only about 6% of pediatric ALL, but are particularly common in Down syndrome ALL, where they have been described in >50% of cases. Most Down syndrome cases have the *P2RY8-CRLF2* rearrangement [149–151].

iAMP21

Amplification of a region of chromosome 21 (*iAMP21*) occurs in approximately 2% of pediatric ALL. The amplification nearly always occurs on an abnormal chromosome 21. It can be detected by FISH for *RUNX1*, which is amplified. The diagnostic criterion for *iAMP21* is the presence of four or more *RUNX1* signals on a single chromosome. However, *RUNX1* may not be the important gene, as it is not overexpressed. Patients with *iAMP21* have a poor prognosis [152,153]. A *P2RY8-CRLF2* rearrangement frequently also is present in these patients [154].

T-lineage ALL

T-lineage ALL (T-ALL) comprises 10–15% of pediatric [155–157] and 14–28% of adult ALL [123,124,140]. It is more frequent in teen-age boys and is associated with a high WBC count. At diagnosis, patients frequently have extramedullary disease, especially mediastinal disease, but also lymphadenopathy and organomegaly [155]. The immunophenotype is CD7+ and cytoplasmic CD3+, with TdT+, CD1a+, CD2+ and CD5+ also common [155,156]. Cytogenetically, normal karyotypes are often reported [158]; however, cryptic rearrangements are common [159]. Rearrangements of the T-cell receptor (*TCR*) loci (*TRA/TRD* at 14q11.2, *TRB* at 7q34 and *TRG* at 7p14) occur in about 35% of T-ALL [160]. They result in overexpression of the genes with which they are rearranged. *TCR* rearrangements with the *HOX* genes are common, but not all *HOX* gene overexpression results from a chromosomal translocation [161]. The recurring t(1;14)(p32;q11.2) results in a fusion oncogene *TRD-TAL1* and occurs in only 3% of ALL, both pediatric and adult. However, a cryptic deletion of chromosome 1 resulting in an *SIL-TAL1* fusion is common, occurring in 15–25% of T-ALL [162,163]. Another abnormality found in T-ALL is amplification of a chimeric *NUP214-ABL1*. *NUP214* is slightly distal to *ABL1* on chromosome 9. The rearrangement is on an episome, which is cytogenetically cryptic, but can be detected by FISH using an *ABL1* probe [164,165]. Tetraploidy occurs in approximately 5% of T-ALL [159].

T-Prolymphocytic leukemia

T-prolymphocytic leukemia (T-PLL) occurs primarily in elderly men. It is a very aggressive disease; survival is poor, with median survival less than 1 year. T-PLL is unique in that the cells require a mitogen for division and usually respond to phytohemagglutinin (PHA). A recurrent abnormality, inv(14)(q11.2q32.1), is present in over 50% of cases. The inv(14) is usually associated with other aberrations, particularly iso(8q) or trisomy 8 [166,167]. There may be an association of T-PLL with t(X;14)(q28;q11.2) and ataxia telangiectasia [167].

Adult T-cell leukemia/lymphoma

Adult T-cell leukemia/lymphoma occurs primarily in Japanese patients, and is caused by the HTLV-1 virus, although some nonendemic cases do not have HTLV-1. They generally have complex karyotypes, and no specific aberrations have been described [168,169].

11.5.2 Chronic lymphocytic neoplasias

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is a B-cell neoplasm. It is the most frequent adult leukemia, comprising up to 30% of all adult leukemias. Approximately 15,680 new cases and over 4580 deaths from the disease were predicted in 2013 in the United States [2]. It occurs in middle-age to elderly patients, and not in children. There is a male predominance [1]. In some cases there is a familial component; and although the *DAPK1* gene at 9q34.1 was implicated in one family [170], the genes involved in most familial cases have not been identified. Patients are often asymptomatic at diagnosis, and the disease may be detected during a routine peripheral blood cell differential. There is wide variation in clinical course, from a very aggressive disease to a long, indolent course. For the cytogenetics lab, CLL is a difficult disease in which to obtain metaphases due to a very low spontaneous proliferative index of the CLL cells and poor response to mitogens. Historically, more than 50% of “successful” analyses were reported as “normal.” This most likely reflected the normal cells, not the neoplastic cells, as FISH has shown up to an 80% abnormality rate [171,172]. This has changed with the introduction of stimulation with CpG oligodeoxynucleotides (ODN) in CLL. CpG ODN are short (20–28 base pairs) single strands of DNA that are not methylated. Utilization of a CpG ODN results in detection of abnormal clones in 70–80% of cases [173–177]. Thus, for conventional metaphase analysis, stimulation with a CpG ODN should be done. (See Protocol 11.3, Bone marrow and leukemic blood culture and harvest procedure using DSP30 CpG oligonucleotide/interleukin-2 for B-cell mitogenic stimulation and Protocol 11.4, Culture of CpG-stimulated peripheral blood and bone marrow in chronic lymphocytic leukemia, for use of CpG ODN as effective agents for stimulation of CLL cells.) Because of the difficulty in obtaining dividing neoplastic cells, FISH is widely utilized to define this disease and predict outcome. The most common abnormalities in CLL when studied by FISH are a deletion of 13q14.3 (*miR-15a* and *miR-16-1*) [178], trisomy 12, deletion 11q22.3 (*ATM*) and deletion 17p13.1 (*TP53*). These abnormalities have prognostic significance with a hierarchy of (best to worst) del(13q), no FISH abnormality, trisomy 12, del(11q) and del(17p) [172,179–181].

B-Prolymphocytic leukemia

B-prolymphocytic leukemia (B-PLL) is a rare disease, occurring primarily in elderly men. Cytogenetics are similar to those of CLL [182].

Hairy cell leukemia

Hairy cell leukemia (HCL) is a rare disorder, which occurs primarily in middle-age to elderly men. It generally has an indolent course. It is a B-cell disease, and cytogenetic analysis generally requires a B-cell mitogen. Stimulation with CpG ODN may be useful in this disease, but that has not been confirmed. Rearrangements of chromosome 14 have reported to be recurrent in HCL, especially with q32 breakpoints. Trisomy 5 is also recurrent [183].

Multiple myeloma

Multiple myeloma (MM) is the second most common hematological cancer in adults. It is a plasma cell neoplasm, resulting from proliferation of immunosecretory plasma cells. It occurs primarily in middle-age to elderly patients and affects African Americans more often than whites. At diagnosis 15–30% of patients are asymptomatic. When symptomatic, symptoms include weakness and fatigue, bone pain, demonstrable monoclonal protein in serum or urine, decreased normal immunoglobulin, anemia, hyperuricemia, bone marrow plasmacytosis, lytic bone lesions, abnormal renal function, and importantly, an increase in the number of plasma cells. MM is more aggressive when immature, blastic plasma cells are present [184,185]. Median survival is 3 years, but with considerable variation among patients.

As with other mature B-cell leukemias, it is very difficult to obtain dividing neoplastic cells in MM. The addition of cytokines may help [186–189]. It appears that stromal cells are essential in stimulating myeloma cell growth. In spite of, or perhaps in part because of these problems, it has been shown that patients with abnormal karyotypes, regardless of the abnormality, have a worse outcome than those with normal karyotypes [190].

Cytogenetics are a primary risk factor in multiple myeloma. Good risk cytogenetics includes hyperdiploidy with 48–74 chromosomes. The extra chromosomes are the “odd numbered” chromosomes, 3, 5, 7, 9, 11, 15, 19 and 21. Patients with t(4;14)(p16.3;q32.3) are considered intermediate risk in some studies and high risk in others. Those with del(17p) or loss of *TP53*, t(14;16)(q32.3;q23) and those with t(14;20)(q32.3;q11;2) are poor risk. These can all be detected by FISH [191,192]. Historically, loss or deletion of chromosome 13 was associated with a poor outcome when

detected by metaphase cytogenetics. However, recent studies have shown that monosomy 13 or del(13q) is strongly associated with other poor prognostic features, and it is not an independent prognostic indicator [193]. Because plasma cells are rare in MM and they do not spontaneously divide, FISH is strongly recommended *on the plasma cells* (CD138+ cells), which are typically rare, so must be enhanced in some manner. There are several methods for enhancing their selection, including magnetic separation (see Protocols 11.5 and 11.6, Plasma cell separation and Harvest procedure for FISH analysis), immuno- or cIg FISH, in which the plasma cells are identified by antibodies (cIg) for Ig kappa and lambda and FISH is counted only on those cells [194].

There is karyotypic instability both in MM and in monoclonal gammopathy of uncertain significance (see Monoclonal gammopathy of uncertain significance next). In spite of the low frequency of abnormalities detected by metaphase cytogenetics, 90% of MM are abnormal by FISH, array-CGH and DNA content, when plasma cells are examined [195]. Thus it is imperative to ensure that plasma cells are examined, particularly for FISH studies in MM.

Monoclonal gammopathy of uncertain significance

Monoclonal gammopathy of uncertain significance (MGUS) is a plasma cell dyscrasia characterized by a monoclonal protein, usually IgG (75%), with kappa light-chain restriction (60%). Cytogenetically, MGUS is very similar to MM [192,196]. It is sometimes a precursor to MM.

Amyloidosis

Amyloidosis is another B-cell neoplasm. Cytogenetically, it has a high incidence of aneuploidy, 14q32.3 translocations and deletions of 13q [197–199].

Waldenstrom macroglobulinemia

Waldenstrom macroglobulinemia (WM), also called lymphoplasmacytic lymphoma, is a lymphoplasmacytic neoplasm that may overlap with lymphoplasmacytic lymphoma. It occurs in middle-age to elderly patients [200]. Recurring cytogenetic abnormalities are trisomy 4, del(6q), del(11q), del(13q), del(17p) and trisomy 18, with del(17p) associated with a poor outcome [201,202]. *IGH* translocations are extremely rare in this tumor, even though it is a B-cell tumor [201].

11.6 Lymphomas

Lymphomas are of the lymphoid lineage and are of two general types: Hodgkin lymphoma (HL) or Hodgkin disease (HD), and non-Hodgkin lymphomas (NHLs). NHL is much more frequent and has several types (Table 11.4). The majority of NHLs are mature B-cell neoplasms, although T-cell and natural killer (NK) cell lymphomas also occur (Table 11.4). Some lymphomas have very typical genetic aberrations, but there is considerable overlap of specific aberrations in lymphomas of different classifications. Approximately 69,740 individuals were estimated to be diagnosed with NHL in 2013, and 19,020 individuals with NHL were predicted to die in 2013 (Table 11.2) [2].

The tissue of choice for cytogenetic study of lymphomas is an involved lymph node. If an involved lymph node is not available, involved bone marrow or other involved tissue may be studied. Table 11.1d lists some recurrent cytogenetic aberrations in lymphomas.

11.6.1 B-cell lymphomas

Diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin lymphoma. It is a heterogeneous neoplasm, with a low incidence of bone marrow involvement (8–35%). Most DLBCL have complex karyotypes. Several different abnormalities recur, frequently involving the *IGH* locus. A t(14;18)(q32.3;q21.3) (*IGH-BCL2*) occurs in approximately 15–25% of the cases [199]. Five to ten per cent of cases have a 3q27 (*BCL6*) rearrangement with a number of partner chromosomes, for example (in order of frequency), 14q32.3, 2p11.2, 22q11.2, 4p13, 6p21, and 11q23. However, the incidence of *BCL6* rearrangements is much higher (30–40%) when molecular methods, including FISH, are employed [203–205]. A t(8;14) (q24.2;q32.3) (*MYC* rearrangement) is seen in 7–10% of DLBCL cases [206]. (See Burkitt lymphoma/leukemia later.) Correlations of outcome with cytogenetic abnormalities in DLBCL have not been established.

Table 11.4 Types and incidences of lymphoma

Non-Hodgkin lymphoma	69,740 estimated new cases in 2013 [2]
B-lineage	Incidence (percent of total) [242]
Diffuse large B-cell lymphoma	30.6
Follicular lymphoma	22.1
Mantle cell lymphoma	6.0
Burkitt/Burkitt-like lymphoma-leukemia	2.5
MALT lymphoma	7.6
Nodal marginal zone B-cell lymphoma	1.8
B-cell CLL/SLL	6.7
Mediastinal large B-cell lymphoma	2.4
Lymphoplasmacytic lymphoma	1.2
T-lineage	
Anaplastic large cell lymphoma	2.4
Mature T-cell lymphoma, not ALCL	7.6
Precursor T lymphoblastic	1.7
Lymphoplasmacytic lymphoma	1.2
Other	7.4
Hodgkin lymphoma	9290 estimated new cases in 2013

Follicular lymphoma

Follicular lymphoma (FL) is the second most common of the B-lineage lymphomas, occurring primarily in older patients. It is derived from germinal center-type cells that commonly exhibit a follicular (nodular) growth pattern in lymph nodes. It usually has an indolent course [207]. The genetic hallmark of FL is t(14;18)(q32.3;q21.3), which results in juxtaposition of the *IGH* and *BCL2* genes and deregulation of *BCL2* [208]. *BCL2* is an anti-apoptotic gene; and as a result of the translocation, it is upregulated and apoptosis is inhibited. This rearrangement occurs in a low number of peripheral blood cells of healthy individuals that do not develop FL, showing that the translocation is insufficient for lymphomagenesis [209,210]. *BCL2* overexpression without the translocation occurs in other normal and neoplastic cells.

Mantle cell lymphoma (including blastic variant)

Mantle cell lymphoma (including blastic variant) (MCL) is uncommon, and comprises only 2–10% of all NHL. It is more common in older patients and has a male predominance. It is aggressive, with a median survival of ≤4 years [211]. The hallmark of MCL, t(11;14)(q13;q32.3) or a variant, occurs in all MCL when tested with FISH or other molecular methods on involved tissue [208]. As a result of the translocation, *CCND1* (cyclin D1) is juxtaposed to one of the immunoglobulin genes, is overexpressed and causes the cells to enter and progress through the cell cycle. Overexpression of cyclin D1 also occurs in other neoplasias. Secondary cytogenetic abnormalities are common in MCL, especially in the blastic variant [212,213].

Burkitt lymphoma/leukemia

Burkitt lymphoma/leukemia (BL) comprises less than 1% of NHL, but its diagnosis is vital, as treatment is unique for this disease. It is endemic in Africa, especially in children, where it is associated with infection with Epstein Barr virus [214]. It occurs both as lymphoma and as a leukemic variant, where the disease is in the marrow, and it comprises less than 5% of pediatric and adult ALL [215,216]. Bone marrow involvement of the lymphoma occurs in 20–35% of cases. BL is characterized by the t(8;14)(q24.2;q32.3) and its variants, t(2;8)(p11.2;q24.2) and t(8;22)(q24.2;q11.2); one of which is present in nearly all cases [208]. The translocations result in overexpression of the *MYC* oncogene, resulting from juxtaposition of *MYC* to one of the immunoglobulin gene control elements [217]. There is also a “Burkitt-like” lymphoma, which is similar to BL.

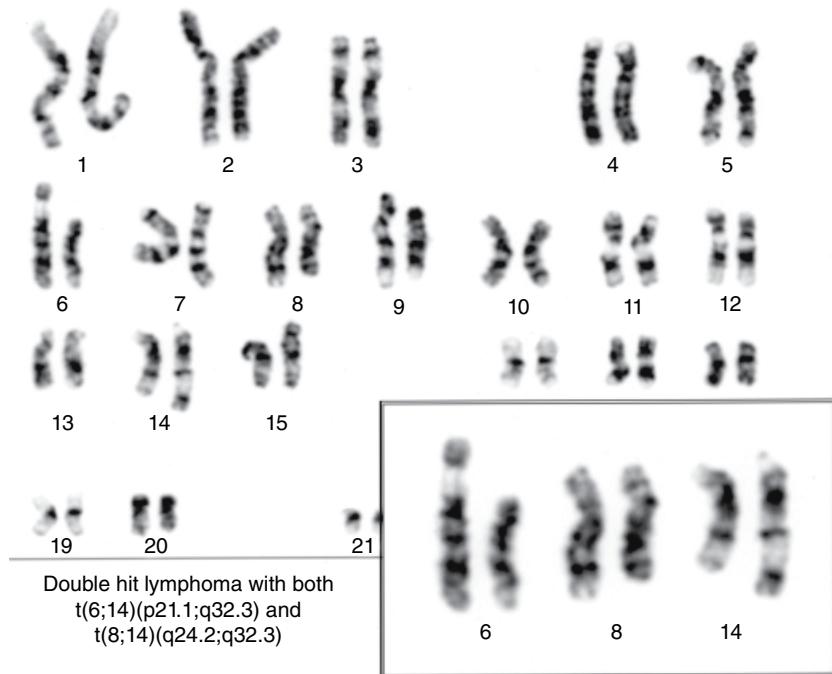


Figure 11.5 Double hit lymphoma t(6;14) and t(8;14). A partial karyogram demonstrating a ‘double hit lymphoma’ with both t(6;14) (p21.1;q32.3) and t(8;14)(q24.2;q32.3). Reproduced with the kind permission of N. Heerema, Department of Pathology, The Ohio State University, Columbus, OH.

Marginal zone B-cell lymphoma

Marginal zone B-cell lymphoma (MZBCL) has three distinct clinicopathological forms: (1) extra-nodal MZBCL of mucosa-associated lymphoid tissue (MALT); (2) splenic MZBCL, known as splenic lymphoma with villous lymphocytes (SLVL); and (3) nodal MZBCL [218,219]. MALT lymphoma is an indolent disease, occurring primarily in the stomach, where it usually follows chronic gastritis due to *Helicobacter pylori* infection. When low-grade, it generally responds to eradication of the infection. The recurring t(11;18)(q22;q21) *BIRC3/API2*-*MALT1* is restricted to gastric MALT lymphoma, and it signifies resistance to *Helicobacter pylori* eradication therapy and has antigen independent growth. High grade tumors are generally more karyotypically complex and may have a t(8;14)(q24.2;q32.3) [219]. In general splenic MZBCL has an indolent course; however, splenic MZBCL with a complex karyotype, 14q aberrations or TP53 deletions has a poor prognosis. Nodal MZBCL has a shorter survival than splenic MZBCL.

B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL – double-hit and triple-hit lymphomas

Non-Burkitt lymphomas with a *MYC* rearrangement often have a second rearrangement of another gene typically associated with lymphomas. These are called “double-hit” (DH) lymphomas, and include *BCL2*+/*MYC*+, *BCL6*+/*MYC* and *CCND1*+/*MYC* cases, as well as others (see Figure 11.5). Triple-hit (TH) lymphomas involving three genes, one of which is *MYC*, also occur. In most cases the *MYC* rearrangement is secondary, it rearranges with one of the immunoglobulin light chains more frequently than in BL, and it rearranges with non-Ig loci. These cases tend to be very aggressive with poor survival. These lymphomas frequently have very complex karyotypes, and FISH can be very useful in identifying them as DH and TH lymphomas [1,220].

Hodgkin lymphoma/disease

Hodgkin lymphoma/disease (HL/HD) comprises 15% of malignant lymphomas and primarily affects young adults. It is a germinal center-derived B-cell tumor, comprised of Reed-Sternberg cells, which are only 0.1–1% of cells. Possibly because there are so few malignant cells, there is a paucity of successful cytogenetic analyses. Highly complex hyperdiploid karyotypes

have been reported to occur in 15% of cases [221], and complex karyotypes are frequent [222,223]. In addition, recurrent rearrangements of the immunoglobulin loci, especially *IGH*, may be present in a significant number of cases, although gains of 2p13-16 and 9p24 are even more frequent than immunoglobulin rearrangements [224].

t(9;14)(p13;q32.3), IGH-PAX5

A t(9;14)(p13;q32.3), an *IGH-PAX5* rearrangement, occurs in many of the B-cell neoplasias, including lymphoplasmacytic lymphoma, multiple myeloma, plasma cell leukemia, CLL and diffuse large B-cell lymphoma. It also occurs occasionally in follicular lymphoma, mantle cell lymphoma and marginal zone lymphoma. The rearrangement leads to dysregulation of B-cell proliferation and differentiation [225].

11.6.2 T-cell lymphomas

Anaplastic large cell lymphoma/ALK1-positive lymphoma

Anaplastic large cell lymphoma/*ALK*-positive lymphoma (ALCL) comprises 5% of NHL in adults and 15% in children, with a male predominance [226]. It is typically found in extranodal sites and has strong Ki-1 antigen (CD30) expression. The most common cytogenetic abnormality is t(2;5)(p23;q35), which is especially common in children [227]. This translocation results in a chimeric oncogene, *NPM1-ALK* [228]. There are many variants, all with rearrangements of the *ALK* gene [229]. FISH for an *ALK* rearrangement can be done to confirm that a sample has a rearrangement of this gene. Fifty to sixty percent of cases, particularly in younger patients, have *ALK* protein expression, which can occur in the absence of an *ALK* gene rearrangement. *ALK*-positive ALCL is an aggressive disease, but has a favorable prognosis, regardless which *ALK* partner is involved, with a 70–80% 5-year survival. *ALK*-negative ALCL cases have a much poorer prognosis, with a 30–40% 5-year survival [230–234].

Primary cutaneous lymphoma – mycosis fungoides and Sézary syndrome

Mycosis fungoides (MF) is the most common form of cutaneous T-cell lymphoma (CTCL). It is derived from mature T lymphocytes and is CD4 positive. There is a male predominance, and it occurs in middle age to elderly individuals. Survival is generally long. Only 30% of cases with cutaneous MF have peripheral blood involvement, and these usually have advanced disease. Bone marrow involvement is less frequent than peripheral blood involvement.

Sézary syndrome (SS) may be the leukemic phase of MF; but in this case, survival is short with a median survival of less than 3 years [235,236]. Peripheral blood involvement occurs in up to 90% of cases with generalized erythroderma, which is characteristic in SS. Stimulation of peripheral blood with a T-cell mitogen results in abnormal karyotypes in about 50% of cases [237,238].

11.7 Laboratory practices

11.7.1 Common cytogenetic aberrations

Some of the more common cytogenetic aberrations in hematopoietic neoplasms are given in the descriptions of the diseases in the preceding sections, some are listed in Table 11.1, and some are shown in Figure 11.2. It is beyond this chapter to describe all recurrent aberrations. The technologist is referred to <http://atlasgeneticsoncology.org> and <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=CancerChromosomes>. The former gives a synopsis of most recurrent cytogenetic aberrations in leukemia and lymphoma; and the latter gives a link to all reported aberrations in cancer, with links to the articles describing the abnormalities.

11.7.2 Cytogenetic methodology

Overview

Table 11.5 gives specific methods used for hematologic malignancies from several labs and detailed procedures from some labs are in the protocol section. An excellent general reference is Hirsh et al., *Genetics in Medicine*, 2005 [239]. The most important thing to remember in doing cancer cytogenetic studies is that the malignant tissue must be studied. In hematologic malignancies, with few exceptions, this is bone marrow for leukemias and involved lymph nodes for lymphomas. A second important dictum is that most malignant cells divide spontaneously and that the typically used mitogens stimulate normal cells and rarely stimulate malignant cells, with the exception of mature B-cell lymphomas. Thus, for most leukemia studies,

Table 11.5 Summary of cytogenetic methods from some laboratories. All methods require incubation at 37 °C, and all laboratories use 3 : 1 methanol-acetic acid for fixation

Laboratory	Media	Supplements	Cell concentration	Culture volume	Culture time	Mitotic inhibitor	Hypotonic	Notes
Institute of Medical and Veterinary Sciences (SA Pathology) Adelaide, AU	RPMI 1640	20% FCS 7.5% 5637 sn (see later) HEPES, L-glutamine, Penicillin, Gentamicin	1 - 2 × 10 ⁶ /mL	10mL	Overnight	Colcemid® @ 0.1 µg/mL for 30min	8–9mL 0.075M KCl for 30 min Add ~1mL cold fix at the end of incubation & mix before centrifugation	Set up 2 cultures one with FdU and uridine; release block with BrdU ² Add 10 µg/mL EtBr 90 min prior to Colcemid® addition. Fixative is cold and freshly prepared
The Ohio State University	RPMI 1640	10% FBS, L-glutamine, Penicillin-Streptomycin + BM Condimed	2 × 10 ⁶ /mL	5mL	Overnight	Colcemid® @ 0.1 µg/mL for 30min	6mL 0.075M KCl for 20 min	
Children's & Women's Health Centre of British Columbia	0.054M KCl 0.075M KCl + 1–2 d ³ Na Heparin + 0.625mL Trypsin-EDTA	Trypsin- EDTA(0.05%) – (0.53 mM)	8 d ³ /tube	10mL	Direct ⁴ (20min)	Colcemid® @ 0.2mL for 10min	See media	
RPMI 1640								
University of North Carolina – Chapel Hill	RPMI 1640	15% FBS 1mL GCT ³ added to 1 culture	1 × 10 ⁶ /mL	10mL	Overnight	Colcemid® @ 0.2mL for 90 min	8–14 mL 0.054M KCl for 15 min	Incubate cells for 20 min in first RPMI, change media
Indiana University	RPMI 1640	20% FBS 1mL GCT ³ 1% L-glutamine, Penicillin-Streptomycin			5–10mL	24h	200 µL EtBr for 90 min 50 µL Colcemid® for 45 min	10mL 0.075M KCl for 12 min
							100 µL Colcemid® for 60 min	10mL 0.075M KCl for 20 min

(Continued)

Table 11.5 (Continued)

Laboratory	Media	Supplements	Cell concentration	Culture volume	Culture time	Mitotic inhibitor	Hypotonic	Notes
Brigham & Women's Hospital, Boston	MarrowMAX™		0.6mL 0.8mL Adjust for very high and low counts	5mL 5mL	24h	50µL EtBr for 90min then add 100µL Colcemid® for 20 min	Two 5.0mL 0.075M KCl hypo periods for a total of 22 min on Hanabi automated harvester	Pediatric ALL; New diag, APML; or other STATs
Seattle Children's Hospital	RPMI 1640	20% FBS, L-glutamine, Penicillin-Streptomycin + 1 mL GCT ⁵	1×10 ⁶ /mL	10mL	Overnight	16µL Colcemid® added at set up, harvested by hand at 7 am the next morning	10mL 0.075M KCl for 20 min	
Allina Northwestern Hospital	RPMI 1640	20% FBS, L-glutamine, Penicillin-Streptomycin + 1 mL BM Condimed	1.5~-2.0×10 ⁶ /mL	10mL	Overnight for STATs	200µL EtBr for 120 min then 0.1mL Colcemid® for 30 min	9.5mL 0.075M KCl 30min	
		20% FBS, L-glutamine, Penicillin-Streptomycin + 1 mL GCT ⁵				0.2mL Colcemid® for 150min at 2-8°C		

¹ 5637 cell line supernatant. Quantmeier H, et al. *Leukaemia Res*, 1997;21(4):343-350.² Webber and Garson, Cancer Genet & Cytogenet, 1983;8:123-132.³ d = drop⁴ Bedside collection⁵ See text chapter Cytogenetic Methodology).

Thanks to contributions from: Sarah Moore, Institute of Medical and Veterinary Sciences, Adelaide AU; Patrice Eydoux and Wendy Duey, Children's & Women's Health Centre of British Columbia; Kathleen Rao, University of North Carolina – Chapel Hill; James Higgins, Spectrum Health, Grand Rapids; Kim Purcell, LabPlus, Aukland Hospital, NZ; Gail Vance, Indiana University; Mary Shago, Hospital for Sick Children Toronto; Paola Dal Cin, Brigham & Women's Hospital Boston; Kent Opheim, Seattle Children's Hospital; Rodney Higgins, Allina Northwestern Hospital, Minneapolis, MN; Nyla Heerema, Allina North Hospital, Minneapolis, MN; Nyla Heerema, The Ohio State University.

Table 11.6 Mitogens used in leukemias/lymphomas

AML	None
APL	None
MDS	None
ALL	None
T-cell lymphomas	None
Chronic myeloproliferative disorders	None
CML	None
CMMI	None
CLL	CpG Oligodeoxynucleotides ¹
B-cell lymphomas (NHL and non-NHL)	CpG Oligodeoxynucleotides
multiple myeloma	IL4 ² and unstimulated
MGUS	IL4 ² and unstimulated
Amyloidosis	IL4 ² and unstimulated
Waldenstrom macroglobulinemia	IL4 ² and unstimulated
B-PLL	B-cell mitogens ³
Hairy cell leukemia	B-cell mitogens ³
T-PLL	T-cell mitogens: PHA, ConA (concanavalin A)
Primary cutaneous lymphoma/mycosis fungoides/Sézary syndrome	T-cell mitogens, PHA, Con A

¹ Various B-cell mitogens have been used in different combinations in the past. It is now recognized that stimulation with a CpG oligodeoxynucleotide is best. (See specific procedures.)

² Various stimulants have been used including IL (interleukin)-2, IL-4 and IL-6. Unstimulated 24- and 72-hour cultures should also be set up. No culture method has been shown to be very successful. (See specific procedures.)

³ Various B-cell mitogens can be used. These include: pokeweed mitogen, TPA (12-O-tetradecanoylphorbol-13-acetate) or PMA (phorbol 12-myristic 13-acetate), LPS (lipopolysaccharide) and TNF α (tumor necrosis factor). Different laboratories have success with different stimulants and combinations of them.

unstimulated bone marrow should be studied. (For the exceptions requiring mitogens, see Table 11.6.) The duration of culture varies, with most laboratories using overnight or 24-hour cultures. Some laboratories have successful direct cultures, although these laboratories frequently are able to go to the bedside to procure the sample. Many laboratories do both a 24-hour (overnight) culture and a 48-hour culture, while others rely on 24-hour (overnight) cultures exclusively. A few laboratories allow longer culture times; but these are not recommended, except in a few diseases in which mitogens are recommended. Although 72-hour unstimulated cultures will sometimes be successful in identifying an abnormal clone, this length of culture time allows nonleukemic cells to take over the culture, and any normal metaphases may result from division of non-malignant cells within the sample.

An adequate sample is a prerequisite for a successful cytogenetic analysis. The sample must contain the leukemia or lymphoma cells. For leukemia, the bone marrow aspirate should be either the first draw or the second draw using a repositioned needle. Later draws frequently will be greatly diluted with blood, and abnormal mitoses will not be obtained. A quick test for an adequate sample is the presence of spicules, but this is not 100% accurate. Samples without spicules can yield adequate metaphases and vice versa.

The sample should be taken in the anti-coagulant sodium heparin, preservative-free, if possible. The amount of sodium heparin should be approximately 20 units per mL of total volume (either bone marrow alone, or bone marrow and transport medium combined). If collected in a green top tube, a smaller sodium heparin tube is preferred [239]. Too much sodium heparin may result in a viscous sample that is nearly impossible to culture and analyze successfully.

The sample should be kept at room temperature and delivered to the lab within 24 hours, although immediate delivery is recommended when possible. Some laboratories prefer to have the sample put into "transport media," usually RPMI 1640, which may help the cells survive. Other laboratories do not find this necessary.

If a bone marrow aspirate is not possible, a bone core may be substituted. However, success rates and numbers of cells obtained from bone cores are nearly always much lower than from bone marrow aspirates.

Another secondary source of leukemia cells is a peripheral blood sample that has a minimum of 15% blasts. Peripheral blood samples for leukemia analyses should not be stimulated, with the exceptions listed in Table 11.6. Even with circulating blasts, it is not always possible to obtain dividing leukemia cells, and “normal analyses” from peripheral bloods should be interpreted with extreme caution.

Specimens must be received with a clinical diagnosis, as culture conditions depend upon this information (see Table 11.7, ICD groupings for hematological diseases for interpreting diagnostic codes). For leukemia cell cultures, most successful laboratories do cell counts using either an electronic cell counter or a hemacytometer (see Chapter 19, section 19.4.1, Determining cell count by using a hemacytometer). Either is acceptable. The concentration of cells most laboratories attempt to obtain is $1\text{--}2 \times 10^6$ cells/mL culture [240]. Typically, laboratories use 10-mL cultures, although some use 5-mL cultures very successfully. The latter is equally successful and more cost effective. If cell counts are low, it is better to set up a culture of lower vol-

Table 11.7 ICD groupings for hematological diseases

CPT (Current Procedural Terminology) codes describe services rendered for billing purposes, and **ICD** (International Classifications of Diseases) codes describe why that service was provided. First developed in 1893 in France by physician Jacques Bertillion, ICD codes were immediately recognized for their usefulness and were adopted by the International Statistical Institute (ISI). The World Health Organization (WHO) took over the responsibility in 1948. Each country tailors the codes to fit their needs; therefore, letters may be added to designate its originating country; for example, ICD-10-CA indicates Canadian origin.

At times, specimen requisitions are received with only an ICD code. The setup technologist must understand these codes in order to identify if a specimen is STAT, requires B- or T-cell stimulation or is a candidate for FISH. The following list has been provided in order to give a sample of ICD-10 codes that are used on hematological referrals for cytogenetic workup. The reader is referred to the most current handbook for interpretation of an order requisition.

ICD-10 coding increased diagnostic codes from 17,000 (in ICD-9-CM) to over 141,000 codes.

Excerpts of ICD-10 codes

C81–C96 malignant neoplasms

C81. Hodgkin Lymphoma

C82. Follicular non-Hodgkin lymphoma (nodular)

C83. Diffuse non-Hodgkin lymphoma

C84. Peripheral and cutaneous T-cell lymphomas

C85. Other and unspecified types of non-Hodgkin lymphoma

C85.0 Lymphosarcoma

C85.1 B-cell lymphoma, unspecified

C90. Multiple myeloma and malignant plasma cell neoplasms

C91. Lymphoid leukemia

C91.0 Acute lymphoblastic leukemia

C91.1 Chronic lymphocytic leukemia

C91.4 Hairy cell leukemia

C92. Myeloid leukemia

C92.0 Acute myeloid leukemia

C92.1 Chronic myeloid leukemia

C92.2 Subacute myeloid leukemia

C92.3 Myeloid sarcoma

C92.4 Acute promyelocytic leukemia

C92.5 Acute myelomonocytic leukemia

C93. Monocytic leukemia

C94. Other leukemias of specified cell type

C95. Leukemia of unspecified cell type

C96. Other and unspecified malignant neoplasms of lymphoid, hematopoietic and related tissue

For a full list, see <http://apps.who.int/classifications/apps/icd/icd10online/>

ume in order to maintain the suggested $1\text{--}2 \times 10^6$ cells/mL. At least two different cultures are recommended. This can be done by using two different media or two different culture times. (Different media used are shown in Table 11.5.) The cells can be cultured in 25-mL flasks or in 15-mL conical centrifuge tubes set at an angle in the incubator to increase surface area for exposure to CO₂. The latter have the advantage that they do not require transferring the culture to centrifuge tubes after mitotic arrest and are more cost effective.

For lymphomas, an involved lymph node is the tissue of choice. If the lymphoma does not present as a nodal disease, for example, an extra-nodal mass or effusion, or cutaneously, the involved tissue is the ideal choice. It is best if these are put into sterile media for transport to the laboratory. These are very fragile, and immediate delivery is a prerequisite for successful analysis. When an involved lymph node is not available, *involved* bone marrow or peripheral blood may be analyzed. The method is the same as for leukemias.

Involved lymph nodes from a lymphoma patient should be gently minced or teased to release malignant cells. The released cells should be treated in the same manner as a bone marrow, being very gentle with the cultures as the cells are quite fragile. Overnight or 24-hour cultures are recommended. As with leukemias, some laboratories have successful direct cultures. Cultures should not be longer than 48 hours, as malignant lymphoma cells will rarely divide after that length of time in culture.

Cells are arrested in metaphase by a mitotic spindle inhibitor, usually Colcemid®, although Velban (typically at a final concentration of 0.0025 µg/mL) is occasionally used. Some laboratories use cell synchronization or a DNA intercalation agent prior to fixation to obtain longer chromosomes, although this may result in a lower mitotic index. Colcemid® time also varies, from 30 minutes to overnight. Generally, 30 minutes are sufficient and result in metaphases of a reasonable quality. Longer Colcemid® times are sometimes used for leukemia types that seem to divide rarely in culture. Although longer Colcemid® time may increase the mitotic index, the chromosomes may be quite short and of inferior quality.

As with constitutional studies, the cells are treated with a hypotonic solution, usually 0.075 M KCl, in order to swell the cells so that chromosomes will adequately spread during slide-making. Other concentrations of KCl are also used. Other hypotonics occasionally used are 0.4% sodium citrate or mixtures of KCl and sodium citrate. The cells are fixed with absolute methanol–glacial acetic acid, usually in a 3 : 1 ratio, although some labs use a 2 : 1 ratio. Slides are prepared as for constitutional studies, aged, banded, usually using trypsin, then stained with Giemsa, Wright or Leishman stain. Q-banding is occasionally used (see Chapter 6, Chromosome stains, for more detailed explanations of staining options).

For all cytogenetic studies, slide-making is extremely important. As with constitutional studies, many different procedures can be used successfully. A significant factor for cancer studies is making the cell concentration on the slides fairly thin. There is a tendency to make the cells on the slides more concentrated, as the mitotic index may be low. But both spreading and banding quality often suffer when the cell concentration on the slides is high. Refer to slide-making chapter for recommendations and troubleshooting.

Analysis of leukemia and lymphoma specimens is an extremely important part of obtaining successful and accurate diagnoses. Importantly, both high-quality and poor-quality metaphases must be examined. Frequently, the poor-quality metaphases are the leukemia cells, and the high-quality cells are normal. Both cultures should be examined. A starting method in many laboratories is to examine the first 20 metaphases seen. Every chromosome should be completely analyzed, band for band. Some abnormalities are very subtle and only realized through careful analysis. If the culture is abnormal, analysis of the first 20 metaphases is adequate, although searching for better quality abnormal cells may be required to correctly identify breakpoints. However, if only normal cells are seen, the technologist should search for abnormal cells. At diagnosis, the mind-set of the technologist should be that the sample is abnormal, and the abnormal cells need to be identified. Unfortunately, in some leukemias, it is extremely difficult to obtain dividing leukemia cells. This is particularly true in hyperdiploid ALL with >50 chromosomes and also frequently in ALL with a t(1;19)(q23;p13.3).

Once at least 20 metaphases have been analyzed and the specimen is found normal, preparing two karyograms would be sufficient. If, however, the specimen was found abnormal, karyograms should be prepared for at least 2 cells from the stemline and one from each sideline (see Chapter 8, section 8.9.2, Neoplastic clonal evolution, for an explanation of stemline and sideline), although it is highly recommended that two cells from each abnormal cell line be karyotyped for a more accurate identification of the breakpoints in structural abnormalities. If a normal cell line is present in an otherwise abnormal specimen, one normal karyogram should be prepared, which would help distinguish any polymorphism from a true acquired abnormality. If nonclonal abnormal cells are found, they too should be karyotyped, as they may recur in a later analysis, indicating their clonality.

If all cells have the same balanced abnormality or one that is potentially constitutional, it is important to rule out a constitutional abnormality. If the abnormality is a recurring abnormality in leukemia, it most likely is not constitutional. However, if there is a sole +21, or +X, a constitutional abnormality should be considered. Stimulated peripheral blood can establish whether the abnormality is constitutional. Similarly, if the standard of the laboratory is to get a follow-up bone marrow, this may rule-out a constitutional abnormality. However, if the patient does not go into remission, a peripheral blood study may still be required. Another possibility is to establish fibroblast cultures from any remaining bone marrow.

Fluorescence in situ hybridization in hematologic malignancies

FISH is widely used in the evaluation of hematologic malignancies. It is recommended that most FISH analyses be done as an adjunct to metaphase analyses. Probes are commercially available for a wide variety of specific aberrations and are offered by various vendors. The probes should be used according to the manufacturer's recommendations.

Different probe strategies are available for the detection of different types of aberrations in hematologic malignancies. These include enumeration probes, such as those for centromeres or for detection of a deletion. There are break-apart probes to detect the rearrangement of specific genes known to be rearranged in some malignancies. These include *IGH*, *KMT2A* and *RUNX1* probes. There are also dual-fusion dual-color probes to detect a specific rearrangement, such as the *BCR-ABL1* [t(9;22)] rearrangement in CML or *RUNX1T1-RUNX1* [t(8;21)] in AML. Extra signal probes, such as for *BCR-ABL1* [t(9;22)] in CML or *ETV6-RUNX1* in ALL are also available. Some probes have "reporter" probes with them, such as a probe for 13q34 with probes for 13q14 to establish the presence of monosomy versus a deleted chromosome in interphase. There are also specific probes to help detect deletions associated with rearrangements, such as the *ASS* probe with the *BCR-ABL1* probes. All probes should be used only after specificity, sensitivity and control reference ranges have been established. Furthermore, they should be applied appropriately. Thus the *RUNX1T1-RUNX1* probes would not be appropriate for a patient with a history of CML.

There are some diagnostic instances in which FISH is particularly useful. These include CML, APL and Burkitt leukemia/lymphoma. It is important to rule-out CML in all patients with a MPN. Furthermore, it is vital that patients with CML be treated appropriately, as therapy is specific for the Philadelphia chromosome. Similarly, both APL and BL require specific treatments, which should be administered immediately. Therefore FISH can be a very useful aid in these diagnoses.

In MM, FISH is often more informative than banded analyses, as it is difficult to get malignant cells to divide in culture in MM. Specific panels of probes to detect clinically relevant abnormalities in MM can be devised. Some aberrations, such as a t(12;21)(p13;q22) in ALL, are cryptic, and FISH or another molecular method is required for their detection.

FISH is also valuable in follow-up of malignancies with known aberrations and in cases for which fresh samples (therefore, dividing cells) are not available or no dividing cells are present. For follow-up samples, the FISH pattern of the diagnostic sample must be established.

FISH is extremely useful in more precisely defining aberrations present by metaphase analysis. Usually, metaphase FISH is required for this. For example, *KMT2A* rearrangements can be very complex, and FISH with a *KMT2A* probe can help to describe the aberration. For many aberrations, RT-PCR is a useful adjunct or alternative method of gaining the information desired.

General procedural notes

See Table 11.5 for specific methods from several surveyed laboratories.

Setup/culture

The media plus supplements should be warmed to 37°C before use. Many labs use only the white cells for culture. These can be obtained by letting the sample settle, by centrifugation, or by using a Ficoll–Hypaque separation. Add the appropriate amount of sample to media + supplements to obtain the desired cell concentration. The cultures should be maintained in a 5% CO₂ atmosphere at 37°C.

If the sample is clotted when received in the laboratory, the clot must be dissociated so the sample can be processed. This usually can be accomplished using collagenase. Occasionally, a clot can be broken-up with scissors or scalpels, in which case viable cells may be released from the clot.

GCT (giant cell tumor) supplemented medium is recommended for myeloid cultures and is available commercially (see Chapter 25, section 25.2.6, Vendor products/equipment and lab support).

Harvest

Cultures should be gently mixed BY HAND after adding the mitotic inhibitor. Cell centrifugation should be at 145–180 g (~800–1000 RPM for most centrifuges). Gently remove supernatant to just above the cell pellet each time a reagent is changed. The cell pellets should be gently resuspended before adding each reagent.

Warm the hypotonic to 37°C prior to adding it to the cell suspension. Add the first mL of hypotonic solution drop by drop slowly down the side of the tube, continually mixing with the cell suspension. The remaining hypotonic can be added

more quickly. Keep the cells in hypotonic at 37°C. After the appropriate amount of time, prefix the cells by adding 1 mL of fixative to the cells in the hypotonic solution. Very cold fixative is recommended.

After about 2 minutes, centrifuge the cells and resuspend the cells in fixative. Leave in the refrigerator or freezer for 30–60 minutes. Repeat fixative changes using 5–10 mL of fixative until supernatant is clear and pellet looks clean (white). These changes can be done either right after one another or the cells may be allowed to set in the fixative.

Hints

1. Make sure cells are thoroughly resuspended before adding any reagent. Mix cells very gently. NEVER vortex.
2. Hypotonic times may be increased 5 to 10 minutes during colder and drier months.
3. Lower concentrations of KCl may facilitate spreading of the chromosomes.
4. The methanol must be very pure. It is best to obtain the highest quality possible.
5. Keep the fixative chilled as much as possible.

Slide-making

Each laboratory has its own method of making slides. The metaphases will spread quite readily if slides are made immediately. If there is excessive spreading, it is best to refrigerate the pellets overnight. If metaphases are present, but spreading is poor, it is best to try again the next day. If there is still no success, the fixative can be changed to 2:1 methanol–acetic acid, which sometimes will help with spreading. It is best not to change the ratio of the fixative of the entire sample, as this can also have disastrous results!

Banding

The same methods of banding that are used for constitutional chromosomal analyses can be used for acquired chromosomal analyses. The typical methods are GTG, GTW, QFQ, and GTL (see Chapter 6, Chromosome stains, for an explanation of these acronyms). In some cases, Wright stain without trypsin is used. The method should be the one best suited to the individual laboratory. Additionally, different samples will band better with different methods, and a trial and error approach may be necessary.

Acknowledgments

Thanks to Carol Cole, Andrew McFaddin and Heather Breidenbach for their help with the manuscript.

Glossary of hematopoietic malignancies

Alkylating agent: organic chemicals that transfer alkyl groups to other molecules, in chemotherapy to DNA.²

Anemia: a condition with less than the normal number of red blood cells or less than the normal quantity of hemoglobin in the blood, decreasing the oxygen-carrying capacity of the blood.¹

Apoptosis: a type of programmed cell death, which involves an orchestrated series of biochemical events leading to a characteristic cell morphology and death.²

Auer rods: clumps of azurophilic granular material that form elongated needles in the cytoplasm seen in the blasts of acute myeloid leukemia. They are composed of fused lysosomes and contain peroxidase, lysosomal enzymes, and large crystalline inclusions.²

Basophilia: an abnormal increase in basophils.

Basophils: white blood cells that stain dark blue to purple with basophilic dyes. They are an important part of the body's immune response.

Blasts: immature blood cells. Leukemic blasts do not grow and age normally; they proliferate wildly and fail to mature.¹

Core binding factors (CBF): a group of heterodimeric transcription factors, composed of a non-DNA-binding CBF β chain and a DNA-binding CBF α chain.² The CBF regulates the transcription of several genes important for hematopoiesis.

CD antigen: (cluster differentiation antigen) any of a number of cell surface markers expressed by leukocytes and used to distinguish cell lineages, developmental stages, and functional subsets. Such markers can be identified by monoclonal antibodies.³

Cytopenia: a reduction of cellular elements in the hematopoietic system.

-cytosis: suffix referring to an excess of cells.

Dyscrasia: abnormal state or disorder of the body, especially of the blood.³

Dysplasia: alteration in size, shape, and organization of adult cells.³

Eosinophil: white blood cells that stain brick-red with eosin, involved with immune, asthma, and allergy reactions.²

Episome: non-chromosomal circular DNA.

Extramedullary: outside of the bone marrow or blood.

Extranodal: outside of the lymph nodes.

Globulin: a class of proteins insoluble in water, but soluble in saline solutions.³

Granulocytic sarcoma: an extramedullary myeloid tumor, composed of immature, malignant white blood cells.

Haploinsufficiency: a single functional copy of a gene that does not produce enough product for normal function.²

Hematopoietic: blood cellular components.²

Hepatomegaly: enlarged liver.

Hepatosplenomegaly: enlarged liver and spleen.

Homeobox genes: genes involved in the regulation of development.

HOX genes: a particular subgroup of homeobox genes that function in patterning the body axis.²

Hyperdiploid: “hyper” = too many; more than 46 chromosomes in human cells.

Hyperuricemia: high levels of uric acid in the blood, which can lead to gout and renal failure.²

Hypodiploid: “hypo” = too few, fewer than 46 chromosomes in human cells.

Immunophenotype: the phenotype of cells defined according to the expression of particular antigens, particularly on the cell surface.

Leukemia: a condition characterized by an accumulation of white blood cells in the bone marrow and blood.

Leukocytopenia: a white blood cell count below normal.

Leukocytosis: a white blood cell count above normal.

Lymphadenopathy: swelling of one or more lymph nodes.²

Lymphoma: a neoplastic disorder of lymphoid, usually lymph nodes, tissue.³

Macroglobulin: a globulin of unusually high molecular weight, in the range of 1,000,000.³

Macroglobulinemia: increased levels of macroglobulins in the blood.³

Mediastinum: a group of structures in the thorax (chest), the central compartment of the thoracic cavity, containing the heart, the great vessels of the heart, esophagus, trachea, thymus, and lymph nodes of the central chest.

MicroRNA (miR): short, 21–25 base pairs, non-coding RNA genes that regulate gene expression both positively and negatively.²

Monoblasts: cells normally found in bone marrow and do not appear in the normal peripheral blood. They mature into monocytes, which in turn develop into macrophages.²

Monosomal karyotype: at least two autosomal monosomies or one monosomy and a structural abnormality.⁵

Multilineage dysplasia: dysplasia affecting several of the blood cell lineages.

Myelodysplastic: dysplasia of myelocytes and other elements of the bone marrow,³

Myelofibrosis: a myeloproliferative disease, characterized by the gradual replacement of the bone marrow by connective tissue.

This reduces the patient’s ability to generate new blood cells resulting in chronic anemia. A prime feature is “extramedullary hematopoiesis,” that is, the remaining blood-forming cells migrate to other sites in the body, for example, the liver or spleen. Patients will typically have an enlarged spleen and liver (hepatosplenomegaly), and examination of the blood cells will show “teardrop” cells.²

Myeloid: having to do with or resembling the bone marrow. May also refer to certain types of hematopoietic (blood-forming) cells found in the bone marrow, sometimes used as a synonym for myelogenous; for example, acute myeloid leukemia and acute myelogenous leukemia are the same disease.⁴ (see Figure 11.1)

Myeloid metaplasia: a clonal disorder arising from the neoplastic transformation of early hematopoietic stem cells, categorized as a chronic myeloproliferative disorder.⁵

Neoplasm: abnormal, disorganized growth in a tissue or organ, usually forming a distinct mass, also known as a tumor. Neoplasia is the correct, scientific term for diseases commonly called cancer, tumor or growth. Neoplasms can be benign or malignant lesions.²

Neutrophilia (or neutrophil leukocytosis): a high number of neutrophil granulocytes.²

Neutrophils: the primary white blood cells that respond to a bacterial infection, so the most common cause of marked neutrophilia is a bacterial infection.

Organomegaly: abnormal enlargement of an organ.²

-philia: a suffix meaning a tendency toward.

Platelet: a blood cell that causes clots to form.

Plasmacytosis: an excess of plasma cells.

Pseudodiploid: “pseudo” = false; normal chromosome number (46 in humans), but with something abnormal, usually, although not always, a structural abnormality.

Remission: abatement or subsiding of the symptoms of a disease. The period during which the symptoms of a disease abate or subside.⁶

Sarcoma: a cancer of the connective or supportive tissue (bone, cartilage, fat, muscle, blood vessels) and soft tissue.²

Secondary disease: an unrelated “second” tumor following certain cancer treatments, including chemotherapy or radiotherapy.²

Spicules: a needle-like structure or part found in the bone marrow, but not in peripheral blood.

Splenomegaly: enlarged spleen.

STAT: immediately; derived from the Latin word *statim*.

Thrombocyte: another term for platelet.

Topoisomerase inhibitor: chemotherapy agents designed to interfere with the action of topoisomerase enzymes (topoisomerase I and II). Because it is coiled, it breaks when DNA replicates in order to relieve the stress that occurs. Topo II is involved in the religation of the DNA; thus Topo II inhibitors prevent the religation of the DNA, resulting in DNA breaks.

Glossary references

1. <http://www.medicinenet.com/leukemia/glossary.htm>
2. <http://en.wikipedia.org/wiki/>
3. <http://medical-dictionary.thefreedictionary.com/>
4. <http://nci.nih.gov/dictionary>
5. <http://www.emedicine.com/MED/topic78.htm>
6. remission. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved August 25, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/remission>

References

1. Swerdlow S, Campo E, Harris N, Jaffe E, Pileri S, Stein H, Thiele J, Vardiman J, eds. *World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th ed. 2008. Lyons: IARC Press.
2. American Cancer Society. *Cancer Facts & Figures 2016*. Atlanta: American Cancer Society; 2016. <http://www.cancer.org/acs/groups/content/@research/documents/document/acspc-047079.pdf>.
3. Appelbaum FR, Gundacker H, Head DR, Slovak ML, Willman CL, Godwin JE, Anderson JE, Petersdorf SH. Age and acute myeloid leukemia. *Blood* 2006; 107(9): 3481–3485.
4. Appelbaum FR, Kopecky KJ, Tallman MS, Slovak ML, Gundacker HM, Kim HT, Dewald GW, Kantarjian HM, Pierce SR, Estey EH. The clinical spectrum of adult acute myeloid leukaemia associated with core binding factor translocations. *Br J Haematol* 2006; 135(2): 165–173.
5. Burnett A, Wetzel M, Lowenberg B. Therapeutic advances in acute myeloid leukemia. *J Clin Oncol* 2011; 29(5): 487–494.
6. Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH, Wheatley K, Harrison CJ, Burnett AK. Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood* 2010; 116(3): 354–365.
7. Breems DA, Van Putten WL, De Greef GE, Van Zelderen-Bhola SL, Gerssen-Schoorl KB, Mellink CH, Nieuwint A, Jotterand M, Hagemeijer A, Beverloo HB, Lowenberg B. Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. *J Clin Oncol* 2008; 26(29): 4791–4797.
8. Byrd JC, Mrozek K, Dodge RK, Carroll AJ, Edwards CG, Arthur DC, Pettenati MJ, Patil SR, Rao KW, Watson MS, Koduru PR, Moore JO, Stone RM, Mayer RJ, Feldman EJ, Davey FR, Schiffer CA, Larson RA, Bloomfield CD. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood* 2002; 100(13): 4325–4336.
9. Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK, Dombret H, Fenaux P, Grimwade D, Larson RA, Lo-Coco F, Naoe T, Niederwieser D, Ossenkoppela GJ, Sanz MA, Sierra J, Tallman MS, Lowenberg B, Bloomfield CD. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* 2010; 115(3): 453–474.

10. Frohling S, Schlenk RF, Kayser S, Morhardt M, Benner A, Dohner K, Dohner H. Cytogenetics and age are major determinants of outcome in intensively treated acute myeloid leukemia patients older than 60 years: results from AMLSG trial AML HD98-B. *Blood* 2006; 108(10): 3280–3288.
11. Slovak ML, Kopecky KJ, Cassileth PA, Harrington DH, Theil KS, Mohamed A, Paitetta E, Willman CL, Head DR, Rowe JM, Forman SJ, Appelbaum FR. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. *Blood* 2000; 96(13): 4075–4083.
12. Head DR. Revised classification of acute myeloid leukemia. *Leukemia* 1996; 10(11): 1826–1831.
13. Caligiuri MA, Strout MP, Gilliland DG. Molecular biology of acute myeloid leukemia. *Semin Oncol* 1997; 24(1): 32–44.
14. Nucifora G and Rowley JD. The AML1 and ETO genes in acute myeloid leukemia with a t(8;21). *Leuk Lymphoma* 1994; 14(5–6): 353–362.
15. Tallman MS, Hakimian D, Shaw JM, Lissner GS, Russell EJ, Variakojis D. Granulocytic sarcoma is associated with the 8;21 translocation in acute myeloid leukemia. *J Clin Oncol* 1993; 11(4): 690–697.
16. Acute myelogenous leukemia with an 8;21 translocation. A report on 148 cases from the Groupe Francais de Cytogenetique Hematologique. *Cancer Genet Cytogenet* 1990; 44(2): 169–179.
17. Raimondi SC, Chang MN, Ravindranath Y, Behm FG, Gresik MV, Steuber CP, Weinstein HJ, Carroll AJ. Chromosomal abnormalities in 478 children with acute myeloid leukemia: clinical characteristics and treatment outcome in a cooperative pediatric oncology group study-POG 8821. *Blood* 1999; 94(11): 3707–3716.
18. Mancini M, Cedrone M, Diverio D, Emanuel B, Stul M, Vranckx H, Brama M, De Cuia MR, Nanni M, Fazi F, Mecucci C, Alimena G, Hagemeijer A. Use of dual-color interphase FISH for the detection of inv(16) in acute myeloid leukemia at diagnosis, relapse and during follow-up: a study of 23 patients. *Leukemia* 2000; 14(3): 364–368.
19. Liu P, Tarle SA, Hajra A, Claxton DF, Marlton P, Freedman M, Siciliano MJ, Collins FS. Fusion between transcription factor CBF beta/PEPB2 beta and a myosin heavy chain in acute myeloid leukemia. *Science* 1993; 261(5124): 1041–1044.
20. Liu PP, Wijmenga C, Hajra A, Blake TB, Kelley CA, Adelstein RS, Bagg A, Rector J, Cotelingam J, Willman CL, Collins FS. Identification of the chimeric protein product of the CBFB-MYH11 fusion gene in inv(16) leukemia cells. *Genes Chromosomes Cancer* 1996; 16(2): 77–87.
21. Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. *Blood Rev* 2004; 18(2): 115–136.
22. Tallman MS, Abutalib SA, Altman JK. The double hazard of thrombophilia and bleeding in acute promyelocytic leukemia. *Semin Thromb Hemost* 2007; 33(4): 330–338.
23. Grimwade D, Gorman P, Duprez E, Howe K, Langabeer S, Oliver F, Walker H, Culligan D, Waters J, Pomfret M, Goldstone A, Burnett A, Freemont P, Sheer D, Solomon E. Characterization of cryptic rearrangements and variant translocations in acute promyelocytic leukemia. *Blood* 1997; 90(12): 4876–4885.
24. Warrell RP Jr, de The H, Wang ZY, Degos L. Acute promyelocytic leukemia. *N Engl J Med* 1993; 329(3): 177–189.
25. Warrell RP Jr, Frankel SR, Miller WH Jr., Scheinberg DA, Itri LM, Hittelman WN, Vyas R, Andreeff M, Tafuri A, Jakubowski A. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid). *N Engl J Med* 1991; 324(20): 1385–1393.
26. Raelson JV, Nervi C, Rosenauer A, Benedetti L, Monczak Y, Pearson M, Pelicci PG, Miller WH Jr. The PML/RAR alpha oncoprotein is a direct molecular target of retinoic acid in acute promyelocytic leukemia cells. *Blood* 1996; 88(8): 2826–2832.
27. Castaigne S, Chomienne C, Daniel MT, Ballerini P, Berger R, Fenaux P, Degos L. All-trans retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. *Blood* 1990; 76(9): 1704–1709.
28. Park J, Jurcic JG, Rosenblat T, Tallman MS. Emerging new approaches for the treatment of acute promyelocytic leukemia. *Ther Adv Hematol* 2011; 2(5): 335–352.
29. Arnould C, Philippe C, Bourdon V, Gregoire MJ, Berger R, Jonveaux P. The signal transducer and activator of transcription STAT5b gene is a new partner of retinoic acid receptor alpha in acute promyelocytic-like leukaemia. *Hum Mol Genet* 1999; 8(9): 1741–1749.
30. Meyer C, Hofmann J, Burmeister T, Groger D, Park TS, Emerencino M, M Pombo de Oliveira, A Renneville, P Villarese, E Macintyre, H Cavé, E Clappier, K Mass-Malo, J Zuna, J Trka, E De Braekeleer, M De Braekeleer, S H Oh,

- G Tsaur, L Fechina, V H J van der Velden, J J M van Dongen, E Delabesse, R Binato, M L M Silva, A Kustanovich, O Aleinikova, M H Harris, T Lund-Aho, V Juvonen, O Heidenreich, J Vormoor, W W L Choi, M Jarosova, A Kolenova, C Bueno, P Menendez, S Wehner, C Eckert, P Talmant, S Tondeur, E Lippert, E Launay, C Henry, P Ballerini, H Lapillone, M B Callanan, J M Cayuela, C Herbaux, G Cazzaniga, P M Kakadiya, S Bohlander, M Ahlmann, J R Choi, P Gameiro, D S Lee, J Krauter, P Cornillet-Lefebvre, G Te Kronnie, B W Schäfer, S Kubetzko, C N Alonso, U zur Stadt, R Sutton, N C Venn, S Izraeli, L Trakhtenbrot, H O Madsen, P Archer, J Hancock, N Cerveira, M R Teixeira, L Lo Nigro, A Möricke, M Stanulla, M Schrappe, L Sedék, T Szczepański, C M Zwaan, E A Coenen, M M van den Heuvel-Eibrink, S Strehl, M Dworzak, R Panzer-Grümayer, T Dingermann, T Klingebiel and R Marschalek. The MLL Recombinome of acute leukemias in 2013. *Leukemia* 2013; 27(11): 2165–2176.
31. Milne TA, Briggs SD, Brock HW, Martin ME, Gibbs D, Allis CD, Hess JL. MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol Cell* 2002; 10(5): 1107–1117.
 32. Muntean AG and Hess JL. The pathogenesis of mixed-lineage leukemia. *Annu Rev Pathol* 2012; 7: 283–301.
 33. Mrozek K, Heinonen K, Lawrence D, Carroll AJ, Koduru PR, Rao KW, Strout MP, Hutchison RE, Moore JO, Mayer RJ, Schiffer CA, Bloomfield CD. Adult patients with de novo acute myeloid leukemia and t(9;11)(p22;q23) have a superior outcome to patients with other translocations involving band 11q23: a cancer and leukemia group B study. *Blood* 1997; 90(11): 4532–4538.
 34. Rubnitz JE, Raimondi SC, Tong X, Srivastava DK, Razzouk BI, Shurtliff SA, Downing JR, Pui CH, Ribeiro RC, Behm FG. Favorable impact of the t(9;11) in childhood acute myeloid leukemia. *J Clin Oncol* 2002; 20(9): 2302–2309.
 35. Hilden JM, Dinndorf PA, Meerbaum SO, Sather H, Villaluna D, Heerema NA, McGlennen R, Smith FO, Woods WG, Salzer WL, Johnstone HS, Dreyer Z, Reaman GH. Analysis of prognostic factors of acute lymphoblastic leukemia in infants: report on CCG 1953 from the Children's Oncology Group. *Blood* 2006; 108(2): 441–451.
 36. Cowell IG and Austin CA. Mechanism of generation of therapy related leukemia in response to anti-topoisomerase II agents. *Int J Environ Res Public Health* 2012; 9(6): 2075–2091.
 37. Godley LA and Larson RA. Therapy-related myeloid leukemia. *Semin Oncol* 2008; 35(4): 418–429.
 38. Bakshi RP, Galande S, Muniyappa K. Functional and regulatory characteristics of eukaryotic type II DNA topoisomerase. *Crit Rev Biochem Mol Biol* 2001; 36(1): 1–37.
 39. Muntean AG, Hess JL. The Pathogenesis of Mixed-Lineage Leukemia. *Annu Rev Pathol Mech Dis* 2012; 7: 283–301.
 40. Smith SM, Le Beau MM, Huo D, Garrison T, Sobecks RM, Anastasi J, Vardiman JW, Rowley JD, Larson RA. Clinical-cytogenetic associations in 306 patients with therapy-related myelodysplasia and myeloid leukemia: the University of Chicago series. *Blood* 2003; 102(1): 43–52.
 41. Larson RA. Etiology and management of therapy-related myeloid leukemia. *Hematology Am Soc Hematol Educ Program* 2007: 453–459.
 42. Aul C, Giagounidis A, Germing U. Epidemiological features of myelodysplastic syndromes: results from regional cancer surveys and hospital-based statistics. *Int J Hematol* 2001; 73(4): 405–410.
 43. Sole F, Espinet B, Sanz GF, Cervera J, Calasanz MJ, Luno E, Prieto F, Granada I, Hernandez JM, Cigudosa JC, Diez JL, Bureo E, Marques ML, Arranz E, Rios R, Martinez Climent JA, Vallespi T, Florensa L, Woessner S. Incidence, characterization and prognostic significance of chromosomal abnormalities in 640 patients with primary myelodysplastic syndromes. Grupo Cooperativo Espanol de Citogenetica Hematologica. *Br J Haematol* 2000; 108(2): 346–356.
 44. Greenberg PL, Tuechler H, Schanz J, Sanz G, Garcia-Manero G, Sole F, Bennett JM, Bowen D, Fenaux P, Dreyfus F, Kantarjian H, Kuendgen A, Levis A, Malcovati L, Cazzola M, Cermak J, Fonatsch C, Le Beau MM, Slovak ML, Krieger O, Luebbert M, Maciejewski J, Magalhaes SM, Miyazaki Y, Pfeilstocker M, Sekeres M, Sperr WR, Stauder R, Tauro S, Valent P, Vallespi T, van de Loosdrecht AA, Germing U, Haase D. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood* 2012; 120(12): 2454–2465.
 45. Rossi G, Pelizzari AM, Bellotti D, Tonelli M, Barlati S. Cytogenetic analogy between myelodysplastic syndrome and acute myeloid leukemia of elderly patients. *Leukemia* 2000; 14(4): 636–641.
 46. Boultwood J, Pellagatti A, McKenzie AN, Wainscoat JS. Advances in the 5q- syndrome. *Blood* 2010; 116(26): 5803–5811.
 47. Wang L, Fidler C, Nadig N, Giagounidis A, Della Porta MG, Malcovati L, Killick S, Gattermann N, Aul C, Boultwood J, Wainscoat JS. Genome-wide analysis of copy number changes and loss of heterozygosity in myelodysplastic syndrome with del(5q) using high-density single nucleotide polymorphism arrays. *Haematologica* 2008; 93(7): 994–1000.

48. Starczynowski DT, Kuchenbauer F, Argiopoulos B, Sung S, Morin R, Muranyi A, Hirst M, Hogge D, Marra M, Wells RA, Buckstein R, Lam W, Humphries RK, Karsan A. Identification of miR-145 and miR-146a as mediators of the 5q- syndrome phenotype. *Nat Med* 2010; 16(1): 49–58.
49. Fuchs O. Important genes in the pathogenesis of 5q- syndrome and their connection with ribosomal stress and the innate immune system pathway. *Leuk Res Treatment* 2012; 1–14.
50. List A, Dewald G, Bennett J, Giagounidis A, Raza A, Feldman E, Powell B, Greenberg P, Thomas D, Stone R, Reeder C, Wride K, Patin J, Schmidt M, Zeldis J, Knight R. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. *N Engl J Med* 2006; 355(14): 1456–1465.
51. Elghetany MT. Myelodysplastic syndromes in children: a critical review of issues in the diagnosis and classification of 887 cases from 13 published series. *Arch Pathol Lab Med* 2007; 131(7): 1110–1116.
52. Germing U, Aul C, Niemeyer CM, Haas R, Bennett JM. Epidemiology, classification and prognosis of adults and children with myelodysplastic syndromes. *Ann Hematol* 2008; 87(9): 691–699.
53. Najfeld V. Clonal origin of leukemia--revisited. A tribute to Philip J Fialkow, MD. *Leukemia* 1998; 12(2): 106–107.
54. Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, Deininger MW, Silver RT, Goldman JM, Stone RM, Cervantes F, Hochhaus A, Powell BL, Gabrilove JL, Rousselot P, Reiffers J, Cornelissen JJ, Hughes T, Agis H, Fischer T, Verhoef G, Shepherd J, Saglio G, Gratwohl A, Nielsen JL, Radich JP, Simonsson B, Taylor K, Baccarani M, So C, Letvak L, Larson RA. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med* 2006; 355(23): 2408–2417.
55. Hughes TP, Kaeda J, Branford S, Rudzki Z, Hochhaus A, Hensley ML, Gathmann I, Bolton AE, van Hoomissen IC, Goldman JM, Radich JP. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. *N Engl J Med* 2003; 349(15): 1423–1432.
56. Quintas-Cardama A, Kantarjian H, Talpaz M, O'Brien S, Garcia-Manero G, Verstovsek S, Rios MB, Hayes K, Glassman A, Bekele BN, Zhou X, Cortes J. Imatinib mesylate therapy may overcome the poor prognostic significance of deletions of derivative chromosome 9 in patients with chronic myelogenous leukemia. *Blood* 2005; 105(6): 2281–2286.
57. Guilbert-Douet N, Morel F, Le Bris MJ, Berthou C, Morice P, Bourquard P, Braekeleer MD. Clonal chromosomal abnormalities in the Philadelphia chromosome negative cells of chronic myeloid leukemia patients treated with imatinib. *Leukemia* 2004; 18(6): 1140–1142.
58. Griesshammer M, Heinze B, Bangerter M, Heimpel H, Fliedner TM. Karyotype abnormalities and their clinical significance in blast crisis of chronic myeloid leukemia. *J Mol Med* 1997; 75(11–12): 836–838.
59. Mohamed AN, Pemberton P, Zonder J, Schiffer CA. The effect of imatinib mesylate on patients with Philadelphia chromosome-positive chronic myeloid leukemia with secondary chromosomal aberrations. *Clin Cancer Res* 2003; 9(4): 1333–1337.
60. Haferlach C, Bacher U, Schnittger S, Weiss T, Kern W, Haferlach T. Similar patterns of chromosome abnormalities in CML occur in addition to the Philadelphia chromosome with or without tyrosine kinase inhibitor treatment. *Leukemia* 2010; 24(3): 638–640.
61. Parikh SA and Tefferi A. Chronic myelomonocytic leukemia: 2012 update on diagnosis, risk stratification, and management. *Am J Hematol* 2012; 87(6): 610–619.
62. Chronic myelomonocytic leukemia: single entity or heterogeneous disorder? A prospective multicenter study of 100 patients. Groupe Francais de Cytogenetique Hematologique. *Cancer Genet Cytogenet* 1991; 55(1): 57–65.
63. Fenaux P, Morel P, Lai JL. Cytogenetics of myelodysplastic syndromes. *Semin Hematol* 1996; 33(2): 127–138.
64. Hasle H, Arico M, Basso G, Biondi A, Cantu Rajnoldi A, Creutzig U, Fenu S, Fonatsch C, Haas OA, Harbott J, Kardos G, Kerndrup G, Mann G, Niemeyer CM, Ptoszkova H, Ritter J, Slater R, Stary J, Stollmann-Gibbels B, Testi AM, van Wering ER, Zimmermann M. Myelodysplastic syndrome, juvenile myelomonocytic leukemia, and acute myeloid leukemia associated with complete or partial monosomy 7. European Working Group on MDS in Childhood (EWOG-MDS). *Leukemia* 1999; 13(3): 376–385.
65. Such E, Cervera J, Costa D, Sole F, Vallespi T, Luno E, Collado R, Calasanz MJ, Hernandez-Rivas JM, Cigudosa JC, Nomdedeu B, Mallo M, Carbonell F, Bueno J, Ardanaz MT, Ramos F, Tormo M, Sancho-Tello R, del Canizo C, Gomez V, Marco V, Xicoy B, Bonanad S, Pedro C, Bernal T, Sanz GF. Cytogenetic risk stratification in chronic myelomonocytic leukemia. *Haematologica* 2011; 96(3): 375–383.

66. Chronic Myelomonocytic Leukemia (CMML) and Juvenile Myelomonocytic Leukemia (JMML). White Plains, NY: Leukemia & Lymphoma Society. <http://www.lls.org/content/nationalcontent/resourcecenter/freedownloadmaterials/leukemia/pdf/cmmljmml.pdf>.
67. Tefferi A and Vardiman JW. Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia* 2008; 22(1): 14–22.
68. Fruchtman SM, Mack K, Kaplan ME, Peterson P, Berk PD, Wasserman LR. From efficacy to safety: a Polycythemia Vera Study group report on hydroxyurea in patients with polycythemia vera. *Semin Hematol* 1997; 34(1): 17–23.
69. Najeau Y and Rain JD. The very long-term evolution of polycythemia vera: an analysis of 318 patients initially treated by phlebotomy or 32P between 1969 and 1981. *Semin Hematol* 1997; 34(1): 6–16.
70. Mesa RA, Silverstein MN, Jacobsen SJ, Wollan PC, Tefferi A. Population-based incidence and survival figures in essential thrombocythemia and agnogenic myeloid metaplasia: an Olmsted County Study, 1976–1995. *Am J Hematol* 1999; 61(1): 10–15.
71. Murphy S. Diagnostic criteria and prognosis in polycythemia vera and essential thrombocythemia. *Semin Hematol* 1999; 36(1 Suppl 2): 9–13.
72. Furgerson JL, Vukelja SJ, Baker WJ, O'Rourke TJ. Acute myeloid leukemia evolving from essential thrombocythemia in two patients treated with hydroxyurea. *Am J Hematol* 1996; 51(2): 137–140.
73. Caramazza D, Begna KH, Gangat N, Vaidya R, Siragusa S, Van Dyke DL, Hanson C, Pardanani A, Tefferi A. Refined cytogenetic-risk categorization for overall and leukemia-free survival in primary myelofibrosis: a single center study of 433 patients. *Leukemia* 2011; 25(1): 82–88.
74. Matano S, Nakamura S, Kobayashi K, Yoshida T, Matsuda T, Sugimoto T. Deletion of the long arm of chromosome 20 in a patient with chronic neutrophilic leukemia: cytogenetic findings in chronic neutrophilic leukemia. *Am J Hematol* 1997; 54(1): 72–75.
75. Pellier I, Le Moine PJ, Rialland X, Francois S, Baranger L, Blanchet O, Larget-Piet L, Ifrah N. Myelodysplastic syndrome with t(5;12)(q31;p12-p13) and eosinophilia: a pediatric case with review of literature. *J Pediatr Hematol Oncol* 1996; 18(3): 285–288.
76. Ma SK, Kwong YL, Shek TW, Wan TS, Chow EY, Chan JC, Chan LC. The role of trisomy 8 in the pathogenesis of chronic eosinophilic leukemia. *Hum Pathol* 1999; 30(7): 864–868.
77. Lepretre S. Eosinophilia in leukemias: a probable leukemic clone. *Haematologica* 2002; 87(8): 785–786.
78. Pardanani A, Ketterling RP, Brockman SR, Flynn HC, Paternoster SF, Shearer BM, Reeder TL, Li CY, Cross NC, Cools J, Gilliland DG, Dewald GW, Tefferi A. CHIC2 deletion, a surrogate for FIP1L1-PDGFR α fusion, occurs in systemic mastocytosis associated with eosinophilia and predicts response to imatinib mesylate therapy. *Blood* 2003; 102(9): 3093–3096.
79. Klion AD, Noel P, Akin C, Law MA, Gilliland DG, Cools J, Metcalfe DD, Nutman TB. Elevated serum tryptase levels identify a subset of patients with a myeloproliferative variant of idiopathic hypereosinophilic syndrome associated with tissue fibrosis, poor prognosis, and imatinib responsiveness. *Blood* 2003; 101(12): 4660–4666.
80. Cools J, DeAngelo DJ, Gotlib J, Stover EH, Legare RD, Cortes J, Kutok J, Clark J, Galinsky I, Griffin JD, Cross NC, Tefferi A, Malone J, Alam R, Schrier SL, Schmid J, Rose M, Vandenbergh P, Verhoef G, Boogaerts M, Wlodarska I, Kantarjian H, Marynen P, Coutre SE, Stone R, Gilliland DG. A tyrosine kinase created by fusion of the PDGFR α and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. *N Engl J Med* 2003; 348(13): 1201–1214.
81. Vandenbergh P, Wlodarska I, Michaux L, Zachee P, Boogaerts M, Van Straelen D, Herregods MC, Van Hoof A, Selleslag D, Roufosse F, Maerevoet M, Verhoef G, Cools J, Gilliland DG, Hagemeijer A, Marynen P. Clinical and molecular features of FIP1L1-PDGFR α (+) chronic eosinophilic leukemias. *Leukemia* 2004; 18(4): 734–742.
82. Apperley JF, Gardembas M, Melo JV, Russell-Jones R, Bain BJ, Baxter EJ, Chase A, Chessells JM, Colombe M, Dearden CE, Dimitrijevic S, Mahon FX, Marin D, Nikolova Z, Olavarria E, Silberman S, Schultheis B, Cross NC, Goldman JM. Response to imatinib mesylate in patients with chronic myeloproliferative diseases with rearrangements of the platelet-derived growth factor receptor beta. *N Engl J Med* 2002; 347(7): 481–487.
83. Harrison CJ. The management of patients with leukaemia: the role of cytogenetics in this molecular era. *Br J Haematol* 2000; 108(1): 19–30.

84. Moorman AV. The clinical relevance of chromosomal and genomic abnormalities in B-cell precursor acute lymphoblastic leukaemia. *Blood Rev* 2012; 26(3): 123–135.
85. Moorman AV, Chilton L, Wilkinson J, Ensor HM, Bown N, Proctor SJ. A population-based cytogenetic study of adults with acute lymphoblastic leukemia. *Blood* 2010; 115(2): 206–214.
86. Third International Workshop on Chromosomes in Leukemia, 1980 (1981): clinical significance of chromosomal abnormalities in acute lymphoblastic leukemia. *Cancer Genet Cytogenet* 1981; 4: 111–137.
87. Heerema NA, Sather HN, Sensel MG, Zhang T, Hutchinson RJ, Nachman JB, Lange BJ, Steinherz PG, Bostrom BC, Reaman GH, Gaynon PS, Uckun FM. Prognostic impact of trisomies of chromosomes 10, 17, and 5 among children with acute lymphoblastic leukemia and high hyperdiploidy (>50 chromosomes). *J Clin Oncol* 2000; 18(9): 1876–1887.
88. Trueworthy R, Shuster J, Look T, Crist W, Borowitz M, Carroll A, Frankel L, Harris M, Wagner H, Haggard M, Mosijszuk A, Pullen J, Steuber P, Land V. Ploidy of lymphoblasts is the strongest predictor of treatment outcome in B-progenitor cell acute lymphoblastic leukemia of childhood: a Pediatric Oncology Group study. *J Clin Oncol* 1992; 10(4): 606–613.
89. Heerema N, Sather H, Reaman G, Hutchinson R, Lange B, Nachman J, Stinherz P, Uckun F, Gaynon P, Trigg M, Arthur D. Cytogenetic studies of acute lymphoblastic leukemia: clinical correlations results from the children's cancer group. *Journal of Association of Genetic Technologists* 1998; 24: 206–212.
90. Secker-Walker LM, Swansbury GJ, Lawler SD, Hardisty RM. Bone marrow chromosomes in acute lymphoblastic leukaemia: a long-term study. *Med Pediatr Oncol* 1979; 7(4): 371–385.
91. Sutcliffe MJ, Shuster JJ, Sather HN, Camitta BM, Pullen J, Schultz KR, Borowitz MJ, Gaynon PS, Carroll AJ, Heerema NA. High concordance from independent studies by the Children's Cancer Group (CCG) and Pediatric Oncology Group (POG) associating favorable prognosis with combined trisomies 4, 10, and 17 in children with NCI Standard-Risk B-precursor Acute Lymphoblastic Leukemia: a Children's Oncology Group (COG) initiative. *Leukemia* 2005; 19(5): 734–740.
92. Moorman AV, Richards SM, Martineau M, Cheung KL, Robinson HM, Jalali GR, Broadfield ZJ, Harris RL, Taylor KE, Gibson BE, Hann IM, Hill FG, Kinsey SE, Eden TO, Mitchell CD, Harrison CJ. Outcome heterogeneity in childhood high-hyperdiploid acute lymphoblastic leukemia. *Blood* 2003; 102(8): 2756–2762.
93. Nachman J, Heerema N, Sather H, Camitta B, Forestier E, Eden T, Otten J, Schrappe M, Pui C-H, Basso G, Silverman L, Janka-Schaub G. Outcome of treatment in children with hypodiploid acute lymphoblastic leukemia. *Blood* 2007; 110(4): 1112–1115.
94. Holmfeldt L, Wei L, Diaz-Flores E, Walsh M, Zhang J, Ding L, Payne-Turner D, Churchman M, Andersson A, Chen SC, McCastlain K, Becksfort J, Ma J, Wu G, Patel SN, Heatley SL, Phillips LA, Song G, Easton J, Parker M, Chen X, Rusch M, Boggs K, Vadodaria B, Hedlund E, Drenberg C, Baker S, Pei D, Cheng C, Huether R, Lu C, Fulton RS, Fulton LL, Tabib Y, Dooling DJ, Ochoa K, Minden M, Lewis ID, To LB, Marlton P, Roberts AW, Raca G, Stock W, Neale G, Drexler HG, Dickins RA, Ellison DW, Shurtliff SA, Pui CH, Ribeiro RC, Devidas M, Carroll AJ, Heerema NA, Wood B, Borowitz MJ, Gastier-Foster JM, Raimondi SC, Mardis ER, Wilson RK, Downing JR, Hunger SP, Loh ML, Mullighan CG. The genomic landscape of hypodiploid acute lymphoblastic leukemia. *Nature Genetics* 2013; 45(3): 242–252.
95. Maia AT, Tussiwand R, Cazzaniga G, Rebulla P, Colman S, Biondi A, Greaves M. Identification of preleukemic precursors of hyperdiploid acute lymphoblastic leukemia in cord blood. *Genes Chromosomes Cancer* 2004; 40(1): 38–43.
96. Uckun FM, Herman-Hatten K, Crotty ML, Sensel MG, Sather HN, Tuel-Ahlgren L, Sarquis MB, Bostrom B, Nachman JB, Steinherz PG, Gaynon PS, Heerema N. Clinical significance of MLL-AF4 fusion transcript expression in the absence of a cytogenetically detectable t(4;11)(q21;q23) chromosomal translocation. *Blood* 1998; 92(3): 810–821.
97. Paulsson K, Panagopoulos I, Knuutila S, Jee KJ, Garwicz S, Fioretos T, Mitelman F, Johansson B. Formation of trisomies and their parental origin in hyperdiploid childhood acute lymphoblastic leukemia. *Blood* 2003; 102(8): 3010–3015.
98. Paulsson K, Morse H, Fioretos T, Behrendtz M, Strombeck B, Johansson B. Evidence for a single-step mechanism in the origin of hyperdiploid childhood acute lymphoblastic leukemia. *Genes Chromosomes Cancer* 2005; 44(2): 113–122.
99. Panzer-Grumayer ER, Fasching K, Panzer S, Hettinger K, Schmitt K, Stockler-Ipsiroglu S, Haas OA. Nondisjunction of chromosomes leading to hyperdiploid childhood B-cell precursor acute lymphoblastic leukemia is an early event during leukemogenesis. *Blood* 2002; 100(1): 347–349.

100. Taub JW, Konrad MA, Ge Y, Naber JM, Scott JS, Matherly LH, Ravindranath Y. High frequency of leukemic clones in newborn screening blood samples of children with B-precursor acute lymphoblastic leukemia. *Blood* 2002; 99(8): 2992–2996.
101. McHale CM and Smith MT. Prenatal origin of chromosomal translocations in acute childhood leukemia: implications and future directions. *Am J Hematol* 2004; 75(4): 254–257.
102. Pui CH, Gaynon PS, Boyett JM, Chessells JM, Baruchel A, Kamps W, Silverman LB, Biondi A, Harms DO, Vilmer E, Schrappe M, Camitta B. Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. *Lancet* 2002; 359(9321): 1909–1915.
103. Pui CH, Chessells JM, Camitta B, Baruchel A, Biondi A, Boyett JM, Carroll A, Eden OB, Evans WE, Gadner H, Harbott J, Harms DO, Harrison CJ, Harrison PL, Heerema N, Janka-Schaub G, Kamps W, Masera G, Pullen J, Raimondi SC, Richards S, Riehm H, Sallan S, Sather H, Shuster J, Silverman LB, Valsecchi MG, Vilmer E, Zhou Y, Gaynon PS, Schrappe M. Clinical heterogeneity in childhood acute lymphoblastic leukemia with 11q23 rearrangements. *Leukemia* 2003; 17(4): 700–706.
104. Shurtleff S, Buijs A, Behm F, Raimondi S, Hancock M, Chan G-F, Pui C, Grosveld G, Downing J. TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. *Leukemia* 1995; 9: 1985–1989.
105. Uckun FM, Pallisgaard N, Hokland P, Navara C, Narla R, Gaynon PS, Sather H, Heerema N. Expression of TEL-AML1 fusion transcripts and response to induction therapy in standard risk acute lymphoblastic leukemia. *Leuk Lymphoma* 2001; 42(1–2): 41–56.
106. Romana SP, Poirel H, Leconiat M, Flexor MA, Mauchauffe M, Jonveaux P, Macintyre EA, Berger R, Bernard OA. High frequency of t(12;21) in childhood B-lineage acute lymphoblastic leukemia. *Blood* 1995; 86(11): 4263–4269.
107. Rubnitz JE, Downing JR, Pui CH, Shurtleff SA, Raimondi SC, Evans WE, Head DR, Crist WM, Rivera GK, Hancock ML, Boyett JM, Buijs A, Grosveld G, Behm FG. TEL gene rearrangement in acute lymphoblastic leukemia: a new genetic marker with prognostic significance. *J Clin Oncol* 1997; 15(3): 1150–1157.
108. Hubner S, Cazzaniga G, Flohr T, van der Velden VH, Konrad M, Potschger U, Basso G, Schrappe M, van Dongen JJ, Bartram CR, Biondi A, Panzer-Grumayer ER. High incidence and unique features of antigen receptor gene rearrangements in TEL-AML1-positive leukemias. *Leukemia* 2004; 18(1): 84–91.
109. Raynaud S, Cave H, Baens M, Bastard C, Cacheux V, Grosgeorge J, Guidal-Giroux C, Guo C, Vilmer E, Marynen P, Grandchamp B. The 12;21 translocation involving TEL and deletion of the other TEL allele: two frequently associated alterations found in childhood acute lymphoblastic leukemia. *Blood* 1996; 87(7): 2891–2899.
110. Jabber Al-Obaidi MS, Martineau M, Bennett CF, Franklin IM, Goldstone AH, Harewood L, Jalali GR, Prentice HG, Richards SM, Roberts K, Harrison CJ. ETV6/AML1 fusion by FISH in adult acute lymphoblastic leukemia. *Leukemia* 2002; 16(4): 669–674.
111. Aguiar RC, Sohal J, van Rhee F, Carapeti M, Franklin IM, Goldstone AH, Goldman JM, Cross NC. TEL-AML1 fusion in acute lymphoblastic leukaemia of adults. M.R.C. Adult Leukaemia Working Party. *Br J Haematol* 1996; 95(4): 673–677.
112. Maloney K, McGavran L, Murphy J, Odom L, Stork L, Wei Q, Hunger S. TEL-AML1 fusion identifies a subset of children with standard risk acute lymphoblastic leukemia who have an excellent prognosis when treated with therapy that includes a single delayed intensification. *Leukemia* 1999; 13: 1708–1712.
113. Rafi SK, El Gebaly H, Qumsiyeh MB. ETV6/CBFA2 fusions in childhood B-cell precursor acute lymphoblastic leukemia with myeloid markers. *Diagn Mol Pathol* 2000; 9(4): 184–189.
114. Borowitz MJ, Rubnitz J, Nash M, Pullen DJ, Camitta B. Surface antigen phenotype can predict TEL-AML1 rearrangement in childhood B-precursor ALL: a Pediatric Oncology Group study. *Leukemia* 1998; 12(11): 1764–1770.
115. Baruchel A, Cayuela JM, Ballerini P, Landman-Parker J, Cezard V, Firat H, Haddad E, Auclerc MF, Valensi F, Cayre YE, Macintyre EA, Sigaux F. The majority of myeloid-antigen-positive (My+) childhood B-cell precursor acute lymphoblastic leukaemias express TEL-AML1 fusion transcripts. *Br J Haematol* 1997; 99(1): 101–106.
116. McHale CM, Wiemels JL, Zhang L, Ma X, Buffler PA, Guo W, Loh ML, Smith MT. Prenatal origin of TEL-AML1-positive acute lymphoblastic leukemia in children born in California. *Genes Chromosomes Cancer* 2003; 37(1): 36–43.
117. Wiemels JL, Cazzaniga G, Daniotti M, Eden OB, Addison GM, Masera G, Saha V, Biondi A, Greaves MF. Prenatal origin of acute lymphoblastic leukaemia in children. *Lancet* 1999; 354(9189): 1499–1503.

118. Raynaud SD, Dastugue N, Zoccola D, Shurtliff SA, Mathew S, Raimondi SC. Cytogenetic abnormalities associated with the t(12;21): a collaborative study of 169 children with t(12;21)-positive acute lymphoblastic leukemia. *Leukemia* 1999; 13(9): 1325–1330.
119. Zuna J, Ford AM, Peham M, Patel N, Saha V, Eckert C, Kochling J, Panzer-Grumayer R, Trka J, Greaves M. TEL deletion analysis supports a novel view of relapse in childhood acute lymphoblastic leukemia. *Clin Cancer Res* 2004; 10(16): 5355–5360.
120. Romana SP, Le Coniat M, Poirel H, Marynen P, Bernard O, Berger R. Deletion of the short arm of chromosome 12 is a secondary event in acute lymphoblastic leukemia with t(12;21). *Leukemia* 1996; 10(1): 167–170.
121. Douet-Guilbert N, Morel F, Le Bris MJ, Herry A, Le Calvez G, Marion V, Abgrall JF, Berthou C, De Brackeleer M. A fluorescence in situ hybridization study of TEL-AML1 fusion gene in B-cell acute lymphoblastic leukemia (1984–2001). *Cancer Genet Cytogenet* 2003; 144(2): 143–147.
122. Crist W, Carroll A, Shuster J, Jackson J, Head D, Borowitz M, Behm F, Link M, Streuber P, Ragab A. Philadelphia chromosome positive childhood acute lymphoblastic leukemia: clinical and cytogenetic characteristics and treatment outcome. A Pediatric Oncology Group study. *Blood* 1990; 76(3): 489–494.
123. Secker-Walker LM, Prentice HG, Durrant J, Richards S, Hall E, Harrison G. Cytogenetics adds independent prognostic information in adults with acute lymphoblastic leukaemia on MRC trial UKALL XA. MRC Adult Leukaemia Working Party. *Br J Haematol* 1997; 96(3): 601–610.
124. Wetzel M, Dodge RK, Mrozek K, Carroll AJ, Tantravahi R, Block AW, Pettenati MJ, Le Beau MM, Frankel SR, Stewart CC, Szatrowski TP, Schiffer CA, Larson RA, Bloomfield CD. Prospective karyotype analysis in adult acute lymphoblastic leukemia: the cancer and leukemia Group B experience. *Blood* 1999; 93(11): 3983–3993.
125. Uckun F, Nachman J, Sather H, Sensel M, Kraft P, Steinherz P, Lange B, Hutchinson R, Reaman G, Gaynon P, Heerema N. Poor treatment outcome of Philadelphia chromosome-positive pediatric acute lymphoblastic leukemia despite intensive chemotherapy. *Leukemia and Lymphoma* 1999; 34(14): 998–1006.
126. Melo JV. The molecular biology of chronic myeloid leukaemia. *Leukemia* 1996; 10(5): 751–756.
127. Wetzel M, Dodge RK, Mrozek K, Stewart CC, Carroll AJ, Tantravahi R, Vardiman JW, Larson RA, Bloomfield CD. Additional cytogenetic abnormalities in adults with Philadelphia chromosome-positive acute lymphoblastic leukaemia: a study of the Cancer and Leukaemia Group B. *Br J Haematol* 2004; 24(3): 275–288.
128. Heerema NA, Harbott J, Galimberti S, Camitta BM, Gaynon PS, Janka-Schaub G, Kamps W, Bassi G, Pui CH, Schrappe M, Auclerc MF, Carroll AJ, Conter V, Harrison CJ, Pullen J, Raimondi SC, Richards S, Riehm H, Sather HN, Shuster JJ, Silverman LB, Valsecchi MG, Arico M. Secondary cytogenetic aberrations in childhood Philadelphia chromosome positive acute lymphoblastic leukemia are nonrandom and may be associated with outcome. *Leukemia* 2004; 18(4): 693–702.
129. Uckun FM, Nachman JB, Sather HN, Sensel MG, Kraft P, Steinherz PG, Lange B, Hutchinson R, Reaman GH, Gaynon PS, Heerema NA. Clinical significance of Philadelphia chromosome positive pediatric acute lymphoblastic leukemia in the context of contemporary intensive therapies: a report from the Children's Cancer Group. *Cancer* 1998; 83(9): 2030–2039.
130. Arico M VM, Camitta B, Scaple M, Chessells J, Baruchel A, Gaynon P, Silverman L, Janka-Schaub G, Kamps W, Pui C-H, Masera G, Conter V, Riehm H, Heerema N, Sallan S, Auclerc M-F, Pullen J, Shuster J, Carroll A, Raimondi S, Richards S. Outcome of treatment in children with Philadelphia chromosome-positive acute lymphoblastic leukemia. *New England Journal of Medicine* 2000; 342: 998–1006.
131. Schultz KR, Bowman WP, Aledo A, Slayton WB, Sather H, Devidas M, Wang C, Davies SM, Gaynon PS, Trigg M, Rutledge R, Burden L, Jorstad D, Carroll A, Heerema NA, Winick N, Borowitz MJ, Hunger SP, Carroll WL, Camitta B. Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. *J Clin Oncol* 2009; 27(31): 5175–5181.
132. Uckun FM, Sensel MG, Sather HN, Gaynon PS, Arthur DC, Lange BJ, Steinherz PG, Kraft P, Hutchinson R, Nachman JB, Reaman GH, Heerema NA. Clinical significance of translocation t(1;19) in childhood acute lymphoblastic leukemia in the context of contemporary therapies: a report from the Children's Cancer Group. *J Clin Oncol* 1998; 16(2): 527–535.
133. Pui CH, Raimondi SC, Hancock ML, Rivera GK, Ribeiro RC, Mahmoud HH, Sandlund JT, Crist WM, Behm FG. Immunologic, cytogenetic, and clinical characterization of childhood acute lymphoblastic leukemia with the t(1;19) (q23; p13) or its derivative. *J Clin Oncol* 1994; 12(12): 2601–2606.

134. Hunger SP, Galili N, Carroll AJ, Crist WM, Link MP, Cleary ML. The t(1;19)(q23;p13) results in consistent fusion of E2A and PBX1 coding sequences in acute lymphoblastic leukemias. *Blood* 1991; 77(4): 687–693.
135. Monica K, LeBrun DP, Dedera DA, Brown R, Cleary ML. Transformation properties of the E2a-Pbx1 chimeric oncprotein: fusion with E2a is essential, but the Pbx1 homeodomain is dispensable. *Mol Cell Biol* 1994; 14(12): 8304–8314.
136. Devaraj PE, Foroni L, Secker-Walker LM. The detection of E2A rearrangement or E2A-PBX1 fusion transcript in t(1;19) leukemia. *Genes Chromosomes Cancer* 1995; 12(2): 157–158.
137. Kantarjian HM, O'Brien S, Smith TL, Cortes J, Giles FJ, Beran M, Pierce S, Huh Y, Andreeff M, Koller C, Ha CS, Keating MJ, Murphy S, Freireich EJ. Results of treatment with hyper-CVAD, a dose-intensive regimen, in adult acute lymphocytic leukemia. *J Clin Oncol* 2000; 18(3): 547–561.
138. Hunger SP, Sun T, Boswell AF, Carroll AJ, McGavran L. Hyperdiploidy and E2A-PBX1 fusion in an adult with t(1;19) + acute lymphoblastic leukemia: case report and review of the literature. *Genes Chromosomes Cancer* 1997; 20(4): 392–398.
139. van Zutven LJ, van Drunen E, de Bont JM, Wattel MM, Den Boer ML, Pieters R, Hagemeijer A, Slater RM, Beverloo HB. CDKN2 deletions have no prognostic value in childhood precursor-B acute lymphoblastic leukaemia. *Leukemia* 2005; 19(7): 1281–1284.
140. Chessells JM, Hall E, Prentice HG, Durrant J, Bailey CC, Richards SM. The impact of age on outcome in lymphoblastic leukaemia; MRC UKALL X and XA compared: a report from the MRC Paediatric and Adult Working Parties. *Leukemia* 1998; 12(4): 463–473.
141. Heerema N, Sather H, Sensel M, Liu-Mares W, Lange B, Bostrom B, Nachman J, Steinherz P, Hutchinson R, Gaynon P, Arthur D, Uckun F. Association of chromosome arm 9p abnormalities with adverse risk in childhood acute lymphoblastic leukemia: A report from the Children's Cancer Group. *Blood* 1999; 94(5): 1537–1544.
142. Calero Moreno TM, Gustafsson G, Garwicz S, Grander D, Jonmundsson GK, Frost BM, Makipernaa A, Rasool O, Savolainen ER, Schmiegelow K, Soderhall S, Vettentranta K, Wesenberg F, Einhorn S, Heyman M. Deletion of the Ink4-locus (the p16ink4a, p14ARF and p15ink4b genes) predicts relapse in children with ALL treated according to the Nordic protocols NOPHO-86 and NOPHO-92. *Leukemia* 2002; 16(10): 2037–2045.
143. Behrendt H, Charrin C, Gibbons B, Harrison CJ, Hawkins JM, Heerema NA, Horschler-Botel B, Huret JL, Lai JL, Lampert F, Nelken B, Perot C, Ritterbach J, Schlegelberger B, Secker-Walker LM, Slater S, Slovak ML, Tanzer J, Van den Akker J. Dicentric (9;12) in acute lymphocytic leukemia and other hematological malignancies: report from a dic(9;12) study group. *Leukemia* 1995; 9(1): 102–106.
144. Clark R, Byatt SA, Bennett CF, Brama M, Martineau M, Moorman AV, Roberts K, Secker-Walker LM, Richards S, Eden OB, Goldstone AH, Harrison CJ. Monosomy 20 as a pointer to dicentric (9;20) in acute lymphoblastic leukemia. *Leukemia* 2000; 14(2): 241–246.
145. Zachariadis V, Schoumans J, Ofverholm I, Barbany G, Halvardsson E, Forestier E, Johansson B, Nordenskjold M, Nordgren A. Detecting dic(9;20)(p13.2;p11.2)-positive B-cell precursor acute lymphoblastic leukemia in a clinical setting using fluorescence in situ hybridization. *Leukemia* 2014;28(1):196–198.
146. Heerema NA, Maben KD, Bernstein J, Breitfeld PP, Neiman RS, Vance GH. Dicentric (9;20)(p11;q11) identified by fluorescence in situ hybridization in four pediatric acute lymphoblastic leukemia patients. *Cancer Genet Cytogenet* 1996; 92(2): 111–115.
147. Zachariadis V, Gauffin F, Kuchinskaya E, Heyman M, Schoumans J, Blennow E, Gustafsson B, Barbany G, Golovleva I, Ehrencrona H, Cavelier L, Palmqvist L, Lonnerholm G, Nordenskjold M, Johansson B, Forestier E, Nordgren A. The frequency and prognostic impact of dic(9;20)(p13.2;q11.2) in childhood B-cell precursor acute lymphoblastic leukemia: results from the NOPHO ALL-2000 trial. *Leukemia* 2011; 25(4): 622–628.
148. An Q, Wright SL, Konn ZJ, Matheson E, Minto L, Moorman AV, Parker H, Griffiths M, Ross FM, Davies T, Hall AG, Harrison CJ, Irving JA, Strefford JC. Variable breakpoints target PAX5 in patients with dicentric chromosomes: a model for the basis of unbalanced translocations in cancer. *Proc Natl Acad Sci USA* 2008; 105(44): 17050–17054.
149. Ensor HM, Schwab C, Russell LJ, Richards SM, Morrison H, Masic D, Jones L, Kinsey SE, Vora AJ, Mitchell CD, Harrison CJ, Moorman AV. Demographic, clinical, and outcome features of children with acute lymphoblastic leukemia and CRLF2 deregulation: results from the MRC ALL97 clinical trial. *Blood* 2011; 117(7): 2129–2136.

150. Mullighan CG, Collins-Underwood JR, Phillips LA, Loudin MG, Liu W, Zhang J, Ma J, Coustan-Smith E, Harvey RC, Willman CL, Mikhail FM, Meyer J, Carroll AJ, Williams RT, Cheng J, Heerema NA, Basso G, Pession A, Pui CH, Raimondi SC, Hunger SP, Downing JR, Carroll WL, Rabin KR. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. *Nat Genet* 2009; 41(11): 1243–1246.
151. Russell LJ, Capasso M, Vater I, Akasaka T, Bernard OA, Calasanz MJ, Chandrasekaran T, Chapiro E, Gesk S, Griffiths M, Gutierrez DS, Haferlach C, Harder L, Heidenreich O, Irving J, Kearney L, Nguyen-Khac F, Machado L, Minto L, Majid A, Moorman AV, Morrison H, Rand V, Strefford JC, Schwab C, Tonnes H, Dyer MJ, Siebert R, Harrison CJ. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. *Blood* 2009; 114(13): 2688–2698.
152. Heerema NA, Carroll AJ, Devidas M, Loh ML, Borowitz MJ, Gastier-Foster JM, Larsen EC, Mattano LA, Jr., Maloney KW, Willman CL, Wood BL, Winick NJ, Carroll WL, Hunger SP, Raetz EA. Intrachromosomal Amplification of Chromosome 21 Is Associated With Inferior Outcomes in Children With Acute Lymphoblastic Leukemia Treated in Contemporary Standard-Risk Children's Oncology Group Studies: A Report From the Children's Oncology Group. *J Clin Oncol* 2013; 31(27): 3397–3402.
153. Moorman AV, Robinson H, Schwab C, Richards SM, Hancock J, Mitchell CD, Goulden N, Vora A, Harrison CJ. Risk-Directed Treatment Intensification Significantly Reduces the Risk of Relapse Among Children and Adolescents With Acute Lymphoblastic Leukemia and Intrachromosomal Amplification of Chromosome 21: A Comparison of the MRC ALL97/99 and UKALL2003 Trials. *J Clin Oncol* 2013; 31(27): 3389–3396.
154. Rand V, Parker H, Russell LJ, Schwab C, Ensor H, Irving J, Jones L, Masic D, Minto L, Morrison H, Ryan S, Robinson H, Sinclair P, Moorman AV, Strefford JC, Harrison CJ. Genomic characterization implicates iAMP21 as a likely primary genetic event in childhood B-cell precursor acute lymphoblastic leukemia. *Blood* 2011; 117(25): 6848–6855.
155. Heerema NA, Sather HN, Sensel MG, Kraft P, Nachman JB, Steinherz PG, Lange BJ, Hutchinson RS, Reaman GH, Trigg ME, Arthur DC, Gaynon PS, Uckun FM. Frequency and clinical significance of cytogenetic abnormalities in pediatric T-lineage acute lymphoblastic leukemia: a report from the Children's Cancer Group. *J Clin Oncol* 1998; 16(4): 1270–1278.
156. Uckun FM, Gaynon PS, Sensel MG, Nachman J, Trigg ME, Steinherz PG, Hutchinson R, Bostrom BC, Sather HN, Reaman GH. Clinical features and treatment outcome of childhood T-lineage acute lymphoblastic leukemia according to the apparent maturational stage of T-lineage leukemic blasts: a Children's Cancer Group study. *J Clin Oncol* 1997; 15(6): 2214–2221.
157. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med* 2004; 350(15): 1535–1548.
158. Harrison CJ and Foroni L. Cytogenetics and molecular genetics of acute lymphoblastic leukemia. *Rev Clin Exp Hematol* 2002; 6(2): 91–113; discussion 200–112.
159. Graux C, Cools J, Michaux L, Vandenberghe P, Hagemeijer A. Cytogenetics and molecular genetics of T-cell acute lymphoblastic leukemia: from thymocyte to lymphoblast. *Leukemia* 2006; 20(9): 1496–1510.
160. Cauwelier B, Dastugue N, Cools J, Poppe B, Herens C, De Paepe A, Hagemeijer A, Speleman F. Molecular cytogenetic study of 126 unselected T-ALL cases reveals high incidence of TCRbeta locus rearrangements and putative new T-cell oncogenes. *Leukemia* 2006; 20(7): 1238–1244.
161. Kees UR, Heerema NA, Kumar R, Watt PM, Baker DL, La MK, Uckun FM, Sather HN. Expression of HOX11 in childhood T-lineage acute lymphoblastic leukaemia can occur in the absence of cytogenetic aberration at 10q24: a study from the Children's Cancer Group (CCG). *Leukemia* 2003; 17(5): 887–893.
162. Janssen JW, Ludwig WD, Sterry W, Bartram CR. SIL-TAL1 deletion in T-cell acute lymphoblastic leukemia. *Leukemia* 1993; 7(8): 1204–1210.
163. Brown L, Cheng JT, Chen Q, Siciliano MJ, Crist W, Buchanan G, Baer R. Site-specific recombination of the tal-1 gene is a common occurrence in human T cell leukemia. *EMBO J* 1990; 9(10): 3343–3351.
164. Barber KE, Martineau M, Harewood L, Stewart M, Cameron E, Strefford JC, Rutherford S, Allen TD, Broadfield ZJ, Cheung KL, Harris RL, Jalali GR, Moorman AV, Robinson HM, Harrison CJ. Amplification of the ABL gene in T-cell acute lymphoblastic leukemia. *Leukemia* 2004; 18(6): 1153–1156.
165. Graux C, Cools J, Melotte C, Quentmeier H, Ferrando A, Levine R, Vermeesch JR, Stul M, Dutta B, Boeckx N, Bosly A, Heimann P, Uyttebroeck A, Mentens N, Somers R, MacLeod RA, Drexler HG, Look AT, Gilliland DG, Michaux L, Vandenberghe P, Wlodarska I, Marynen P, Hagemeijer A. Fusion of NUP214 to ABL1 on amplified episomes in T-cell acute lymphoblastic leukemia. *Nat Genet* 2004; 36(10): 1084–1089.

166. Matutes E. T-cell Prolymphocytic Leukemia. *Cancer Control Journal* 1998; 5(1): 19–24.
167. Maljaei SH, Brito-Babapulle V, Hiorns LR, Catovsky D. Abnormalities of chromosomes 8, 11, 14, and X in T-prolymphocytic leukemia studied by fluorescence in situ hybridization. *Cancer Genet Cytogenet* 1998; 103(2): 110–116.
168. Itoyama T, Chaganti RS, Yamada Y, Tsukasaki K, Atogami S, Nakamura H, Tomonaga M, Ohshima K, Kikuchi M, Sadamori N. Cytogenetic analysis and clinical significance in adult T-cell leukemia/lymphoma: a study of 50 cases from the human T-cell leukemia virus type-1 endemic area, Nagasaki. *Blood* 2001; 97(11): 3612–3620.
169. Tsukasaki K, Krebs J, Nagai K, Tomonaga M, Koeffler HP, Bartram CR, Jauch A. Comparative genomic hybridization analysis in adult T-cell leukemia/lymphoma: correlation with clinical course. *Blood* 2001; 97(12): 3875–3881.
170. Raval A, Tanner SM, Byrd JC, Angerman EB, Perko JD, Chen SS, Hackanson B, Grever MR, Lucas DM, Matkovic JJ, Lin TS, Kipps TJ, Murray F, Weisenburger D, Sanger W, Lynch J, Watson P, Jansen M, Yoshinaga Y, Rosenquist R, de Jong PJ, Coggill P, Beck S, Lynch H, de la Chapelle A, Plass C. Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. *Cell* 2007; 129(5): 879–890.
171. Dohner H, Stilgenbauer S, Dohner K, Bentz M, Lichter P. Chromosome aberrations in B-cell chronic lymphocytic leukemia: reassessment based on molecular cytogenetic analysis. *J Mol Med* 1999; 77(2): 266–281.
172. Dohner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L, Dohner K, Bentz M, Lichter P. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000; 343(26): 1910–1916.
173. Dicker F, Schnittger S, Haferlach T, Kern W, Schoch C. Immunostimulatory oligonucleotide-induced metaphase cytogenetics detect chromosomal aberrations in 80% of CLL patients: A study of 132 CLL cases with correlation to FISH, IgVH status, and CD38 expression. *Blood* 2006; 108(9): 3152–3160.
174. Mayr C, Speicher MR, Kofler DM, Buhmann R, Strehl J, Busch R, Hallek M, Wendtner CM. Chromosomal translocations are associated with poor prognosis in chronic lymphocytic leukemia. *Blood* 2006; 107(2): 742–751.
175. Muthusamy N, Breidenbach H, Andritsos L, Flynn J, Jones J, Ramanunni A, Mo X, Jarjoura D, Byrd JC, Heerema NA. Enhanced detection of chromosomal abnormalities in chronic lymphocytic leukemia by conventional cytogenetics using CpG oligonucleotide in combination with pokeweed mitogen and phorbol myristate acetate. *Cancer Genet* 2011; 204(2): 77–83.
176. Put N, Konings P, Rack K, Jamar M, Van Roy N, Libouton JM, Vannuffel P, Sartenaer D, Ameye G, Speleman F, Herens C, Poirel HA, Moreau Y, Hagemeijer A, Vandenberghe P, Michaux L. Improved detection of chromosomal abnormalities in chronic lymphocytic leukemia by conventional cytogenetics using CpG oligonucleotide and interleukin-2 stimulation: A Belgian multicentric study. *Genes Chromosomes Cancer* 2009; 48(10): 843–853.
177. Struski S, Gervais C, Helias C, Herbrecht R, Audhuy B, Mauvieux L. Stimulation of B-cell lymphoproliferations with CpG-oligonucleotide DSP30 plus IL-2 is more effective than with TPA to detect clonal abnormalities. *Leukemia* 2009; 23(3): 617–619.
178. Calin GA, Cimmino A, Fabbri M, Ferracin M, Wojcik SE, Shimizu M, Taccioli C, Zanesi N, Garzon R, Aqeilan RI, Alder H, Volinia S, Rassenti L, Liu X, Liu CG, Kipps TJ, Negrini M, Croce CM. MiR-15a and miR-16–1 cluster functions in human leukemia. *Proc Natl Acad Sci USA* 2008; 105(13): 5166–5171.
179. Byrd JC, Smith L, Hackbarth ML, Flinn IW, Young D, Proffitt JH, Heerema NA. Interphase cytogenetic abnormalities in chronic lymphocytic leukemia may predict response to rituximab. *Cancer Res* 2003; 63(1): 36–38.
180. Lin TS, Flinn IW, Lucas MS, Porcu P, Sickler J, Moran ME, Lucas DM, Heerema NA, Grever MR, Byrd JC. Filgrastim and alemtuzumab (Campath-1H) for refractory chronic lymphocytic leukemia. *Leukemia* 2005; 19(7): 1207–1210.
181. Byrd JC, Gribben JG, Peterson BL, Grever MR, Lozanski G, Lucas DM, Lampson B, Larson RA, Caligiuri MA, Heerema NA. Select high-risk genetic features predict earlier progression following chemoimmunotherapy with fludarabine and rituximab in chronic lymphocytic leukemia: justification for risk-adapted therapy. *J Clin Oncol* 2006; 24(3): 437–443.
182. Dearden C. B- and T-cell prolymphocytic leukemia: antibody approaches. *Hematology Am Soc Hematol Educ Program* 2012; 2012: 645–651.
183. Sambani C, Trafalis DT, Mitsoulis-Mentzikoff C, Poulakidas E, Makropoulos V, Pantelias GE, Mecucci C. Clonal chromosome rearrangements in hairy cell leukemia: personal experience and review of literature. *Cancer Genet Cytogenet* 2001; 129(2): 138–144.

184. Kawano Y, Fujiwara S, Wada N, Izaki M, Yuki H, Okuno Y, Iyama K, Yamasaki H, Sakai A, Mitsuya H, Hata H. Multiple myeloma cells expressing low levels of CD138 have an immature phenotype and reduced sensitivity to lenalidomide. *Int J Oncol* 2012; 41(3): 876–884.
185. Kyle RA and Rajkumar SV. Multiple myeloma. *Blood* 2008; 111(6): 2962–2972.
186. Lai JL, Zandecki M, Mary JY, Bernardi F, Izquierdo V, Flactif M, Morel P, Jouet JP, Bauters F, Facon T. Improved cytogenetics in multiple myeloma: a study of 151 patients including 117 patients at diagnosis. *Blood* 1995; 85(9): 2490–2497.
187. Brigaudeau C, Trimoreau F, Gachard N, Rouzier E, Jaccard A, Bordessoule D, Praloran V. Cytogenetic study of 30 patients with multiple myeloma: comparison of 3 and 6 day bone marrow cultures stimulated or not with cytokines by using a miniaturized karyotypic method. *Br J Haematol* 1997; 96(3): 594–600.
188. Nilsson T, Lenhoff S, Turesson I, Rylander L, Mitelman F, Westin J, Hoglund M, Johansson B. Cytogenetic features of multiple myeloma: impact of gender, age, disease phase, culture time, and cytokine stimulation. *Eur J Haematol* 2002; 68(6): 345–353.
189. Hernandez JM, Gutierrez NC, Almeida J, Garcia JL, Sanchez MA, Mateo G, Rios A, San Miguel JF. IL-4 improves the detection of cytogenetic abnormalities in multiple myeloma and increases the proportion of clonally abnormal metaphases. *Br J Haematol* 1998; 103(1): 163–167.
190. Badros AZ. In the age of novel therapies, what defines high-risk multiple myeloma? *J Natl Compr Canc Netw* 2010; 8(Suppl. 1): S28–34.
191. Munshi NC, Anderson KC, Bergsagel PL, Shaughnessy J, Palumbo A, Durie B, Fonseca R, Stewart AK, Harousseau JL, Dimopoulos M, Jagannath S, Hajek R, Sezer O, Kyle R, Sonneveld P, Cavo M, Rajkumar SV, San Miguel J, Crowley J, Avet-Loiseau H. Consensus recommendations for risk stratification in multiple myeloma: report of the International Myeloma Workshop Consensus Panel 2. *Blood* 2011; 117(18): 4696–4700.
192. Rajkumar SV. Multiple myeloma: 2013 update on diagnosis, risk-stratification, and management. *Am J Hematol* 2013; 88(3): 226–235.
193. Fonseca R, Bergsagel PL, Drach J, Shaughnessy J, Gutierrez N, Stewart AK, Morgan G, Van Ness B, Chesi M, Minvielle S, Neri A, Barlogie B, Kuehl WM, Liebisch P, Davies F, Chen-Kiang S, Durie BG, Carrasco R, Sezer O, Reiman T, Pilarski L, Avet-Loiseau H. International Myeloma Working Group molecular classification of multiple myeloma: spotlight review. *Leukemia* 2009; 23(12): 2210–2221.
194. Fonseca R, Bailey RJ, Ahmann GJ, Rajkumar SV, Hoyer JD, Lust JA, Kyle RA, Gertz MA, Greipp PR, Dewald GW. Genomic abnormalities in monoclonal gammopathy of undetermined significance. *Blood* 2002; 100(4): 1417–1424.
195. Slovak ML, Bedell V, Pagel K, Chang KL, Smith D, Somlo G. Targeting plasma cells improves detection of cytogenetic aberrations in multiple myeloma: phenotype/genotype fluorescence in situ hybridization. *Cancer Genet Cytogenet* 2005; 158(2): 99–109.
196. Bergsagel PL and Kuehl WM. Molecular pathogenesis and a consequent classification of multiple myeloma. *J Clin Oncol* 2005; 23(26): 6333–6338.
197. Harrison CJ, Mazzullo H, Ross FM, Cheung KL, Gerrard G, Harewood L, Mehta A, Lachmann HJ, Hawkins PN, Orchard KH. Translocations of 14q32 and deletions of 13q14 are common chromosomal abnormalities in systemic amyloidosis. *Br J Haematol* 2002; 117(2): 427–435.
198. Hayman SR, Bailey RJ, Jalal SM, Ahmann GJ, Dispenzieri A, Gertz MA, Greipp PR, Kyle RA, Lacy MQ, Rajkumar SV, Witzig TE, Lust JA, Fonseca R. Translocations involving the immunoglobulin heavy-chain locus are possible early genetic events in patients with primary systemic amyloidosis. *Blood* 2001; 98(7): 2266–2268.
199. Zhang HW, Cheng NL, Chen ZW, Wang JF, Li SH, Bai W. Clinical impact of t(14;18) in diffuse large B-cell lymphoma. *Chin J Cancer Res* 2011; 23(2): 160–164.
200. Dimopoulos MA, Panayiotidis P, Moulopoulos LA, Sfikakis P, Dalakas M. Waldenstrom's macroglobulinemia: clinical features, complications, and management. *J Clin Oncol* 2000; 18(1): 214–226.
201. Schop RF, Van Wier SA, Xu R, Ghobrial I, Ahmann GJ, Greipp PR, Kyle RA, Dispenzieri A, Lacy MQ, Rajkumar SV, Gertz MA, Fonseca R. 6q deletion discriminates Waldenstrom macroglobulinemia from IgM monoclonal gammopathy of undetermined significance. *Cancer Genet Cytogenet* 2006; 169(2): 150–153.

202. Nguyen-Khac F, Lambert J, Chapiro E, Grelier A, Mould S, Barin C, Daudignon A, Gachard N, Struski S, Henry C, Penther D, Mossafa H, Andrieux J, Eclache V, Bilhou-Nabera C, Luquet I, Terre C, Baranger L, Mugneret F, Chiesa J, Mozziconacci MJ, Callet-Bauchu E, Veronese L, Blons H, Owen R, Lejeune J, Chevret S, Merle-Beral H, Leblondon V. Chromosomal aberrations and their prognostic value in a series of 174 untreated patients with Waldenstrom's macroglobulinemia. *Haematologica* 2013; 98(4): 649–654.
203. Bastard C, Deweindt C, Kerckaert JP, Lenormand B, Rossi A, Pezzella F, Fruchart C, Duval C, Monconduit M, Tilly H. LAZ3 rearrangements in non-Hodgkin's lymphoma: correlation with histology, immunophenotype, karyotype, and clinical outcome in 217 patients. *Blood* 1994; 83(9): 2423–2427.
204. Offit K, Lo Coco F, Louie DC, Parsa NZ, Leung D, Portlock C, Ye BH, Lista F, Filippa DA, Rosenbaum A, Ladanyi M, Jhanwar SC, Dalla-Favera R, Chaganti RSK. Rearrangement of the bcl-6 gene as a prognostic marker in diffuse large-cell lymphoma. *N Engl J Med* 1994; 331(2): 74–80.
205. Ye BH, Lista F, Lo Coco F, Knowles DM, Offit K, Chaganti RS, Dalla-Favera R. Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large-cell lymphoma. *Science* 1993; 262(5134): 747–750.
206. Horn H, Ziepert M, Becher C, Barth TF, Bernd HW, Feller AC, Klapper W, Hummel M, Stein H, Hansmann ML, Schmelzter C, Moller P, Cogliatti S, Pfleiderer M, Schmitz N, Trumper L, Siebert R, Loeffler M, Rosenwald A, Ott G. MYC status in concert with BCL2 and BCL6 expression predicts outcome in diffuse large B-cell lymphoma. *Blood* 2013; 121(12): 2253–2263.
207. Dave SS, Wright G, Tan B, Rosenwald A, Gascoyne RD, Chan WC, Fisher RI, Braziel RM, Rimsza LM, Grogan TM, Miller TP, LeBlanc M, Greiner TC, Weisenburger DD, Lynch JC, Vose J, Armitage JO, Smeland EB, Kvaloy S, Holte H, Delabie J, Connors JM, Lansdorp PM, Ouyang Q, Lister TA, Davies AJ, Norton AJ, Muller-Hermelink HK, Ott G, Campo E, Montserrat E, Wilson WH, Jaffe ES, Simon R, Yang L, Powell J, Zhao H, Goldschmidt N, Chiorazzi M, Staudt LM. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. *N Engl J Med* 2004; 351(21): 2159–2169.
208. Offit K and Chaganti RS. Chromosomal aberrations in non-Hodgkin's lymphoma. Biologic and clinical correlations. *Hematol Oncol Clin North Am* 1991; 5(5): 853–869.
209. Roulland S, Lebailly P, Lecluse Y, Heutte N, Nadel B, Gauduchon P. Long-term clonal persistence and evolution of t(14;18)-bearing B cells in healthy individuals. *Leukemia* 2006; 20(1): 158–162.
210. de Jong D. Molecular pathogenesis of follicular lymphoma: a cross talk of genetic and immunologic factors. *J Clin Oncol* 2005; 23(26): 6358–6363.
211. Mantle Cell Lymphoma Facts No. 4 in a series providing the latest information for patients, caregivers and healthcare professionals. Leukemia & Lymphoma Society: White Plains, NY. <http://www.lls.org/content/nationalcontent/resourcecenter/freeeducationmaterials/lymphoma/pdf/mantlecelllymphoma.pdf>
212. Cuneo A, Bigoni R, Rigolin GM, Roberti MG, Bardi A, Piva N, Milani R, Bullrich F, Veronese ML, Croce C, Birg F, Dohner H, Hagemeijer A, Castoldi G. Cytogenetic profile of lymphoma of follicle mantle lineage: correlation with clinicobiologic features. *Blood* 1999; 93(4): 1372–1380.
213. de Boer CJ, van Krieken JH, Schuurings E, Kluin PM. Bcl-1/cyclin D1 in malignant lymphoma. *Annals of Oncology* 1997; 8(Suppl 2): 109–117.
214. Bouffet E, Frappaz D, Pinkerton R, Favrot M, Philip T. Burkitt's lymphoma: a model for clinical oncology. *Eur J Cancer* 1991; 27(4): 504–509.
215. Preudhomme C, Dervite I, Wattel E, Vanrumbeke M, Flactif M, Lai JL, Hecquet B, Coppin MC, Nelken B, Gosselin B, Fenaux P. Clinical significance of p53 mutations in newly diagnosed Burkitt's lymphoma and acute lymphoblastic leukemia: a report of 48 cases. *J Clin Oncol* 1995; 13(4): 812–820.
216. Soussain C, Patte C, Ostronoff M, Delmer A, Rigal-Huguet F, Cambier N, Leprise PY, Francois S, Cony-Makhoul P, Harousseau JL, Janvier M, Chauvenet L, Witz F, Jose P. Small noncleaved cell lymphoma and leukemia in adults. A retrospective study of 65 adults treated with the LMB pediatric protocols. *Blood* 1995; 85(3): 664–674.
217. ar-Rushdi A, Nishikura K, Erikson J, Watt R, Rovera G, Croce CM. Differential expression of the translocated and the untranslocated c-myc oncogene in Burkitt lymphoma. *Science* 1983; 222(4622): 390–393.
218. Thieblemont C. Clinical presentation and management of marginal zone lymphomas. *Hematology Am Soc Hematol Educ Program* 2005: 307–313.

219. Zinzani PL. The many faces of marginal zone lymphoma. *Hematology Am Soc Hematol Educ Program* 2012; 426–432.
220. Aukema SM, Siebert R, Schuuring E, van Imhoff GW, Kluin-Nelemans HC, Boerma EJ, Kluin PM. Double-hit B-cell lymphomas. *Blood* 2011; 117(8): 2319–2331.
221. Schlegelberger B, Weber-Mathiesen K, Himmeler A, Bartels H, Sonnen R, Kuse R, Feller AC, Grote W. Cytogenetic findings and results of combined immunophenotyping and karyotyping in Hodgkin's disease. *Leukemia* 1994; 8(1): 72–80.
222. Joos S, Granzow M, Holtgreve-Grez H, Siebert R, Harder L, Martin-Subero JI, Wolf J, Adamowicz M, Barth TF, Lichter P, Jauch A. Hodgkin's lymphoma cell lines are characterized by frequent aberrations on chromosomes 2p and 9p including REL and JAK2. *Int J Cancer* 2003; 103(4): 489–495.
223. Drexler HG. Recent results on the biology of Hodgkin and Reed-Sternberg cells. I. Biopsy material. *Leuk Lymphoma* 1992; 8(4–5): 283–313.
224. Martin-Subero JI, Klapper W, Sotnikova A, Callet-Bauchu E, Harder L, Bastard C, Schmitz R, Grohmann S, Hoppner J, Riemke J, Barth TF, Berger F, Bernd HW, Claviez A, Gesk S, Frank GA, Kaplanskaya IB, Moller P, Parwaresch RM, Rudiger T, Stein H, Kuppers R, Hansmann ML, Siebert R. Chromosomal breakpoints affecting immunoglobulin loci are recurrent in Hodgkin and Reed-Sternberg cells of classical Hodgkin lymphoma. *Cancer Res* 2006; 66(21): 10332–10338.
225. Ohno H, Ueda C, Akasaka T. The t(9;14)(p13;q32) translocation in B-cell non-Hodgkin's lymphoma. *Leuk Lymphoma* 2000; 36(5–6): 435–445.
226. Benharoch D, Meguerian-Bedoyan Z, Lamant L, Amin C, Brugieres L, Terrier-Lacombe MJ, Haralambieva E, Pulford K, Pileri S, Morris SW, Mason DY, Delsol G. ALK-positive lymphoma: a single disease with a broad spectrum of morphology. *Blood* 1998; 91(6): 2076–2084.
227. Skinnider BF, Connors JM, Sutcliffe SB, Gascoyne RD. Anaplastic large cell lymphoma: a clinicopathologic analysis. *Hematol Oncol* 1999; 17(4): 137–148.
228. Morris SW, Kirstein MN, Valentine MB, Dittmer K, Shapiro DN, Look AT, Saltman DL. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. *Science* 1995; 267(5196): 316–317.
229. Pulford K, Morris SW, Turturro F. Anaplastic lymphoma kinase proteins in growth control and cancer. *J Cell Physiol* 2004; 199(3): 330–358.
230. Gascoyne RD, Aoun P, Wu D, Chhanabhai M, Skinnider BF, Greiner TC, Morris SW, Connors JM, Vose JM, Viswanatha DS, Coldman A, Weisenburger DD. Prognostic significance of anaplastic lymphoma kinase (ALK) protein expression in adults with anaplastic large cell lymphoma. *Blood* 1999; 93(11): 3913–3921.
231. Attard-Montalto SP, Saha V, Norton AJ, Kingston JE, Eden OB. Anaplastic large cell lymphoma in childhood. *Med Pediatr Oncol* 1993; 21(9): 665–669; discussion 669–670.
232. Vecchi V, Burnelli R, Pileri S, Rosito P, Sabattini E, Civino A, Pericoli R, Paolucci G. Anaplastic large cell lymphoma (Ki-1+/CD30+) in childhood. *Med Pediatr Oncol* 1993; 21(6): 402–410.
233. Romaguera JE, Manning JT Jr, Tornos CS, Rodriguez J, Brooks TE, Pugh WC, Ordonez NG, Goodacre AM, Cabanillas F. Long-term prognostic importance of primary Ki-1 (CD30) antigen expression and anaplastic morphology in adult patients with diffuse large-cell lymphoma. *Ann Oncol* 1994; 5(4): 317–322.
234. Sandlund JT, Pui CH, Roberts WM, Santana VM, Morris SW, Berard CW, Hutchison RE, Ribeiro RC, Mahmoud H, Crist WM, Heim M, Raimondi SC. Clinicopathologic features and treatment outcome of children with large-cell lymphoma and the t(2;5)(p23;q35). *Blood* 1994; 84(8): 2467–2471.
235. Siegel RS, Pandolfino T, Guitart J, Rosen S, Kuzel TM. Primary cutaneous T-cell lymphoma: review and current concepts. *J Clin Oncol* 2000; 18(15): 2908–2925.
236. Zackheim HS, Amin S, Kashani-Sabet M, McMillan A. Prognosis in cutaneous T-cell lymphoma by skin stage: long-term survival in 489 patients. *J Am Acad Dermatol* 1999; 40(3): 418–425.
237. Thangavelu M, Finn WG, Yelavarthi KK, Roenigk HH, Jr., Samuelson E, Peterson L, Kuzel TM, Rosen ST. Recurring structural chromosome abnormalities in peripheral blood lymphocytes of patients with mycosis fungoides/Sezary syndrome. *Blood* 1997; 89(9): 3371–3377.
238. Karenko L, Hytytinne E, Sarna S, Ranki A. Chromosomal abnormalities in cutaneous T-cell lymphoma and in its premalignant conditions as detected by G-banding and interphase cytogenetic methods. *J Invest Dermatol* 1997; 108(1): 22–29.

239. Hirsh B, Brothman AR, Jacky PB, Rao KW, Wolff DJ. Section E6 of the ACMG technical standards and guidelines: chromosome studies for acquired abnormalities. *Genet Med* 2005; 7(7): 509–513.
240. Dewald G, Ketterling RP, Wyatt WA, Stupca PJ. Cytogenetic Studies in Neoplastic Hematologic Disorders in McClatchey KD, ed. *Clinical Laboratory Medicine* 2nd ed 2001; Philadelphia: Lippincott, Williams and Wilkins.
241. Howlader N, Noone AM, Krapcho M, Miller D, Bishop K, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer Statistics Review, 1975–2013, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2013/, based on November 2015 SEER data submission, posted to the SEER web site, April 2016.
242. Jaffe E, Harris N, Stein H, Vardiman J, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues*. 2001; Lyon: IARC Press.
243. Balgobind BV, Raimondi SC, Harbott J, Zimmermann M, Alonso TA, Aufrignon A, Beverloo HB, Chang M, Creutzig U, Dworzak MN, Forestier E, Gibson B, Hasle H, Harrison CJ, Heerema NA, Kaspers GJ, Leszl A, Litvinko N, Nigro LL, Morimoto A, Perot C, Pieters R, Reinhardt D, Rubnitz JE, Smith FO, Stary J, Stasevich I, Strehl S, Taga T, Tomizawa D, Webb D, Zemanova Z, Zwaan CM, van den Heuvel-Eibrink MM. Novel prognostic subgroups in childhood 11q23/MLL-rearranged acute myeloid leukemia: results of an international retrospective study. *Blood* 2009; 114(12): 2489–2496.
244. Slovak ML, Gundacker H, Bloomfield CD, Dewald G, Appelbaum FR, Larson RA, Tallman MS, Bennett JM, Stirewalt DL, Meshinchi S, Willman CL, Ravindranath Y, Alonso TA, Carroll AJ, Raimondi SC, Heerema NA. A retrospective study of 69 patients with t(6;9)(p23;q34) AML emphasizes the need for a prospective, multicenter initiative for rare ‘poor prognosis’ myeloid malignancies. *Leukemia* 2006; 20(7): 1295–1297.
245. Tasaka T, Matsuhashi Y, Uehara E, Tamura T, Kakazu N, Abe T, Nagai M. Secondary acute monocytic leukemia with a translocation t(8;16)(p11;p13): case report and review of the literature. *Leuk Lymphoma* 2004; 45(3): 621–625.
246. Fleischman EW, Reshmi S, Frenkel MA, Konovalova WI, Guleva GP, Kulagina OE, Konstantinova LN, Tupitsyn NN, Rowley JD. MLL is involved in a t(2;11)(p21;q23) in a patient with acute myeloblastic leukemia. *Genes Chromosomes Cancer* 1999; 24(2): 151–155.
247. Labis E. t(11;16)(q23;p13.3). *Atlas Genet Cytogenet Oncol Haematol* 2009: <http://AtlasGeneticsOncology.org/Anomalies/t1116q23p13ID1120.html>.
248. Peniket A, Wainscoat J, Side L, Daly S, Kusec R, Buck G, Wheatley K, Walker H, Chatters S, Harrison C, Boultwood J, Goldstone A, Burnett A. del(9q) AML: clinical and cytological characteristics and prognostic implications. *Br J Haematol* 2005; 129(2): 210–220.
249. Bernstein J, Dastugue N, Haas OA, Harbott J, Heerema NA, Huret JL, Landman-Parker J, LeBeau MM, Leonard C, Mann G, Pages MP, Perot C, Pirc-Danoewinata H, Roitzheim B, Rubin CM, Slociak M, Viguerie F. Nineteen cases of the t(1;22)(p13;q13) acute megakaryoblastic leukaemia of infants/children and a review of 39 cases: report from a t(1;22) study group. *Leukemia* 2000; 14(1): 216–218.
250. Slater RM, von Drunen E, Kroes WG, Weghuis DO, van den Berg E, Smit EM, van der Does-van den Berg A, van Wering E, Hahlen K, Carroll AJ, Raimondi SC, Beverloo HB. t(7;12)(q36;p13) and t(7;12)(q32;p13)—translocations involving ETV6 in children 18 months of age or younger with myeloid disorders. *Leukemia* 2001; 15(6): 915–920.
251. Sanada M, Uike N, Ohyashiki K, Ozawa K, Lili W, Hangaishi A, Kanda Y, Chiba S, Kurokawa M, Omine M, Mitani K, Ogawa S. Unbalanced translocation der(1;7)(q10;p10) defines a unique clinicopathological subgroup of myeloid neoplasms. *Leukemia* 2007; 21(5): 992–997.
252. Slovak ML, O'Donnell M, Smith DD, Gaal K. Does MDS with der(1;7)(q10;p10) constitute a distinct risk group? A retrospective single institutional analysis of clinical/pathologic features compared to -7/del(7q) MDS. *Cancer Genet Cytogenet* 2009; 193(2): 78–85.
253. Loss of the Y chromosome from normal and neoplastic bone marrows. United Kingdom Cancer Cytogenetics Group (UKCCG). *Genes Chromosomes Cancer* 1992; 5(1): 83–88.
254. Wiktor A, Rybicki BA, Piao ZS, Shurafa M, Barthel B, Maeda K, Van Dyke DL. Clinical significance of Y chromosome loss in hematologic disease. *Genes Chromosomes Cancer* 2000; 27(1): 11–16.
255. Rubnitz JE, Camitta BM, Mahmoud H, Raimondi SC, Carroll AJ, Borowitz MJ, Shuster JJ, Link MP, Pullen DJ, Downing JR, Behm FG, Pui CH. Childhood acute lymphoblastic leukemia with the MLL-ENL fusion and t(11;19)(q23;p13.3) translocation. *J Clin Oncol* 1999; 17(1): 191–196.

256. Heerema NA, Sather HN, Sensel MG, Lee MK, Hutchinson R, Lange BJ, Bostrom BC, Nachman JB, Steinherz PG, Gaynon PS, Uckun FM. Clinical significance of deletions of chromosome arm 6q in childhood acute lymphoblastic leukemia: a report from the Children's Cancer Group. *Leuk Lymphoma* 2000; 36(5–6): 467–478.
257. Russell LJ, Akasaka T, Majid A, Sugimoto KJ, Loraine Karan E, Nagel I, Harder L, Claviez A, Gesk S, Moorman AV, Ross F, Mazzullo H, Strefford JC, Siebert R, Dyer MJ, Harrison CJ. t(6;14)(p22;q32): a new recurrent IGH@ translocation involving ID4 in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). *Blood* 2008; 111(1): 387–391.
258. Heerema NA, Nachman JB, Sather HN, La MK, Hutchinson R, Lange BJ, Bostrom B, Steinherz PG, Gaynon PS, Uckun FM. Deletion of 7p or monosomy 7 in pediatric acute lymphoblastic leukemia is an adverse prognostic factor: a report from the Children's Cancer Group. *Leukemia* 2004; 18(5): 939–947.
259. Familiades J, Bousquet M, Lafage-Pochitaloff M, Bene MC, Beldjord K, De Vos J, Dastugue N, Coyaud E, Struski S, Quelen C, Prade-Houdellier N, Dobbeltstein S, Cayuela JM, Soulier J, Grardel N, Preudhomme C, Cave H, Blanchet O, Lheritier V, Delannoy A, Chalandon Y, Ifrah N, Pigneux A, Brousset P, Macintyre EA, Huguet F, Dombret H, Broccardo C, Delabesse E. PAX5 mutations occur frequently in adult B-cell progenitor acute lymphoblastic leukemia and PAX5 haploinsufficiency is associated with BCR-ABL1 and TCF3-PBX1 fusion genes: a GRAALL study. *Leukemia* 2009; 23(11): 1989–1998.
260. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, Girtman K, Mathew S, Ma J, Pounds SB, Su X, Pui CH, Relling MV, Evans WE, Shurtleff SA, Downing JR. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 2007; 446(7137): 758–764.
261. Heerema NA, Sather HN, Sensel MG, Lee MK, Hutchinson R, Nachman JB, Lange BJ, Steinherz PG, Bostrom B, Gaynon PS, Uckun FM. Prognostic significance of cytogenetic abnormalities of chromosome arm 12p in childhood acute lymphoblastic leukemia: a report from the Children's Cancer Group. *Cancer* 2000; 88(8): 1945–1954.
262. Lundin C, Heidenblad M, Strombeck B, Borg A, Hovland R, Heim S, Johansson B. Tiling resolution array CGH of dic(7;9)(p11 approximately 13;p11 approximately 13) in B-cell precursor acute lymphoblastic leukemia reveals clustered breakpoints at 7p11.2 approximately 12.1 and 9p13.1. *Cytogenet Genome Res* 2007; 118(1): 13–18.
263. Gastier-Foster JM, Carroll AJ, Ell D, Harvey R, Chen I-M, Ketterling R, Meloni-Ehrig A, Opheim KE, Patil S, Pettenati M, Rao K, Wu S, Heerema NA. Two Distinct Subsets of dic(9;12)(p12;p11.2) among Children with B-Cell Precursor Acute Lymphoblastic Leukemia (ALL): PAX5-ETV6 and ETV6-RUNX1 Rearrangements: A Report from the Children's Oncology Group. *ASH Annual Meeting Abstracts* 2007; 110(11): 1439.
264. Strehl S, Konig M, Dworzak MN, Kalwak K, Haas OA. PAX5/ETV6 fusion defines cytogenetic entity dic(9;12) (p13;p13). *Leukemia* 2003; 17(6): 1121–1123.
265. Messinger YH, Higgins RR, Devidas M, Hunger SP, Carroll AJ, Heerema NA. Pediatric acute lymphoblastic leukemia with a t(8;14)(q11.2;q32): B-cell disease with a high proportion of Down syndrome: a Children's Oncology Group study. *Cancer Genet* 2012; 205(9): 453–458.
266. Huret JL. t(5;14)(q31;q32). *Atlas Genet Cytogenet Oncol Haematol* 1999; <http://AtlasGeneticsOncology.org/Anomalies/t514ID1111.html>.
267. Moorman AV, Richards SM, Robinson HM, Strefford JC, Gibson BE, Kinsey SE, Eden TO, Vora AJ, Mitchell CD, Harrison CJ. Prognosis of children with acute lymphoblastic leukemia (ALL) and intrachromosomal amplification of chromosome 21 (iAMP21). *Blood* 2007; 109(6): 2327–2330.
268. Hebert J, Cayuela J, Berkeley J, Sigaux F. Candidate tumor-suppressor genes MTS1 (p16INK4A) and MTS2 (p15INK4B) display frequent homozygous deletions in primary cells from T- but not from B-cell lineage acute lymphoblastic leukemias. *Blood* 1994; 84(12): 4038–4044.
269. Soulier J, Clappier E, Cayuela JM, Regnault A, Garcia-Peydro M, Dombret H, Baruchel A, Toribio ML, Sigaux F. HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). *Blood* 2005; 106(1): 274–286.
270. Bash RO, Crist WM, Shuster JJ, Link MP, Amylon M, Pullen J, Carroll AJ, Buchanan GR, Smith RG, Baer R. Clinical features and outcome of T-cell acute lymphoblastic leukemia in childhood with respect to alterations at the TAL1 locus: a Pediatric Oncology Group study. *Blood* 1993; 81(8): 2110–2117.
271. Clappier E, Cuccuini W, Kalota A, Crinquette A, Cayuela JM, Dik WA, Langerak AW, Montpellier B, Nadel B, Walrafen P, Delattre O, Aurias A, Leblanc T, Dombret H, Gewirtz AM, Baruchel A, Sigaux F, Soulier J. The C-MYB

- locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. *Blood* 2007; 110(4): 1251–1261.
272. Boyer J. t(8;14)(q24;q11). *Atlas Genet Cytogenet Oncol Haematol* 2001; <http://AtlasGeneticsOncology.org/Anomalies/t0814ID1061.html>.
 273. Ferrando AA, Neuberg DS, Dodge RK, Paietta E, Larson RA, Wiernik PH, Rowe JM, Caligiuri MA, Bloomfield CD, Look AT. Prognostic importance of TLX1 (HOX11) oncogene expression in adults with T-cell acute lymphoblastic leukaemia. *Lancet* 2004; 363(9408): 535–536.
 274. Marks DI, Paietta EM, Moorman AV, Richards SM, Buck G, DeWald G, Ferrando A, Fielding AK, Goldstone AH, Ketterling RP, Litzow MR, Luger SM, McMillan AK, Mansour MR, Rowe JM, Tallman MS, Lazarus HM. T-cell acute lymphoblastic leukemia in adults: clinical features, immunophenotype, cytogenetics, and outcome from the large randomized prospective trial (UKALL XII/ECOG 2993). *Blood* 2009; 114(25): 5136–5145.
 275. Boehm T, Foroni L, Kaneko Y, Perutz MF, Rabbits TH. The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. *Proc Natl Acad Sci USA* 1991; 88(10): 4367–4371.
 276. Ballerini P, Blaise A, Busson-Le Coniat M, Su XY, Zucman-Rossi J, Adam M, van den Akker J, Perot C, Pellegrino B, Landman-Parker J, Douay L, Berger R, Bernard OA. HOX11L2 expression defines a clinical subtype of pediatric T-ALL associated with poor prognosis. *Blood* 2002; 100(3): 991–997.
 277. Cave H, Suciu S, Preudhomme C, Poppe B, Robert A, Uyttebroeck A, Malet M, Boutard P, Benoit Y, Mauvieux L, Lutz P, Mechinaud F, Grardel N, Mazingue F, Dupont M, Margueritte G, Pages MP, Bertrand Y, Plouvier E, Brunie G, Bastard C, Plantaz D, Vande Velde I, Hagemeijer A, Speleman F, Lessard M, Otten J, Vilmer E, Dastugue N. Clinical significance of HOX11L2 expression linked to t(5;14)(q35;q32), of HOX11 expression, and of SIL-TAL fusion in childhood T-cell malignancies: results of EORTC studies 58881 and 58951. *Blood* 2004; 103(2): 442–450.
 278. van Grotel M, Meijerink JP, Beverloo HB, Langerak AW, Buys-Gladdines JG, Schneider P, Poulsen TS, den Boer ML, Horstmann M, Kamps WA, Veerman AJ, van Wering ER, van Noesel MM, Pieters R. The outcome of molecular-cytogenetic subgroups in pediatric T-cell acute lymphoblastic leukemia: a retrospective study of patients treated according to DCOG or COALL protocols. *Haematologica* 2006; 91(9): 1212–1221.
 279. Ballerini P, Busson M, Fasola S, van den Akker J, Lapillonne H, Romana SP, Marynen P, Bernard OA, Landman-Parker J, Berger R. NUP214-ABL1 amplification in t(5;14)/HOX11L2-positive ALL present with several forms and may have a prognostic significance. *Leukemia* 2005; 19(3): 468–470.
 280. Dreyling MH, Schrader K, Fonatsch C, Schlegelberger B, Haase D, Schoch C, Ludwig W, Loffler H, Buchner T, Wormann B, Hiddemann W, Bohlander SK. MLL and CALM are fused to AF10 in morphologically distinct subsets of acute leukemia with translocation t(10;11): both rearrangements are associated with a poor prognosis. *Blood* 1998; 91(12): 4662–4667.
 281. Asnafi V, Radford-Weiss I, Dastugue N, Bayle C, Leboeuf D, Charrin C, Garand R, Lafage-Pochitaloff M, Delabesse E, Buzyn A, Troussard X, Macintyre E. CALM-AF10 is a common fusion transcript in T-ALL and is specific to the TCRgammadelta lineage. *Blood* 2003; 102(3): 1000–1006.
 282. Graux C, Stevens-Kroef M, Lafage M, Dastugue N, Harrison CJ, Mugneret F, Bahloul K, Struski S, Gregoire MJ, Nadal N, Lippert E, Taviaux S, Simons A, Kuiper RP, Moorman AV, Barber K, Bosly A, Michaux L, Vandenberghe P, Lahortiga I, De Keersmaecker K, Wlodarska I, Cools J, Hagemeijer A, Poirel HA. Heterogeneous patterns of amplification of the NUP214-ABL1 fusion gene in T-cell acute lymphoblastic leukemia. *Leukemia* 2009; 23(1): 125–133.
 283. Cripe LD and Hromas R. Malignant disorders of megakaryocytes. *Semin Hematol* 1998; 35(3): 200–209.
 284. Cervantes F, Barosi G, Demory JL, Reilly J, Guarnone R, Dupriez B, Pereira A, Montserrat E. Myelofibrosis with myeloid metaplasia in young individuals: disease characteristics, prognostic factors and identification of risk groups. *Br J Haematol* 1998; 102(3): 684–690.
 285. Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G, Sanz M, Vallespi T, Hamblin T, Oscier D, Ohyashiki K, Toyama K, Aul C, Mufti G, Bennett J. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood* 1997; 89(6): 2079–2088.
 286. Gohring G, Michalova K, Beverloo HB, Betts D, Harbott J, Haas OA, Kerndrup G, Sainati L, Bergstraesser E, Hasle H, Stary J, Trebo M, van den Heuvel-Eibrink MM, Zecca M, van Wering ER, Fischer A, Noellke P, Strahm B, Locatelli F, Niemeyer CM, Schlegelberger B. Complex karyotype newly defined: the strongest prognostic factor in advanced childhood myelodysplastic syndrome. *Blood* 2010; 116(19): 3766–3769.

287. Fink SR, Paternoster SF, Smoley SA, Flynn HC, Geyer SM, Shanafelt TD, Lee YK, Jelinek DF, Kay NE, Dewald GW. Fluorescent-labeled DNA probes applied to novel biological aspects of B-cell chronic lymphocytic leukemia. *Leuk Res* 2005; 29(3): 253–262.
288. Cuneo A, Rigolin GM, Bigoni R, De Angeli C, Veronese A, Cavazzini F, Bardi A, Roberti MG, Tammiso E, Agostini P, Ciccone M, Della Porta M, Tieghi A, Cavazzini L, Negrini M, Castoldi G. Chronic lymphocytic leukemia with 6q- shows distinct hematological features and intermediate prognosis. *Leukemia* 2004; 18(3): 476–483.
289. Huret J. t(2;19)(p12;q13) IGK/BCL3; t(14;19)(q32;q13) IGH/BCL3; t(19;22)(q13;q11) BCL3/IGL. *Atlas Genet Cytogenet Oncol Haematol* 2008; <http://AtlasGeneticsOncology.org/Anomalies/t1419ID2050.html>.
290. Wierda W, O'Brien S, Wen S, Faderl S, Garcia-Manero G, Thomas D, Do KA, Cortes J, Koller C, Beran M, Ferrajoli A, Giles F, Lerner S, Albitar M, Kantarjian H, Keating M. Chemoimmunotherapy with fludarabine, cyclophosphamide, and rituximab for relapsed and refractory chronic lymphocytic leukemia. *J Clin Oncol* 2005; 23(18): 4070–4078.
291. Van Den Neste E, Robin V, Francart J, Hagemeijer A, Stul M, Vandenberghe P, Delannoy A, Sonet A, Deneys V, Costantini S, Ferrant A, Robert A, Michaux L. Chromosomal translocations independently predict treatment failure, treatment-free survival and overall survival in B-cell chronic lymphocytic leukemia patients treated with cladribine. *Leukemia* 2007; 21(8): 1715–1722.
292. Bosch F, Lopez-Guillermo A, Campo E, Ribera JM, Conde E, Piris MA, Vallespi T, Woessner S, Montserrat E. Mantle cell lymphoma: presenting features, response to therapy, and prognostic factors. *Cancer* 1998; 82(3): 567–575.
293. Rodriguez MA and Pugh WC. Mantle cell lymphomas. *Cancer Treat Res* 1996; 85: 41–50.
294. Knutsen T. Cytogenetic mechanisms in the pathogenesis and progression of follicular lymphoma. *Cancer Surv* 1997; 30: 163–192.
295. Barrans SL, Evans PA, O'Connor SJ, Kendall SJ, Owen RG, Haynes AP, Morgan GJ, Jack AS. The t(14;18) is associated with germinal center-derived diffuse large B-cell lymphoma and is a strong predictor of outcome. *Clin Cancer Res* 2003; 9(6): 2133–2139.
296. Montoto S, Lopez-Guillermo A, Colomer D, Esteve J, Bosch F, Ferrer A, Villamor N, Moreno C, Campo E, Montserrat E. Incidence and clinical significance of bcl-2/IgH rearrangements in follicular lymphoma. *Leuk Lymphoma* 2003; 44(1): 71–76.
297. Ohno H. Pathogenetic role of BCL6 translocation in B-cell non-Hodgkin's lymphoma. *Histol Histopathol* 2004; 19(2): 637–650.
298. Lossos IS, Akasaka T, Martinez-Climent JA, Siebert R, Levy R. The BCL6 gene in B-cell lymphomas with 3q27 translocations is expressed mainly from the rearranged allele irrespective of the partner gene. *Leukemia* 2003; 17(7): 1390–1397.
299. Ott G, Katzenberger T, Greiner A, Kalla J, Rosenwald A, Heinrich U, Ott MM, Muller-Hermelink HK. The t(11;18) (q21;q21) chromosome translocation is a frequent and specific aberration in low-grade but not high-grade malignant non-Hodgkin's lymphomas of the mucosa-associated lymphoid tissue (MALT-) type. *Cancer Res* 1997; 57(18): 3944–3948.
300. Rosenwald A, Ott G, Stilgenbauer S, Kalla J, Bredt M, Katzenberger T, Greiner A, Ott MM, Gawin B, Dohner H, Muller-Hermelink HK. Exclusive detection of the t(11;18)(q21;q21) in extranodal marginal zone B cell lymphomas (MZBL) of MALT type in contrast to other MZBL and extranodal large B cell lymphomas. *Am J Pathol* 1999; 155(6): 1817–1821.
301. Farinha P, Gascoyne RD. Molecular pathogenesis of mucosa-associated lymphoid tissue lymphoma. *J Clin Oncol* 2005; 23(26): 6370–6378.
302. Streubel B, Vinatzer U, Lamprecht A, Raderer M, Chott A. T(3;14)(p14.1;q32) involving IGH and FOXP1 is a novel recurrent chromosomal aberration in MALT lymphoma. *Leukemia* 2005; 19(4): 652–658.
303. Du MQ, Peng H, Liu H, Hamoudi RA, Diss TC, Willis TG, Ye H, Dogan A, Wotherspoon AC, Dyer MJ, Isaacson PG. BCL10 gene mutation in lymphoma. *Blood* 2000; 95(12): 3885–3890.
304. Cuneo A, Bigoni R, Roberti MG, Milani R, Agostini P, Cavazzini F, Minotto C, De Angeli C, Bardi A, Tammiso E, Negrini M, Cavazzini P, Castoldi G. Molecular cytogenetic characterization of marginal zone B-cell lymphoma: correlation with clinicopathologic findings in 14 cases. *Haematologica* 2001; 86(1): 64–70.

305. Kujawski LA, Longo WL, Williams EC, Turman NJ, Brandt N, Mosher DF, Eickhoff JC, Kahl BS. A 5-drug regimen maximizing the dose of cyclophosphamide is effective therapy for adult Burkitt or Burkitt-like lymphomas. *Cancer Invest* 2007; 25(2): 87–93.
306. Yustein JT, Dang CV. Biology and treatment of Burkitt's lymphoma. *Curr Opin Hematol* 2007; 14(4): 375–381.
307. Amakawa R, Ohno H, Fukuhara S. t(9;14)(p13;q32) involving the PAX-5 gene: a unique subtype of 14q32 translocation in B cell non-Hodgkin's lymphoma. *Int J Hematol* 1999; 69(2): 65–69.
308. Poppe B, De Paepe P, Michaux L, Dastugue N, Bastard C, Herens C, Moreau E, Cavazzini F, Yigit N, Van Limbergen H, De Paepe A, Praet M, De Wolf-Peeters C, Wlodarska I, Speleman F. PAX5/IGH rearrangement is a recurrent finding in a subset of aggressive B-NHL with complex chromosomal rearrangements. *Genes Chromosomes Cancer* 2005; 44(2): 218–223.

Contributed protocols section

IMPORTANT: No protocol included in this manual should be used clinically unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements of all chemicals used in the laboratory.

See additional protocols within the chapter.

Protocol 11.1 Cancer cytogenetics procedure

Contributed by Kathleen Rao, PhD, University of North Carolina, Cytogenetics Laboratory, Chapel Hill, NC Adapted from a procedure created for UNC by A. Fitzgibbon and M. Vigil, reviewed by Kathleen Rao.

I. Principle

The goal in performing cancer cytogenetics is to produce analyzable metaphases to aid in the diagnosis and treatment of the patient. Bone marrow aspirate, leukemic blood, bone core biopsy, and lymph nodes are the sample types. The sample is grown as a short-term culture. Cells are arrested at metaphase using Colcemid®. Following arrest, the cells are swollen in a hypotonic solution, 0.075 M KCl, which facilitates the separation of individual chromosomes. The cells are fixed in a modified Carnoy's fixative and stored at 4°C. Microscopic analysis is then performed on 20 metaphases.

II. Materials

A. Reagents

1. Complete Growth medium is assembled steriley. Test for sterility. Complete medium is used for 3 weeks and stored at 4°C.
2. Components of 15% RPMI medium/Bone Marrow collection medium (500 mL)
 - a. RPMI 1640, 410mL, Gibco® #21870-076
 - b. Fetal bovine serum, 75 mL (no long term storage), Hyclone #SH-30070.03
 - c. Penicillin/Strep, 5 mL, Gibco® #15070-063
 - d. L-Glutamine, 5 mL, Irvine #9317-100
 - e. Sodium heparin, 5 mL 1000 units/mL (for collection media only)
3. For bone marrow collection medium, aliquot 5-mL boluses into 15-mL sterile centrifuge tubes. Store at -15°C to -20°C. Expires in 6 months.
4. Aliquot 250 mL at a time with the remainder to be frozen with parafilm around screw top. Thaw when needed, but use all of the remaining media at first thaw.
5. GCT conditioned medium, Origen #50-0815. Aliquot 5-mL into centrifuge tubes. Store tubes at -10°C to -30°C. Expiration date is on bottle label. This label is transferred to the holding rack when aliquots are prepared.
6. Collagenase
 - a. Combine the following:
 - i. lyophilized collagenase Type 1, 100 mg, Gibco® #17100-017
 - ii. alpha MEM basal medium, 100 mL, Gibco® #12571-071

- iii. penicillin/Strep, 2 mL, Gibco® #15070-063
 - iv. sodium heparin, 1000 units/mL, 2 mL, hospital pharmacy
 - v. aliquot 5 mL of collagenase medium into 15-mL sterile centrifuge tubes. Store at -10 °C to -30 °C. Expires in 6 months.
7. 0.075 M KCl, 5.6 g of KCl/L dH₂O, Fisher #P217-500. Store at 2–8 °C. Expires in 1 month.
 8. Modified Carnoy's fixative. Freshly prepare a solution of 1 part glacial acetic acid, Fisher #A38-500 to 3 parts methanol, Fisher #A412-500. Chill to 4 °C. Expires daily.
 9. Colcemid®, 10 µg/mL, Gibco® #20-3706AV. Store at 4 °C. Expiration on bottle.
 10. Ethidium Bromide (EB), 500 µg/mL aqueous solution, Sigma #E-1385. Room temperature storage. Use directly from bottle until expiration date on bottle.

NOTE: Ethidium bromide is toxic. To clean up spills, refer to Safety Data Sheet.

III. Method

Specimen collection

To select the appropriate procedure, consider the indication and type of specimen. If no indication is provided and there is no indication given, the doctor must be contacted before proceeding. (See Table 11.8.)

Routine Bone Marrow or Peripheral Blood Setup [NOTE: ALL Bone Marrow and PBC samples must be adjusted for cell density per this protocol]

1. Note the WBC on all bone marrow or peripheral blood for cancer samples that arrive in the laboratory. If the WBC is less than $10 \times 10^6/\text{mL}$ (10 million cells per milliliter), it will need to be spun. Centrifuge at 1000 RPM for 10 minutes. While it is spinning, determine the total number of white cells in the specimen and the indication. These two factors will determine how the specimen is to be set up.
2. To determine the total number of WBCs in a specimen, multiply the WBC count by the total volume. The count is usually expressed in thousand cells per microliter, therefore 10^3 on the form (e.g., 3.5×10^3 per microliter). We express the count in million cells per milliliter (mL), thereby multiplying the numerator and the denominator by 1000. Thus $3.5 \times 10^3/\text{microliter} = 3.5 \times 10^6/\text{mL}$. If there were 5 mL, the total number of WBCs would be $3.5 \times 10^6/\text{mL} \times 5 \text{ mL} = 17.5 \times 10^6$ cells.
3. 10–15 million cells are needed for a 10-mL T-25 flask (1 million cells/1 mL of medium is optimal). Half that total is required for a 5-mL T₁₂ flask. If the *total* number of cells is less than 20 million and more than 5 million, then two T₁₂ flasks are set. One T-12 flask is set if the total count is less than 5 million. (See Table 11.9.)

WBC counts can be obtained through the Core Lab by sending a properly labeled 0.5-mL aliquot of mixed marrow or blood to them for analysis. Note: WBCs for neoplastic peripheral bloods can be obtained by checking the WBC count for that day in the hospital electronic records systems.

Sample calculations

Keep in mind you need $10 \times 10^6 \rightarrow 15 \times 10^6$ total WBCs in each flask

1. Low WBC = $2.48 \times 10^3/\mu\text{L}$; this translates to $2.48 \times 10^6/\text{mL}$
Total volume of specimen of dilute bone marrow = 6.0 mL

Table 11.8 Routine bone marrow and peripheral blood setup (GCT)

- | | |
|---|--|
| <ul style="list-style-type: none"> • CML • AML • ALL • MPN • MDS • ANLL • Lymph node culture • Bone core biopsy • Lymphomas (unless specified as mature B or T-cell lineage or MM) | <ul style="list-style-type: none"> • Anemia • ITP (idiopathic thrombocytopenia) • Neutropenia • Leukopenia • Myelofibrosis • Polycythemia vera (PCV) • Thrombocytosis |
|---|--|

Table 11.9 Setting guidelines for low–adequate count routine cancer specimens

Total no. of cells in specimen tube (WBC × Vol)		
No. of cultures to set		
**<3 million	Set in centrifuge tube with appropriate volume of medium	Place aliquot of media in a color-coded, labeled tube so that the volume can be brought up to 5mL before the addition of EB and Colcemid®. Place in refrigerator.
**3 to<5 million	One reduced volume GCT mini	Place aliquot of media in a color-coded, labeled tube so that the volume can be brought up to 5mL before the addition of EB and Colcemid®. Place in refrigerator.
**5 million	One 5mL GCT mini	
**6–9 million	Two reduced volume minis	Place aliquot of media in a color-coded, labeled tube so that the volume can be brought up to 5mL before the addition of EB and Colcemid®. Place in refrigerator.
10–19 million	Two 5mL minis	
20–30 million	Two 10mL flasks	

** Contact BM Tech/Director so that a backup PBC can be requested. (If Tech/Director is not available, page the attending physician and request a PBC.)

$$\begin{array}{r}
 2.48 \times 10^6 / 1 \text{ mL} \\
 \times \quad 6 \text{ mL} \\
 \hline
 14.88 \times 10^6 \text{ total cell number}
 \end{array}$$

Therefore, pellet must be concentrated – centrifuge at 1000 RPM. Remove supernatant to volume of 2 mL, resuspend and dispense equal volumes into two mini flasks (14.88×10^6 cells in 2 mL).

2. Adequate WBC = $12.3 \times 10^3/\mu\text{L} = 12.3 \times 10^6/\text{mL}$
Just remove 1 mL of specimen and add to a 10 mL flask.
3. High WBC = $40 \times 10^3/\mu\text{L} = 40 \times 10^6/\text{mL}$
Need $\frac{1}{4}$ number of WBCs. Remove 0.25 mL and dispense in a 10-mL flask.

Routine setup

NOTE: Typically, flasks with and without conditioned media are set, unless total WBC count is below 5×10^6 .

1. Using sterile technique, prepare:

10 mL cultures

Flask A = 1 mL specimen, 1 mL of GCT, and 8 mL of 15% RPMI

Flask B = 1 mL specimen and 9 mL of 15% RPMI

5 mL cultures

Flask A = 1 mL specimen, 0.5 mL of GCT, and 3.5 mL of 15% RPMI

Flask B = 1 mL specimen and 4 mL of 15% RPMI

NOTE: If the white count is low, T-12 flask(s) may be used. Keep in mind; use one half of amounts normally used for GCT, EB, Colcemid®, etc.

2. Mix and incubate overnight to 48 hours at 37°C and in 5% CO₂.

B. Receiving, setting and harvesting diagnostic bone marrow/bone core specimens

A bone marrow/bone core specimen qualifies as diagnostic if any of the following conditions apply:

1. The patient has no previous history in hospital medical record computer systems.

2. The patient has a history in hospital medical record computer systems, but that history is not related to cancer or the initial specimen was of a different tissue type, including lymph node, peripheral blood, tumor, CSF, pleural effusion, etc.
3. The samples are described as pretreatment on the test requisition and either of the above conditions applies. No results were obtained previously and the patient has a cancer history in the hospital medical record computer systems.

Receiving/setup

1. If a diagnostic bone core or a bone marrow specimen is received and the total cell count is less than 10 million cells, please contact a bone marrow technologist or director immediately so that they can request a backup peripheral blood specimen to be sent to the laboratory. If there are no bone marrow technologists or directors available, the rotating technologist must contact the attending physician immediately to request a peripheral blood be sent to the laboratory before the patient begins treatment. The person requesting the peripheral blood sample in this case must flag the case to track the specimen.
2. In the event that a diagnostic bone marrow and bone core are received on the same patient on the same day and the cell count on the bone marrow is such that only a reduced volume mini flask could be set, combine the bone marrow and bone core into a single flask and set up as appropriate.

Protocol for pediatric ALL

First time patients or those in relapse with adequate WBC should have a third flask cultured for 24 hours with no stimulation.

1. Flask A = 1 mL GCT, 1 mL of specimen, and 8 mL of 15% RPMI medium
2. Flask B = 1 mL of specimen and 9 mL of 15% RPMI medium
3. Flask C = 1 mL of the specimen and 9 mL of 15% RPMI medium

Mix and incubate overnight to 48 hours at 37°C and in 5% CO₂.

Lymph node biopsy

With some lymphoproliferative disorders, such as non-Hodgkin lymphoma, the lymph node is the source of abnormal cells present, and the bone marrow may not be infiltrated. In order to perform cytogenetic analysis, the lymph node requires special handling as delineated in the following protocols.

Lymph node transport collection

Lymph node should be placed in a sterile 15-mL centrifuge tube with 5 mL of bone marrow collection medium and sent to the Cytogenetics basket in the Core Lab for pick up by Cytogenetics laboratory personnel.

CAUTION! Follow universal precautions while handling lymph node. Wear gloves, process the specimen in the hood through first fixation and clean work areas with bleach. Notify supervisor immediately of any direct contact with the sample.

Setup for lymph node biopsy

Set up one T-12 mini flask*

1. Remove lymph node by either pouring out sample into the 60 mm dish or using a 1 mL pipette to hold and pull sample out.
2. The transport medium is removed and centrifuged at 1000 RPM for 10 minutes. This pellet will be added to the T12 flask.
3. Add 1 mL of growth media to the 60 mm dish.
4. Mince the sample with a #10 scalpel into very fine pieces.
5. Remove the 1 mL fluid only from the dish, with the tissue pieces clumped away to prevent them from being removed, and place the fluid into the culture flask.

6. Wash the material in the dish for a second rinse with 0.3 mL of GCT and 0.7 mL growth media and repeat step 5.
7. Wash the material in the dish for a third rinse with 1 mL of growth medium. Use this 1 mL to re-suspend the spun down pellet from step 2 and place in flask for a total of 3 mL* of fluid in the culture flask.
*NOTE: If an insufficient amount of material is released from the lymph node upon mincing, the portion remaining can be treated overnight with collagenase.
8. Incubate 37°C for 24 hours.
*The amount of material in the flask may be adjusted upward to 5 mL based on how cellular the suspension is. If need be, as little as 2 mL can be placed in a centrifuge tube, adjusting the washes accordingly.

Bone core biopsy

Specimen collection

Usually a bone core biopsy is obtained when the white cell count of the marrow is inadequate for cytogenetic studies. The bone biopsy is transported to the laboratory as soon as possible, at room temperature, in bone marrow collection medium. Because the number of cells retrieved is very small due to the size of the bone core biopsy, we are currently mincing it up and combining it with the bone marrow, if one is concurrently received. If the total number of cells in the bone marrow is adequate for two regular mini cultures, then the bone core is set up as an independent third culture, following the Setup for bone core biopsy procedure outlined next.

Setup for bone core biopsy (see Table 11.10)

Set up one T-12 mini flask (see Setup for lymph node biopsy).

Table 11.10 amounts are for a 10 mL (T-25) culture. When setting a 5 mL mini (T-12) flask or centrifuge tube culture, use ½ the amount of EB and Colcemid®.

Pouring and harvesting

1. Following the incubation period and after EB and Colcemid® addition, mix flasks and transfer to 15 mL centrifuge tubes. To maintain quality control, two technologists are involved in the transfer of specimens from flasks to tubes. Each technologist is responsible for only one of the two flasks set on any given patient. Ex: P96-288BC has an A and B flask in a single harvest. Tech 1 will pour the A flask and Tech 2 will pour the B flask. This applies to harvests that contain more than one flask on a particular patient.
NOTE: Pour one flask at a time.
 - a. Check label by cross-checking with the harvest list information
 - b. Transfer sample from flask to tube.
 - c. Remove label from flask and affix to tube.
 - d. Place date and your initials on tape using VWR LAB Marker.
 - e. Color code lid of tubes with tape color matching label.
 - f. Centrifuge tubes for 8 minutes at 1200 RPM or 10 minutes at 1000 RPM.
2. Aspirate all but a few drops of supernatant. Re-suspend the pellet by lightly tapping the centrifuge tube. Add 10 mL pre-warmed KCl slowly, drop by drop, with constant gentle agitation for the first 2 mL. Mix and place in 37°C water bath for 12 min.

Table 11.10 Mitotic arrest and harvest

	Ethidium bromide	Ethidium bromide time	Colcemid®	Colcemid® time
Routine (includes PBC, LPS, IL4 and 72-hour cultures)	200 µL	90 min	50 µL	45 min
Bone core biopsy			50 µL for T ₂₅	90 min
Lymph nodes	200 µL	90 min	50 µL	90 min

3. Remove from bath and add 1 mL of fresh cold 3:1 modified Carnoy's fixative. Mix gently and centrifuge 8–10 min at 1000–1200 RPM.
4. Aspirate supernatant. Mix cell pellet well but gently. Add 10 mL fresh cold fixative drop by drop, with constant gentle agitation for the first 2 mL. Mix gently and incubate at room temperature for at least 10 minutes or overnight at 4°C.
5. Mix tubes and centrifuge for 5 minutes at 1200 RPM or 10 min at 1000 RPM. A single fix is performed for a bone biopsy harvest (proceed to step 7). If it is a weekend or holiday, the harvest may be stopped after the first fix and stored at 4°C.
6. Repeat step 4 one more time or until the cell pellet is white and the supernatant is clear. There doesn't need to be a waiting period after the second addition of fix.
7. Add enough fixative to obtain a moderately cloudy suspension.
8. See Chapter 2, Slide-making, for preparing slides from suspension cultures.

Microscope work

Leukemia/Cancer Cytogenetics

1. Count and analyze 20 cells.
2. Image enough of these cells to represent all abnormalities.
3. Prepare two karyograms per stem line and at least one per sideline (subclone).
4. Print and analyze cells representative of all abnormalities.

Protocol 11.2 Bone marrow/leukemic peripheral blood setup and harvest procedure

Contributed by Kent Opheim, PhD, Seattle Children's Hospital, Cytogenetics Laboratory, Seattle, WA

I. Principle

Bone marrow cells are processed by direct and/or short term cultures for chromosome analysis of hematologic disorders and diseases, such as leukemia. Peripheral blood from a patient in blast crisis can be cultured for chromosome analysis as an alternative to a bone marrow sample in the diagnosis of leukemia.

Specimen requirements

1–2 mL bone marrow into preservative free sodium heparinized tube or 2 mL peripheral blood, which has at least 5% circulating blast cells, into a sodium heparin tube.

Safety

Lab coat and gloves should be worn while working with blood products.

Quality control and assurance

1. Quality control items mentioned in procedural text are indicated with QC.
2. Quality assurance items mentioned in procedural text are indicated with QA.

II. Materials

A. Supplies

1. 15 mL sterile plastic centrifuge tubes with screw caps (Falcon #2099)
2. Corning 25 cm² tissue culture flasks (CMS #366-302)
3. Sterile disposable serological pipettes: 1 mL, 5 mL, and 10 mL

4. Micro-centrifuge tubes
5. Pasteur pipettes
6. Sterile gauze

B. Equipment

1. Biogard sterile tissue culture hood
2. Double-chambered water-jacketed incubator set at 37°C, 5% CO₂
3. Centrifuge: IEC HN-SII; rotor #958
4. MarrowMAX™ complete bone marrow media (Invitrogen Corp); stored at 2–8°C.

C. Reagents and/or media

NOTE

- All new lots of reagents must be tested before use for adequate growth and mitotic index by overlapping in test cultures. **QC**
- Always check reagent/media expiration dates prior to using. **QA**

For setup

1. MarrowMAX™ Complete Bone Marrow Media (Invitrogen); stored at 2–8°C.

For harvest

2. Colcemid® (Gibco® KaryoMAX™ #15212-012), 10 µg/mL.
3. 0.075 M KCl hypotonic solution; pre-warm small amounts to 37°C.
4. 3 : 1 Modified Carnoy's fixative (3 parts absolute methanol : 1 part glacial acetic acid). Make fresh.

III. Method

A. Setup

ALWAYS mix the BM specimen or diluted specimen thoroughly prior to drawing from sample. QA

If more than one sample (same source, same patient, same date) was delivered, use the sample with the highest WBC. Combine the samples when none of the samples has 20×10^6 cells or greater.

1. The sample tube should be labeled with the draw number during the aspirate procedure. Record the tube numbers on the BM/LPB Lab Data Sheet (LDS).
2. Place a patient label on a sterile 15 mL Falcon tube.
3. The ideal concentration for tissue culture inoculation is 1×10^6 cells/mL or 10×10^6 cells per 10 mL culture. Take a white blood cell count (WBC) of the sample and calculate the amount of bone marrow sample needed to inoculate each 10 mL culture with 10×10^6 cells.
4. Add appropriate amount of BM sample calculated from step 3 to two T-25 flasks, labeled A and B.
5. Set up flasks as described:

A = 24 hour/overnight culture – 10-mL culture with MarrowMAX™ complete bone marrow medium.

B = 24 hour/overnight culture – 10-mL culture with MarrowMAX™ complete bone marrow medium.

If the WBC is too low and/or there is only enough inoculate to set up one 10mL flask, instead set up two 5-mL flasks and document it so the reagents of both flasks will be halved at harvest time.

B. Harvest

1. Label one sterile 15-mL Falcon tube for each culture to be harvested with a patient label and culture (A or B culture).
2. Remove the B cultures from the B incubator and gently mix the cultures by inverting or rocking the flasks. Make sure that all of the cells attached to the bottom of the flasks are in suspension.
3. Transfer the B cultures into their respectively labeled tubes.

4. Add 200 µL of Colcemid® to the B cultures and place in 2–8 °C refrigerator for 2.5 hours.
5. After the B cultures have been in the refrigerator for 1.5 hours, prepare the A cultures for the additions by repeating steps 2 and 3 with the A cultures.
6. Add 100 µL of Colcemid® to the A tubes and place back into the A incubator for 30 minutes.
7. After 30 minutes, centrifuge all the tubes at 1100 RPM for 10 minutes.
8. **Change the Pasteur pipette between patients;** aspirate supernatant, being careful not to aspirate the pellet.
9. Gently resuspend the pellet.
10. Add enough pre-warmed 0.075 M KCl hypotonic to bring volume up to 10 mL.
11. Place in 37°C incubator for 28 minutes.
12. After incubation, add 0.5 mL modified Carnoy's fixative and re-suspend cells.
13. Centrifuge for 8 minutes at 1100 RPM.
14. **Change the Pasteur pipette between patients;** re-suspend in 10 mL fixative and let stand for 30 minutes.
15. Wash cells *three more times* in 6, 4, and 2 mL of modified Carnoy's fixative, centrifuging, and aspirating supernatant in between. **The same Pasteur pipette may be used for all tubes as now it is in a “hard pellet” stage.** Fixed cells can be stored in suspension in the refrigerator at this point.
16. Make slides.

C. Direct harvest

1. Add 100 µL Colcemid® and place in incubator for 30 minutes.
2. After 30 minutes, transfer all BM/LPB cultures into their respectively labeled tubes and centrifuge at 1100 RPM for 10 minutes.
3. Proceed to step 8 of the Harvest (B) above.

Protocol 11.3 Bone marrow and leukemic blood culture and harvest procedure using DSP30 CPG oligonucleotide/interleukin-2 for B-cell mitogenic stimulation

Contributed by Patricia Crowley Larsen and Patricia Minehart Miron, PhD, UMass Memorial Medical Center, Cytogenetics Laboratory, Worcester, MA

I. Principle

Chronic lymphocytic leukemia (CLL) is a B-cell malignancy. CLL cells are difficult to culture because they rapidly apoptose ex vivo, they do not spontaneously divide, and they have a very weak response to standard B-cell mitogens. Oligonucleotides (ON) are short single strands of DNA or RNA, usually 19-25 base pairs. They can be natural or synthesized. The CpG ON used to stimulate CLL cells are rich in unmethylated CG sequences. CpG ON activate cells of the immune system in a sequence-dependent manner.

II. Materials

Supplies

1. MarrowMAX™ bone marrow media, Gibco® #12260-014
2. Colcemid® solution, 10 µg/mL, Gibco® #1512-012
3. Potassium chloride solution (KCl), Sigma #P3911
4. Distilled water, Invitrogen #15230-147
5. Methanol, Mallinckrodt #3016-02
6. Glacial acetic acid, Fisher #A38-500
7. Clean, dry slides (previously stored in 100% ethanol, 2–8 °C)
8. 1, 5, and 10 mL sterile pipettes, Fisher #13678-11B, -11D, -11E
9. 9-inch sterile Pasteur pipettes, Fisher #22-230-490
10. Sterile 15 mL centrifuge tubes, Fisher #055395
11. Oligonucleotide 27 mer synthesis scale 0.20 µmol, TIB MOLBIOL # DSP30: 131 nmol yield
12. UltraPure distilled water, Invitrogen #10977-015

13. Interleukin-2, Sigma #I2644 – 10 µg size
14. Hanks' balanced salt solution (HBSS), Invitrogen #14170-120
15. Isoton solution, Beckman Coulter #8547194

Equipment

1. Coulter counter, Beckman
2. Micropipette, 1000 µL and 2-20 µL, Fisher #S304665 and S304663
3. Micropipette, 20–200 µL, Transferpette #2704178
4. Micropipette tips, Fisher #02681163 and 02707501
5. MCT graduated Eppendorf™ tube, 1.5 mL, Fisher #05-408-129
6. Spinchron DLX centrifuge, Beckman Coulter #365303
7. Vacuum flask
 - a. Filter Flask, Fisher # FB-300-2000
 - b. Millex FH Vacuum Line Protection Filter, Millipore/Fisher #SLFH05010
 - c. Saint-Gobain Tygon tubing, Fisher #14-169-8G
 - d. One-hole rubber stoppers, size 6, Fisher #14-135J

Reagents



CAUTION: All reagents are potentially hazardous. Use appropriate safety procedures when handling these materials. Avoid contact with skin and mucous membranes. For an explanation of chemical safety considerations, see Chapter 20, Safety data sheets (SDS).

1. **MarrowMAX™ medium:** Thaw a 100-mL bottle of MarrowMAX™ media overnight in the refrigerator. Next day, label with date opened, date expired and technologist initials. Store at 2–8 °C. Expiration date: 2 months from date of preparation.
2. **Colcemid® stock solution** (10 µg/mL): Label with date opened and store at 2–8 °C. Expiration date is 3 months after opening.
3. **Dilute Colcemid® solution** (1 : 100 dilution): To prepare 1 : 100 dilute Colcemid® solution: Add 0.1 mL of the stock Colcemid® to 9.9 mL of sterile, distilled water in a 15-mL sterile centrifuge tube or other container. Label with reagent name, concentration, quantity, date prepared, expiration date. Store at 2–8 °C. Expiration date is 3 months after date prepared.
4. **Potassium Chloride (0.075 M solution) (KCl):** Add 5.6 g of KCl to 1 L of Invitrogen distilled water. Mix well, label with reagent name, quantity, date prepared and expiration date and store at room temp. Expiration date is 1 month after date prepared.
5. **Modified Carnoy's Fixative** (3 : 1) (anhydrous methanol–glacial acetic acid): Using a fume hood, prepare a 100-mL bottle of fixative by mixing 75 mL of anhydrous methanol with 25 mL of glacial acetic acid. Prepare each bottle fresh as needed. Label and store at room temperature with cap closed tightly (*stock bottles expire 5 days after opening*).
6. **0.1 M acetic acid:** Mix 5.7 µL of factory glacial acetic acid (17.4 M) with 994 µL of distilled water in a small eppendorf tube or other conical tube.
7. **Interleukin-(IL)-2 stock solution:** Centrifuge vial briefly in microcentrifuge to ensure all lyophilite is at the bottom of the vial. Add 100 µL of 0.1 M acetic acid solution to the vial and mix. Centrifuge vial for 5 seconds. Add 900 µL of Hanks' balanced salt solution to dilute to a 1-mL stock solution. Invert to mix. Centrifuge vial for 5 seconds. Proceed to MarrowMAX™ culture media with oligonucleotide-IL2 (OL-IL2) (step 9).
8. **Oligonucleotide (27 mer) 131 nmol yield stock viles:** Centrifuge vials for 5 seconds. Reconstitute four viles of stock oligonucleotide with 1 ml of UltraPure distilled water each. Allow to sit for 10 minutes, invert gently, and centrifuge and proceed to step 9.
9. **MarrowMAX™ Culture Media with Oligonucleotide-IL2 (OL-IL2):**
 - a. Fill 25 (15 mL) centrifuge tubes each with 10 mL of MarrowMAX™ media.
 - b. Add 40 µL of stock IL-2 solution into each tube of MarrowMAX™ medium.
 - c. Invert oligonucleotide tubes. Centrifuge the four stock vials for 5 seconds. Add 153 µL of stock oligonucleotide solution to each tube of MarrowMAX™ medium containing the IL2. Mix well. Label tubes with reagent name, date prepared, date expired (6 months), storage conditions (–20 °C), volume, and technologist initials.

Procedure

1. Two cultures are routinely established: one overnight dilute Colcemid® (ONC) culture and one 72-hour culture with DSP30 Oligo/Interleukin-2 (OL-IL2). **For very fatty bone marrow specimens**, see Note 1.
2. Each culture is inoculated with 1×10^6 white blood cells per mL of culture media. Invert tubes to mix well.
3. Place cultures in a slanted position, split between two 37°C incubators, with caps tightened. At the end of the day, add 100 µL of dilute (1 : 100) Colcemid® solution to the 10 mL of ONC culture. Mix well. Re-incubate at 37°C in a slanted position (closed system). The OL-IL2 culture is harvested after a 72-hour culture period following steps 4–15.
4. The following morning, add 100 µL of stock Colcemid® (10 mg/mL) to the ONC culture to be harvested. Reduce this amount proportionately for smaller culture volumes. Mix well and re-incubate at 37°C for 20 minutes.
5. Place appropriate amount of 0.075 M KCl in a 37°C water bath to use in step 7.
6. Centrifuge culture for 10 minutes at 1000 RPM.
7. Aspirate off the supernatant and add 10 mL KCl (0.075 M) (37°C).
8. Mix well and let stand for 20 minutes in an upright position in a 37°C incubator.
9. Add 1 mL of modified Carnoy's fixative. Mix well and let stand at room temperature for 10 minutes.
10. Centrifuge at 1000 RPM for 10 minutes.
11. Aspirate supernatant. Re-suspend pellet by flicking and add 10 mL modified Carnoy's fixative and let stand for 20 minutes at 2–8°C. Tubes can be left longer at this step in the refrigerator.
12. Centrifuge at 1000 RPM for 10 minutes.
13. Aspirate supernatant, and re-suspend by flicking bottom of tube. Add 10 mL fresh fixative.
14. Repeat steps 12 and 13 but resuspend with 5 mL of fresh room-temperature fixative.
15. Store fixed cell pellets at 2–8°C or proceed to slide preparation. If dropping slides, centrifuge for 10 minutes at 1000 RPM.

III. Notes

1. **Very Fatty Bone Marrow Specimens:** Wash specimen in 5 mL of Hanks' BSS, centrifuge and remove serum layer with fat, prior to performing cell count on resuspended specimen.

IV. Additional readings

1. Muthusamy N, Breidenbach H, Andritsos L, Flynn J, Jones J, Ramanunni A, Mo X, Jarjoura D, Byrd JC, Heerema NA. Enhanced detection of chromosomal abnormalities in chronic lymphocytic leukemia by conventional cytogenetics using CpG oligonucleotide in combination with pokeweed mitogen and phorbol myristate acetate. *Cancer Genetics*, 2011; 204:77–83.
2. Put N, Konings P, Rack K, Jamar M, Van Roy N, Libouton J-M, Vannuffel P, Sartenaer D, Ameye G, Speleman F, Herens C, Poirel HA, Moreau Y, Hagemeijer A, Vandenberghe P, Michaux L. Improved detection of chromosomal abnormalities in chronic lymphocytic leukemia by conventional cytogenetics using CpG oligonucleotide and Interleukin-2 stimulation: A Belgian multicentric study. *Genes Chromosomes and Cancer* 2009; 48: 843–853.
3. Decker T, Schneller F, Hipp S, Miethig C, Jahn T, Duyster J, Peschel C. Cell cycle progression of chronic lymphocytic leukemia cells is controlled by cyclin D2, cyclin D3, cyclin-dependent kinase (cdk) 4 and the cdk inhibitor p27. *Leukemia* 2002; 16: 327–334.
4. Dicker F, Schnittger S, Haferlach T, Kern W, Schoch C. Immunostimulatory oligonucleotide-induced metaphase cytogenetics detect chromosomal aberrations in 80% of CLL patients: a study of 132 CLL cases with correlation to FISH, IgVH status, and CD38 expression. *Blood* 2006; 108: 3152–3160.

Protocol 11.4 Culture of CpG-stimulated peripheral blood and bone marrow in chronic lymphocytic leukemia

Contributed by Nyla A. Heerema, PhD, The Ohio State University (OSU), Cytogenetics Laboratory, Columbus, Ohio

I. Principle

Chronic lymphocytic leukemia (CLL) is a B-cell malignancy. CLL cells are difficult to culture because they rapidly apoptose ex vivo, they do not spontaneously divide, and they have a very weak response to standard B-cell mitogens. Oligodeoxynucleotides (ODNs) are short single strands of DNA or RNA, usually 19–25 base pairs. They can be natural or synthesized. The CpG ODN used to stimulate CLL cells are rich in unmethylated CG sequences. CpG ODNs activate cells of the immune system in a sequence-dependent manner. These CpGs can also be used to stimulate other B-cell malignancies, such as non-Hodgkin lymphomas.

Specimen

Peripheral blood

Aseptically collect 10 to 20 mL of blood in one or two sodium heparin Vacutainer® tubes. Immediately invert the tubes several times to mix the sample and prevent clotting. Samples collected in EDTA, citrate or lithium heparin may be rescued by washing the cells in several changes of medium to dilute out the offending anticoagulant. Because it can be difficult to predict when a given culture may be unsuccessful, however, always attempt to culture all specimens and immediately notify physician of the possible need for a repeat sample.

Bone marrow

For baseline studies, obtain the marrow sample before treatment. Samples may be obtained to assess remission status. However, recent chemotherapy (days) may decrease culture success by decreasing the number of cells in metaphase as immature cells are eliminated. Aseptically collect 1–2 mL or more of marrow aspirate in a sodium heparin Vacutainer® tube. Gently invert the tube immediately several times to mix the sample and prevent clotting. Samples collected in EDTA, sodium citrate or lithium heparin are unacceptable but may be rescued by washing the cells in several changes of medium to dilute out the offending anticoagulant. Attempt to rescue clotted marrow aspirates. See notes section for suggestions for clotted specimens. However, because it can be difficult to predict when a given culture may be unsuccessful, always attempt to culture all specimens and immediately notify the physician of possible need for a repeat sample. Excessive heparin in the sample may lend a gelatinous quality to the specimen. Hypocellular marrows or aspirates contaminated with peripheral blood (dry tap) may result in unsuccessful studies. A peripheral blood culture or bone core biopsy may be helpful in these circumstances.

Handling conditions

Deliver specimens at room temperature as soon as possible after collection. Do not refrigerate or freeze!

II. Materials

A. Supplies

1. 10-mL pipettes
2. 15 mL sterile Sarstedt screw cap tubes, Sarstedt #62.554.205, (non-pyrogenic according to FDA LAL guidelines. Non-cytotoxic according to ISO 10993-5; Elution method)

B. Equipment

3. Eppendorf™ pipettor
4. Pipetaid
5. Pipetman
6. AND balance
7. Coulter AcT
8. Biological Safety Cabinet
9. Precision water bath (2)
10. Incubator
11. Forma incubator
12. Centrifuge
13. Hettich centrifuge
14. Refrigerator

C. Reagents

15. RPMI 1640 Medium, BioWhittaker, 500 mL, Fisher Scientific, #BW12-702 F
 - a. Store at 2–8°C.
 - b. Date and initial bottle when opened.
 - c. Discard in sink with running water at manufacturer's expiration date.

16. L-glutamine (GlutaMAX™), Gibco® Invitrogen #35050-061
 - a. Aseptically dispense in 5-mL aliquots. Label each aliquot as "L-glut". Place aliquots in a 1-L plastic beaker.
 - b. Label beaker as "L-glutamine" with lot number, dates of preparation and expiration, and preparer's initials.
 - c. Store frozen between -5 °C and -30 °C.
 - d. Thaw completely and mix well before use.
 - e. Discard in sink with running water at manufacturer's expiration date.
17. Fetal Bovine Serum, Hyclone Laboratories #SH30070.02
 - a. Store frozen between -5 °C and -30 °C.
 - b. Thaw completely and mix well before use.
 - c. Discard biohazard waste container at manufacturer's expiration date.
 - d. Avoid repeated freezing and thawing.
 - e. Avoid exposure to light.
18. Penicillin-Streptomycin 5000 units/mL, Gibco® Invitrogen #150700-63
 - a. Aseptically dispense in 5-mL aliquots. Label each aliquot as "PenStrep". Place aliquots in a 1-L plastic beaker.
 - b. Label beaker as "Pen-Strep", with lot number, dates of preparation and expiration, and preparer's initials.
 - c. Store frozen between -5 °C and -30 °C.
 - d. Thaw completely and mix well before use.
 - e. Discard in biohazard waste container at manufacturer's expiration date.
19. RPMI growth medium preparation
MEDIUM IS PREPARED ASEPTICALLY IN LAMINAR FLOW HOOD
 - a. Aseptically add to sterile, capped, 750-mL Falcon® 3028 tissue culture flask, Fisher #08-772-1A
 - i. 400 mL of RPMI 1640
 - ii. 10 mL of L-glutamine (GlutaMAX™)
 - iii. 85 mL of fetal bovine serum
 - iv. 10 mL of penicillin-streptomycin
 - b. Label as "RPMI," with batch number, dates of preparation and expiration, and initials of technologist who made the medium.
 - c. Record lot numbers of RPMI medium, antibiotics, fetal bovine serum, and L-glutamine.
 - d. Store at 2–8 °C.
 - e. After testing the Growth Medium for 48 hours, add 10 mL of penicillin-streptomycin.
 - f. Discard unused portion of prepared medium in sink with running water after 3 weeks or at manufacturer's expiration date, whichever comes first.
20. Lectin Pokeweed Mitogen (PKW), Sigma Aldrich #L8777
 - a. Each bottle contains 5 mL of (lyophilized) pokeweed mitogen (PKW).
 - b. Store at 2–8 °C.
 - c. Thaw completely to room temperature before reconstituting.
 - d. Using a 5-mL syringe, add 5 mL of 1× phosphate buffered saline through rubber stopper on vial.
 - e. Mix gently to dissolve and label with reconstitution date.
 - f. Before using PKW, allow it to come to room temperature. Using a 1-mL syringe add 50 µL to each 5 mL culture (final concentration = 10 µL/mL) for 72–96 hours.
 - g. Use in conjunction with Phorbol 12-myristate 13-acetate (PMA).
 - h. Phorbol 12-myristate 13-acetate (PMA), Sigma Aldrich #P8139.
21. Prepare stock solution PMA by diluting Phorbol 12-myristate 13-acetate (PMA) powder as follows:
 - a. 1 mg of PMA/1 mL of DMSO (0.00162 M), Sigma #D2650.
 - b. Prepare working solution by mixing 0.1 mL of PMA stock solution with 10 mL RPMI 1640 medium. Use 20 µL of this dilution in a 5 mL culture. (0.04 µg PMA/mL = 40 ng PMA/mL solution final concentration.)
 - c. PMA is usually used in conjunction with pokeweed mitogen. Use 50 µL PWM and 20 µL PMA in culture for 72–96 hours to stimulate B-cells.



**PMA is a potent tumor promoter of known carcinogenesis.
HARMFUL IF ABSORBED THROUGH SKIN.
USE EXTREME CAUTION.**

22. CpG ODN 685
We have this "custom oligonucleotide" synthesized by Sigma Aldrich, Inc. Other companies will synthesize as well, but we have only tried Sigma Aldrich.
Sequence: 5'-TCG TCG ACG TCG TTC GTT CTC-3' [3]

- a. Reconstitute to working concentration with sterile saline.
 - i. Volume of saline (in mL) to reconstitute powder = $0.1 \times \text{wgt (in mg)}$ of CpG ODN 685, i.e., 39 mg CpG ODN 685 is reconstituted in 3.9 mL of saline
 - ii. Use 20 μL of working concentration per 10 mL of culture.
- 23. Colcemid[®] 10 $\mu\text{g/mL}$ in HBSS, Gibco[®] #152100-040
 - a. Label with date opened and expiration date.
 - b. Store at 2–8 °C.
 - c. Discard open bottle in biohazard waste after one month or at manufacturer's expiration date, whichever comes first.
- 24. Potassium Chloride (KCl), Sigma #P-3911
 - a. Preparation of stock solution: (0.075 M KCl)
 - i. Dissolve 5.59 g of KCl in 1 L of double-distilled demineralized water in a 1000-mL volumetric flask.
 - ii. Pour mixture into tightly capped 1-L plastic bottle.
 - iii. Label as "0.075 M KCl", with dates of preparation and expiration, and preparer's initials.
 - iv. Store at 2–8 °C.
 - v. Discard any unused stock in sink with running water after 1 week.
- 25. Modified Carnoy's fixative
 - a. Methanol, analytical reagent grade (Mallinkrodt)
 - b. Glacial acetic acid, analytical reagent grade (Mallinkrodt)



Corrosive

FIXATIVE IS CORROSIVE AND FLAMMABLE. Wear gloves and protective clothing to avoid contact with skin. Prepare in fume hood.



Flammable materials

- a. Preparation of working solution:
 - i. Measure with a 100 mL graduated cylinder, 1 part (i.e., 25 mL) glacial acetic acid to 3 parts (i.e., 75 mL) methanol.
 - ii. Pour into 100 mL glass bottle and cap tightly.
 - iii. Label as "Fix" with date of preparation, preparer's initials, and warning: **CORROSIVE/FLAMMABLE**
- b. Prepare fresh daily.
- c. Always keep bottle capped and in freezer when not in use.
- d. Discard daily any unused portion into safety canister according to guidelines according to your laboratory's Chemical Management Guidelines.

III. Method [1,2]



ALL SPECIMENS MUST BE CONSIDERED POTENTIALLY INFECTIOUS; therefore, handle accordingly. Perform in laminar flow hood.

Caution: Wear gloves and protective clothing during entire culture and harvest procedures.

RPMI – Culture 1 (also used for FISH)	20 μL PMA/50 μL PKW/10 μL CpG ODN 685	72 hours
RPMI – Culture 2	only 10 μL CpG ODN 685	72 hours

Peripheral blood and bone marrow setup

1. Allow RPMI growth media to warm in a 37 °C water bath for 10 minutes or longer.
2. Assess marrow sample for volume, and presence or absence of spicules. Record on processing sheet. Note time and date of culture initiation on processing sheet.
3. Assess sample for TNCC (total nucleated cell count). Record on processing sheet. If less than 10×10^3 , spin tube at 1000 RPM for 8 minutes and remove buffy coat. If greater than or equal to 10×10^3 , do not spin.
4. Using a sterile pipette, add 5 mL of room temperature RPMI media to each of two sterile, screw-topped 15 mL conical Sarstedt centrifuge tubes. Using a permanent marker, label tubes with patient's name and log number, and designate as #1, #2.
5. Dispense appropriate amount of unspun peripheral blood, bone marrow or buffy coat according to guidelines in Table 11.11 into each of two tubes of media. Discard pipette in biohazard waste.
6. Record on processing sheet lot number and type of media used in each tube, and harvesting conditions and stimulation method.

Table 11.11 Specimen inoculation chart 5-mL culture tubes

TNCC	No. of WBC added	Specimen vol. (mL)
4.0 or below		*
4.0–10.0		**
11.0	6.6 million	0.6
12.0	7.2 million	0.6
13.0	7.8 million	0.6
14.0	7.0 million	0.5
15.0	7.5 million	0.5
16.0	8.0 million	0.5
17.0	6.8 million	0.4
18.0	7.2 million	0.4
19.0	7.6 million	0.4
20.0	6.0 million	0.3
21.0	6.3 million	0.3
22.0	6.6 million	0.3
23.0–30.0	6.9–9.0 million	0.3
31.0–50.0	7.7–12.5 million	0.25
51.0–75.0	10.2–15.6 million	0.2
76.0–100.0***	7.6–10 million	0.1

* (1–4 TNCC) Spin down and set up 1 culture tube with entire buffy
 ** (4–10 TNCC) Spin down and set up 2 culture tubes with $\frac{1}{2}$ buffy in each.
 *** Dilute any specimens >100 TNCC. Use dilution to set up cultures.

7. Place appropriate processing label on each tube and circle appropriate mitogen, Colcemid® time, and harvest day as necessary.
8. Retain extra specimen in refrigerator until adequacy of culture is proven. Write requisition number on outside of tube. Discard in biohazard waste container after report is finalized.
9. Add media to tubes.
10. Add mitogen(s) to each tube.
11. Add appropriate amount of specimen to each tube.
12. Mix and incubate cultures, loosely capped, at 37°C in 5% CO₂ incubator for the following times before continuing with Routine Harvest Procedure.
 - a. Tube #1: RPMI (PKW + PMA + CpG ODN 685), ~72 hours, then 30 min. Colcemid®.
 - b. Tube #2: RPMI (CpG ODN 685), ~72 hours, then 30 minutes Colcemid®.

Note: If the TNCC is too low, set up only culture #1 (see Table 11.11).

Harvest

1. Add, using Eppendorf™ pipettor, 50 µL of Colcemid® to each culture tube containing 5 mL of medium. Specimens set up in 10 mL or 2 mL of media receive 1000 µL and 20 µL, respectively. Discard pipette tip into biohazard waste container.
2. Cap tightly and mix well, but gently, by inversion. Incubate for 30 minutes at 37°C in 5% CO₂ incubator.
3. Place bottle of 0.075 M KCl in 37°C water bath.
4. Prepare modified Carnoy's fixative. Keep in freezer, tightly capped.
5. After 30 minutes, remove culture tubes from incubator and centrifuge at 1000 RPM (183g) for 8 minutes.
6. Aspirate supernatant using Pasteur pipette attached to vacuum aspirator assembly until 1/8 to 1/4 inch supernatant remains above cell pellet. Try not to disturb cell pellet.
7. Change the Pasteur pipette after aspirating each patient.

8. Replace cap on culture tube and evenly resuspend cells by gently flicking bottom of the tube with finger. Make sure entire pellet is resuspended.
9. Add 1 mL warmed 0.075 M KCl very slowly down side of tube, mixing with cell suspension. Add additional 5 mL KCl to bring volume to approximately 6 mL.
10. Cap tightly, and invert gently several times.
11. Place culture tube in 37°C water bath for 15–20 minutes.
12. Following incubation, slowly add 1–2 mL fresh fixative to culture tube using Pasteur pipette or squeeze bottle.
13. Cap tightly, and invert gently several times.
14. Centrifuge at 1000 RPM (183g) for 8 minutes.
15. Aspirate supernatant using Pasteur pipette attached to vacuum aspirator assembly until 1/8 to 1/4 inch supernatant remains above cell pellet. Try not to disturb cell pellet.
16. Change the Pasteur pipette after aspirating each patient.
17. Replace cap on culture tube and evenly resuspend cells by gently flicking bottom of the tube with finger. Make sure entire pellet is resuspended.
18. Add fixative to culture tube slowly, with gentle agitation, to bring the volume up to the 10 mL mark on the tube.
19. Place culture tube in refrigerator for 30 minutes to overnight.
20. Centrifuge at 1000 RPM (183g) for 8 minutes.
21. Aspirate supernatant using Pasteur pipette attached to vacuum aspirator assembly until 1/8 to 1/4 inch supernatant remains above cell pellet. Try not to disturb cell pellet.
22. Change the Pasteur pipette after aspirating each patient.
23. Replace cap on culture tube and re-suspend cells by gently flicking bottom of the tube with finger. Make sure entire pellet is evenly resuspended.
24. Add fixative to culture tube slowly, with gentle agitation, to bring the volume approximately up to the 5 mL mark on the tube.
25. Replace cap on culture tube, invert gently to mix and centrifuge at 1000 RPM (183g) for 8 minutes.
26. Aspirate supernatant using Pasteur pipette attached to vacuum aspirator assembly until 1/8 to 1/4 inch supernatant remains above cell pellet. Try not to disturb cell pellet.
27. Change the Pasteur pipette after aspirating each patient.
28. Repeat steps 24–28 until cell pellet is white and supernatant is clear.
29. Dilute cell pellet with fixative to obtain a moderately cloudy suspension.

IV. Notes

1. Hypotonic times may be increased 5–10 minutes during colder and drier months.
2. Keep fixative chilled as much as possible.
3. Lower concentrations of KCl may facilitate spreading of mitotic figures.
4. Higher concentrations of glacial acetic acid (e.g., 2 : 1) may facilitate spreading of mitotic figures.
5. Discard contents of vacuum aspirator assembly flask when full by carefully pouring into plastic safety disposal can. Wear gloves and protective clothing.
6. Clotted specimens should be manually cut and the clot squeezed with scalpels to release cells. Do TNCC on Coulter AcT on liquid fraction of sample. If clot is large and resists releasing cells, treat with collagenase for 1–2 hours to disrupt and use product to set up culture tube. Do not attempt to run a TNCC as it is likely to clog the AcT.

V. Additional readings

1. Priest JH: *Medical Cytogenetics and Cell Culture*, 2nd ed. Philadelphia: Lea and Febiger, 1977.
2. Liang X, Moseman EA, Farrar MA, Bachanova V, Weisdorf DJ, Blazar BR, Chen W. Toll-like receptor 9 signaling by CpG-B oligodeoxynucleotides induces an apoptotic pathway in human chronic lymphocytic leukemia B cells. *Blood* 2010; 115(24):5041–5052.

Protocol 11.5 Plasma cell separation and harvest procedure for FISH analysis

Contributed by Patricia Crowley Larsen and Patricia Minehart Miron, PhD, UMass Memorial Medical Center, Cytogenetics Laboratory, Worcester, MA

I. Principle

Plasma cell disorders, which include monoclonal gammopathy of unknown significance (MGUS), plasmacytoma, and plasma cell myeloma, are characterized by the monoclonal proliferation of plasma cells. Metaphase chromosome analysis of these disorders is severely limited by the extremely low in vitro proliferation rate of plasma cells until advanced disease is present. Interphase FISH allows detection of targeted abnormalities in non-dividing cells, but is still hampered by the small number of plasma cells in bone marrow specimens and the patchy nature of disease. MACS, Magnetic Cell Sorting (Miltenyi Biotec) apparatus, can improve FISH detection of abnormalities by isolating human plasma cells from bone marrow or peripheral blood by enrichment through positive selection of magnetically labeled CD138+ cells using magnetic separation column. The isolated cells can then be used for fluorescence in situ hybridization (FISH).

Specimen collection and storage

2 mL of peripheral blood, treated with sodium heparin

0.5–2 mL of bone marrow, treated with sodium heparin

Note: Store specimen overnight at room temperature if cell separation process cannot be completed the same day.

II. Materials

Equipment

1. MiniMACS Separator Starting Kit (magnet and stand), Miltenyi Biotec #130-090-312
2. MACS Sterile Pre-Separation Filters, Miltenyi Biotec, #130-041-407
3. MACS MS Sterile Separation Columns, Miltenyi Biotec, #130-042-201
4. Beckman Spinchron Centrifuge DLX, #365303

Reagents and reagent preparation

1. PBS pH 7.2, Invitrogen #20012-027
 2. PBS Suspension Buffer:
 - a. Mix: 500 mL pH 7.2 PBS with 1% FBS (5 mL) and 2 mM EDTA (2 mL).
 - b. Store at 2–8 °C for up to two weeks.
 3. PBS Wash Buffer (cold)
 - a. Mix: 500 mL of PBS with 0.5% BSA (2.5 mL) and 2 mM EDTA (2 mL).
 - b. Store at 2–8 °C for up to 2 weeks.
 4. EDTA, Invitrogen #15575-020
 5. Ficoll–Paque Plus (1.077 density), GE Healthcare Life Sciences #17-1440-02
 6. MACS CD138 (Syndecan-1) MicroBeads Miltenyi Biotec, #130-051-301
 7. Fetal Bovine Serum, Invitrogen #26140-079
 8. Bovine Serum Albumin (BSA) Fraction V, Invitrogen #15260
 9. Potassium Chloride (KCl), Sigma #P3911-500G, 0.075 M Solution:
 - a. Mix 2.8 g potassium chloride with 500 mL of sterile distilled water.
 - b. Store at room temperature for up to two weeks.
- Modified Carnoy's Fixative: Methyl Alcohol Anhydrous, Fisher #3016-02; Glacial Acetic Acid, Fisher #A38-500
- a. Mix 3 parts anhydrous methanol with 1 part glacial acetic acid. Prepare fresh as needed.
 - b. Store at room temperature.

III. Method

A. Separation procedure

Bring Ficoll and Suspension Buffer to room temperature prior to use.

1. Add bone marrow containing 10×10^6 cells to a 15 mL centrifuge tube.
2. To the same centrifuge tube, add 10 × the sample volume of PBS Suspension Buffer. (Example: If using 0.4 mL of bone marrow, mix with 4 mL of PBS Suspension Buffer for a total of 4.4 mL of cell suspension.)

3. To a new 15-mL centrifuge tube, add a volume of Ficoll-Paque that is half the volume of the cell suspension, then SLOWLY layer diluted cells on top of Ficoll-Paque, being careful not to mix the Ficoll with cells. There will be two visibly distinct layers. (Example: Layer 4.4 mL of diluted bone marrow over 2.2 mL of Ficoll-Paque.)
4. Centrifuge for 20 minutes at 1324 RPM or (RCF Max of 400 g), using brake setting of 1 or 2.
5. Carefully pipet up the cloudy “pinkish” layer that appears between the top supernatant and the ficoll layer and transfer to a new centrifuge tube.
6. Wash twice in 10 mL PBS Suspension Buffer. Centrifuge for 10 minutes at 1146 RPM (or RCF Max of 300 \times g), using brake setting of 1 or 2.
7. Carefully remove the supernatant completely.
8. Add 20 μ L of MACS micro beads. Mix well by flicking tube and incubate for 15 minutes at 4°C (shake every 2–3 minutes).
9. Add 2 mL COLD PBS wash solution (PBS with 0.5% BSA and 2 mm EDTA). Centrifuge at 1000 RPM for 10 minutes.
10. While sample is spinning, set up magnetic column plus pre-separation filter and prime it by adding 0.5 mL of COLD PBS wash solution through the filter.
11. Remove supernatant completely and resuspend cell pellet in 1 mL COLD PBS wash solution.
12. Add cell suspension to filter/column and allow cells to pass through completely.
13. Rinse tube with 0.5 mL cold PBS wash and allow any residual cells to pass through column.
14. Wash three more times with 0.5 mL cold PBS wash. *NOTE: This flow-through is the un-labeled fraction that you DO NOT want.*
15. Discard yellow filter. Place a new 15 mL centrifuge tube under column, pipet 1 mL PBS Wash Buffer on top of column, then carefully remove column from magnet.
16. Gently insert plunger through top of column to elute cells into the 15 mL centrifuge tube.
17. Proceed to harvesting the elution or store overnight for harvest the next day.

Harvest procedure

1. Centrifuge CD138+ cells for 10 minutes at 1000 RPM.
2. Remove supernatant.
3. Add 1 mL 37°C hypotonic (0.075 M KCl).
4. Incubate for 10 minutes at 37°C.
5. Add 10 mL modified Carnoy's fixative directly to the tube following incubation.
6. Centrifuge for 10 minutes at 1000 RPM.
7. Remove as much supernatant as possible without disturbing the very small (invisible) cell pellet.
8. Resuspend pellet in 100 μ L fixative.
9. Add approximately 20 μ L of cell suspension to each of four hybridization areas for FISH. Check under inverted phase for appropriate cell concentration. Add more cells if required to ensure adequate concentration.

IV. Additional readings

1. Nilsson T, Lenhoff S, Turesson I, Rylander L, Mitelman F, Westin J, Höglund M, Johansson B. Cytogenetic features of multiple myeloma: impact of gender, age, disease phase, culture time, and cytokine stimulation. *Eur J Haematol* 2002; 68:345–353.
2. Shaughnessy J, Jacobson J, Sawyer J, McCoy J, Fassas A, Zhan F, Bumm K, Epstein J, Anaissie E, Jagannath S, Vesole D, Siegel D, Desikan R, Munshi N, Badros A, Tian E, Zangari M, Tricot G, Crowley J, Barlogie B. Continuous absence of metaphase-defined cytogenetic abnormalities, especially of chromosome 13 and hypodiploidy, ensures long-term survival in multiple myeloma treated with Total Therapy I: interpretation in the context of global gene expression. *Blood* 2003; 101(10): 3849–3856.
3. *MACS Magnetic Cell Sorting User Manual*. Miltenyi Biotec Inc., Auburn, CA, 95602, USA.

Protocol 11.6 Plasma cell separation and harvest procedure for FISH

Contributed by Nyla A. Heerema, PhD, The Ohio State University, Cytogenetics Laboratory, Columbus, Ohio.

I. Principle

Multiple Myeloma is a lymphoproliferative disorder characterized by the monoclonal proliferation of plasma cells. The CD138/syndecan-1 antigen is expressed on plasma cells, multiple myeloma cells and certain other tumor cells. The majority of mitotic cells in myeloma patients are often nonmalignant cells rather than plasma cells. As a result, it may be difficult to identify an abnormal clone unless a large number of cells are analyzed. The EasySep human whole blood CD138 selection kit can isolate human plasma cells from bone marrow or peripheral blood by magnetically labeling CD138+ cells, followed by isolation using a positive selection magnetic separation. By using the EasySep procedure, target cells are specifically labeled with dextran-coated magnetic nanoparticles using bispecific Tetrameric Antibody Complexes (TAC). These complexes recognize both dextran and the target cell surface antigen. The small size of the magnetic dextran iron particles allows for efficient binding to the TAC-labeled cells. The magnetically labeled cells are then separated from unlabeled cells with the Big Easy magnet, using the EasySep procedure. These isolated cells can then be used for protocols such as FISH analysis.

Specimen collection and storage

For baseline studies, obtain the bone marrow sample before treatment. Samples may be obtained to assess remission status. Aseptically collect 1–2 mL or more of marrow aspirate in a sodium heparin Vacutainer® tube. Gently invert the tube immediately several times to mix the sample and prevent clotting. Excessive heparin in the sample may lend a gelatinous quality to the specimen. Hypocellular marrows or aspirates contaminated with peripheral blood (dry tap) may result in unsuccessful studies. Peripheral blood samples are not recommended and bone core biopsies are unacceptable for cell separation. A bone marrow aspirate is preferred. Specimen should be stored at room temperature overnight if cell separation cannot be completed the same day.

II. Materials

Reagents

1. EasySep Human Whole Blood CD138 Positive Selection Cocktail (Stem Cell Technologies #18387). Store at 4°C.
2. EasySep Whole Blood Magnetic Nanoparticles (Stem Cell Technologies #18180). Store at 4°C.
3. EasySep RBC Lysis Buffer 10× Concentrate (Stem Cell Technologies #20110). Store at room temp for 2 years. Prepare 1× lysis buffer by adding 1 part 10× lysis buffer to 9 parts distilled water and store at 4°C for 3 months.
4. Robosep Buffer (Stem Cell Technologies #20104). Store at 4°C.
5. Modified Carnoy's Fixative (3:1 v:v methanol : glacial acetic acid) Prepare fresh daily.
6. Distilled Water
7. RPMI 1640
8. Potassium Chloride (KCl) 0.075 M

Supplies

1. 14 mL Polystyrene round bottom tubes (Becton Dickinson #352057)
2. 10–100 µL pipettes
3. 10-100 µL pipette tips
4. 2-welled, 15 mm circle microscope slides
5. Timer
6. Graduated cylinder
7. Forceps
8. Non-sterile pipettes
9. 1 mL, 5 mL, 10 mL pipettes

Equipment

1. “Big Easy” EasySep Magnet (Stem Cell Technologies #18001)
2. 37°C Precision water bath
3. Rotina 35 bench-top centrifuge
4. Biological safety hood

III. Method

Plasma cell separation

1. Transfer desired amount of specimen (250 µL to 4 mL) to a 14 mL polystyrene tube. Volume of specimen is based on TNCC, usually 2 mL for counts > 4. For specimens with a low TNCC (< 4), spin sample tube at 1000 RPM for 8 min. and use buffy coat.
2. Add 1× EasySep RBC lysis buffer at a ratio of 1 part RBC lysis buffer to 1 part whole blood/bone marrow sample (e.g., to 2 mL sample, add 2 mL Lysis Buffer for final volume of 4 mL). Mix well and proceed immediately to next step.
3. Add EasySep CD138 **Positive Selection Cocktail** at 25 µL/mL whole blood or bone marrow/lysis buffer mixture (e.g., for 4 mL of whole blood/lysis buffer mixture, add 100 µL of cocktail). Mix well and incubate at room temp for 15 minutes.
4. Mix EasySep **Whole Blood Magnetic Nanoparticles** well by pipetting up and down vigorously more than 5 times (*do NOT vortex*). Add Nanoparticles at 25 µL/mL whole blood/lysis buffer mixture (e.g., for 4 mL of whole blood/lysis buffer mixture, add 100 µL of Nanoparticles). Mix well and incubate at room temp for 10 minutes.
5. If the total volume is less than 2.5 mL, add **Robosep Buffer** to 5 mL; otherwise, add **Robosep Buffer** to 10 mL. Mix the cells in the tube by gently inverting tube 2-3 times.
6. Place tube, WITHOUT THE CAP, into the magnet. Set aside at room temperature for 5 minutes.
7. Pick up the EasySep magnet, and in one continuous motion, invert the magnet and tube, pouring off the supernatant fraction. Leave the magnet and tube in an inverted position for 2-3 seconds, and then return to upright position. Do NOT shake or blot off any drops that may remain hanging from tube.
8. Remove the tube from the magnet and add 10 mL **Robosep Buffer**. Mix well by gently pipetting up and down 2-3 times. Place tube back in the magnet, without the cap, and set aside for 5 minutes.
9. Repeat steps 7 and 8 (inverting magnet and tube, adding 10 mL buffer, incubating 5 minutes) and then step 7 once more, for a total of three 5-minute separations in the magnet.
10. Remove tube from magnet and resuspend cells in 5 mL of prewarmed RPMI. Proceed to harvest procedure.

Harvest

1. Centrifuge CD138+ cells for 10 minutes at 900-1000 RPM.
2. Remove supernatant (you won't see a pellet; draw down to line on tube).
3. Add 1 mL 37°C hypotonic (0.075 M KCl) and gently mix.
4. Incubate for 10 minutes at 37°C.
5. Add 5 mL fixative DIRECTLY to the tube, and mix gently following incubation.
6. Centrifuge for 10 minutes at 900–1000 RPM.
7. Remove as much supernatant as possible without disturbing the very small (invisible) cell pellet.
8. Pipet 10 µL of the separated specimen onto each of 5 hybridization areas, using 2-welled slides for the standard Multiple Myeloma FISH panel.*
9. Allow slides to completely air dry.
10. Again pipet 10 µL of the separated specimen onto each of the 5 hybridization areas and allow to air dry completely and then place onto the hot plate for 5 minutes.*
11. Proceed to FISH procedure (see Chapter 16, Protocol Section).

*Amount may need to be adjusted dependent upon cell concentration.

Protocol 11.7 Bone marrow GTG-banding

Contributed by Urvashi Surti, PhD, Pittsburgh Cytogenetics Laboratory, Magee-Women's Hospital of UPMC, Pittsburgh, PA

For additional banding methods, see protocols in Chapter 6.

I. Principle

Aged slides containing cells fixed in the metaphase or prometaphase stage of cell division are pretreated with trypsin and stained with Giemsa. The trypsin digests certain proteins on the chromosomes and produces a characteristic pattern of light and dark bands on each chromosome that is the same in all humans. Certain chromosomes contain polymorphic regions when G-banded (i.e., satellites, qh+ regions) that may be useful in determining parental origin of chromosomes. Slides stained by the G-banding method can be analyzed for abnormalities in chromosome number or structure.

II. Materials

General laboratory supplies

1. 4 glass Coplin jars
2. Forceps
3. Scale for weighing out trypsin
4. Timer

Reagents

5. 5 mL trypsin (1:250) 0.25% 1× solution (Fisher #25-050-C1] in HBSS (w/o calcium and magnesium salts and w/o sodium bicarbonate. Fisher #MT21-021-CV)
6. Diluent 2 (Hematol; Cardinal Health #B3157-12B, or equivalent), stored at room temperature
7. Fetal bovine serum (Irvine Scientific #3003)
8. Gurr's buffer (pH 6.8 buffer tablet dissolved in 1 liter deionized water - Biomedical Specialties #33199)
9. Giemsa stain (Sigma #GS-500)
10. Deionized water
11. Hanks' Balanced Salt Solution (HBSS) (Fisher #MT21-021-CV or equivalent)

III. Method

Bone marrows and cancer bloods

1. Set up the Coplin jars as follows:
 Jar #1: 5 mL of trypsin/45 mL Diluent 2 solution
 Jar #2: 3 mL of thawed fetal bovine serum added to 47 mL of HBSS
 Jar #3: 50 mL Gurr's buffer
 Jar #4: 7 mL Giemsa stain added to 70 mL Gurr's buffer, mixed thoroughly.
 Note: Clean jars and precise attention to time exposures will give the best results.
2. Begin banding by making one or more initial test runs in order to determine the optimum trypsin and staining times. Slides on cases that have already been completed should be used for the test run(s), if available. If none are available, use a slide from a case or cases on which several slides are available for staining on that day. Prior to banding, all slides should be dried in a 60°C oven overnight or a 90°C oven for about 1 hour 15 minutes to 1 hour 30 minutes prior to G-banding.
3. Trypsin time should be in the vicinity of 45 sec.; however, consult the banding log from the previous day to determine optimal amount of time in trypsin solution. Place a slide into jar #1.
4. Using forceps, remove slide from jar #1 and blot end on paper towel. Place into jar #2 for a minimum of 1 minute and blot end on paper towel.
5. Place slide into jar #3 for a minimum of 1 minute. Remove and blot end on paper towel.
6. Place slide into jar #4 for 2–7 minutes (check previous day data in logbook). Blot end on paper towel.
7. Rinse slide under running tap water.
8. Promptly blow the slides dry with the in-house air supply. Carefully evaluate the slides under the microscope, noting conditions of under- or overtrypsinization and under- or overstaining.
9. Adjust the amount of time in trypsin solution or Giemsa stain solution as necessary (see Notes for troubleshooting tips) until a test slide produces acceptable banding results. Run the remaining slides through the banding protocol, using times determined by test run(s). Record the trypsin and Giemsa times in the appropriate log.
 Note: *Each case* must be carefully checked and the banding and staining times individually adjusted. Also note if a case has a suboptimal number or quality of metaphase spreads. If necessary, ask for slides to be redropped.

IV. Notes

1. If there are a large number of slides, make a fresh jar #2 part of the way through the banding.
2. If slides appear fuzzy, decrease amount of time in trypsin solution, or make a fresh jar #2 to inactivate the trypsin completely.
3. If slides appear underbanded (solid stained), increase time in trypsin solution.

4. If slides appear banded but too light/dark, increase/decrease amount of time in Giemsa stain. Alternately, more/less Giemsa may be added to the staining solution and a test slide run through at the original time.

Protocol 11.8 GTW banding procedure (G-bands by trypsin using Wright stain)

Contributed by Nyla A. Heerema, PhD, The Ohio State University (OSU), Cytogenetics Laboratory, Columbus, Ohio

Additional banding procedures can be found in Chapter 6, Chromosome stains.

I. Principle

This banding technique allows individual identification and differentiation of each chromosome pair. Banding refers to the appearance of the chromosomes with horizontal, sequential light and dark interfaces or cross-bands along their length. This banding pattern may be induced by pre-treating slides in a dilute trypsin solution followed by staining with Wright stain.

The mechanism for this banding is not yet fully understood. Trypsin effectively helps remove histone and non-histone proteins. This enables the banding process. Banding is, in part, dependent on the spatial arrangement of the DNA allowing hydrogen bonding with the Wright stain to occur. If longitudinally separate sites are brought into close proximity to one another by folds in the DNA, hydrogen bonding can occur and banding is produced. In regions where the DNA chains become sufficiently dispersed, hydrogen bonding is prevented and no banding is produced. It is also postulated that the dark staining regions are rich in bases adenine-thymine (A-T). This technique is routinely used for detecting and defining chromosomal abnormalities. See Chapter 2, Slide-making, for slide preparation procedures.

II. Materials

Reagents

1. Trypsin solution 0.25%, Gibco® Invitrogen #KC21270
 - a. Aliquot 40 mL into 50 mL plastic capped conical tubes.
 - b. Label each aliquot "TRY" and place in 1 liter plastic beaker.
 - c. Label beaker as "0.25% Trypsin GIBCO," with lot number, dates of preparation and expiration, and preparer's initials.
 - d. Store frozen at -10°C to -20°C.
 - e. Discard in sink with running water at manufacturer's expiration date.
2. Isotonic buffered saline (sodium azide-free), Isoton II, 20 L, Beckman Coulter #8546719
 - a. Store at room temperature.
 - b. Discard in sink with running water at manufacturer's expiration date.
3. Double-distilled demineralized water
4. Gurr buffer tablets, pH 6.8, Liter, Fisher Scientific #NC9753709
 - a. Store indefinitely at room temperature.
 - b. Preparation of working solution: Dissolve one tablet in 1 liter double-distilled demineralized water in 1 liter volumetric flask.
 - c. Store at room temperature in stoppered volumetric flask.
 - d. Label as "Gurr buffer", with lot number, dates of preparation and expiration, and preparer's initials.
 - e. Discard in sink with running water after one week.
5. Fisherbrand filter paper, Fisher Scientific, P8-creped, #09-790-12G
6. Wright stain MCB, Sigma Aldrich #W0625-100G
 - a. Store at room temperature.
 - b. Discard in hazardous waste container at manufacturer's expiration date. Mark date, type and concentration of compound, quantity discarded, and initials on tag attached to safety can.
 - c. Preparation of stock solution
 - i. Dissolve 0.3 grams Wright stain in 200 mL absolute methanol in 500 mL bottle.
 - ii. Let stand for 9.5 minutes at room temperature.
 - iii. Filter using 18.5 cm Fisherbrand filter paper, filter into 500 mL dark (brown) bottle.
 - d. Label as "Wright stain", with lot number, dates of preparation and expiration, and preparer's initials.
 - e. Store in dark bottle at room temperature in OSHA-approved safety cabinet.
 - f. Discard stock solution after 2 months in hazardous waste container. Mark date, type and concentration of compound, quantity discarded, and initials on tag attached to safety can.

Equipment

7. AND balance
8. Pipetman
9. Mistral 2000 centrifuge, Rotina 35 centrifuge
10. Coplin jars
11. Staining dishes (500 mL)

III. Method

Trypsin banding

1. Dry slides on metal trays in 90 °C oven for 60 minutes before staining.
2. Fill three 500 mL staining dishes designated #1, #2, and #4 as follows:
 - #1. 40 mL 0.25% Gibco® trypsin solution + 360 mL isotonic buffered saline
 - #2. 400 mL isotonic buffered saline
 Fill Coplin jar designated #3 as follows:
 - #3. 10 mL Wright stain stock solution + 40 mL Gurr buffer pH 6.8
 - a. Make just before needed.
 - b. After 20 minutes, discard in hazardous waste container. Mark date, type and concentration of compound, quantity discarded, and initials on tag attached to safety can.
3. Select representative slides to be stained from each culture.
4. Place slide in dish #1 for 1-10 seconds; note time.
5. Dip slide five times in dish #2.
6. Place slide in Coplin jar #3 for 1 to 2 minutes; note time.
7. Dip slide ten times in dish #4.
8. Blow slides dry with stream of air from air valve fitted with rubber tubing.
9. Check banding quality microscopically using 80× high dry objective. Refer to NOTES section of this procedure for troubleshooting.
10. If no metaphases (suitable or unsuitable) are found after scanning the first 5 slides from each culture, cease scanning. Report as culture failure and complete Quality Control – Problem Investigation form.

IV. Notes

1. Checking stain quality
 - a. Check the first representative test slide stained using high-dry 80X objective on microscope to assess banding quality.
 - b. Tolerance limit: Strive for three dimensional chromosomes that demonstrate crisp banding patterns with variable contrast between light and dark regions of each chromosome.
 - c. Adjust drying and/or trypsin and/or staining times using another representative test slide.
 - d. Check slide microscopically after each succeeding adjustment.
 - e. Staining factors are influenced by type of specimen, length of chromosomes, humidity, temperature and length of time in drying oven.
2. Trypsin time adjustment
 - a. Increase or decrease trypsin time in increments of one second as needed.
 - b. Use a longer trypsin time if chromosomes have no visible bands.
 - c. Use a longer trypsin time if there is not enough contrast between light and dark regions. Note: If slide was dried too long, a longer trypsin time will not be effective, and crisp banding cannot be achieved.
 - d. Use a shorter trypsin time if chromosomes appear eaten away at edges or chromosomes are “puffy.”
 - e. Note: If trypsin time gets too short, dry slides longer.
3. Stain adjustment
 - a. Increase or decrease staining time in increments of 15 seconds as needed.
 - b. Use a longer staining time if contrast looks adequate, but chromosomes are too pale.
 - c. Use a shorter staining time if banding patterns are present, but there is poor contrast between dark and light regions.

4. Drying adjustment

- a. Increase or decrease drying time in increments of 30 minutes as needed.
- b. Use a longer drying time if chromosomes demonstrate undefined edges (fuzzy and bloated) with little or no banding.
- c. Use a shorter drying time if chromosome preparations require long trypsin times.
- d. Use a shorter drying time if chromosomes stain darkly with no bands visible.

V. Additional readings

1. Seabright M. A rapid banding technique for human chromosomes. *Lancet* 1971 (ii): 971–972.
2. Sumner AT, Evans HJ. Mechanisms involved in the banding of chromosomes with quinacrine and Giemsa. *Exp Cell Res* 1973; 81: 223–226.
3. Yunis JJ, Ranchez O. G-banding and chromosome structure. *Chromosoma* 1973; 44: 15–23.
4. Priest JH. *Medical Cytogenetics and Cell Culture*, 2nd ed. Philadelphia: Lea and Febiger; 1977.

CHAPTER 12

Cytogenetic methods and findings in human solid tumors

Marilyn Nelson

Human Genetics Laboratories, Munroe-Meyer Institute, University of Nebraska Medical Center, Omaha, NE, USA

12.1 Introduction*

12.1.1 Historical review

Scientists have attempted to unravel the mysteries of tumor pathogenesis for over a century. As early as 1914, German embryologist Theodor Boveri provided an amazingly insightful clue when he hypothesized that the defects causing cancer resided in chromosomes. He proposed that a disruption of the normal chromosomal balance in cancer cells might lead to malignant tumors [1,2]. It is now clear that tumor cells are characterized by genetic alterations that affect the regulation of normal cell development, cell proliferation, and other fundamental cellular activities. These alterations often manifest themselves cytogenetically as numerical or structural abnormalities. The Philadelphia (Ph) chromosome in chronic myelogenous leukemia (CML), first discovered in 1960 and later redefined in 1973, represented the earliest described nonrandom chromosomal abnormality in a human malignancy [3,4]. The 1960s and 1970s were a time of significant discoveries in solid tumor cytogenetics, with the detection of double minutes (dmin) and homogeneously staining regions (hsr) (see Figure 12.1) representing gene amplification in neuroblastoma [5,6], and the identification of a recurrent loss of chromosome 22 in meningioma [7,8]. In the 1980s the first nonrandom translocation in a solid tumor was identified in Ewing sarcoma, much like those seen in hematological malignancies [9,10]. Since then, cytogenetic studies have contributed significantly to our understanding of carcinogenesis, impacting both diagnostic classifications and prognostic parameters [11,12]. Solid tumors comprise a large portion of all human malignancies, but even with the tremendous advances that have been made in the past decades, only a small percentage of them have been cytogenetically catalogued. By mid-2006, clonal chromosomal aberrations had been reported in over 50,000 human malignancies; however, less than one-third of those were from solid tumors [13].

Cytogenetic characterization of solid tumors has lagged significantly behind that of leukemia and lymphoma primarily because of the feasibility of obtaining tissue and the technical problems associated with culturing them. These difficulties and the limited data available have restricted the use of cytogenetics in the clinical diagnostic study of solid tumors. Fortunately, alterations and adaptations of existing conventional cytogenetic protocols, and the development of molecular cytogenetic techniques, have significantly advanced our knowledge of solid tumor cytogenetics. Several pediatric tumors are associated with disease or prognosis-specific cytogenetic abnormalities. The most recent guidelines from the American College of Medical Genetics (ACMG) recommends cytogenetic testing for the majority of pediatric tumors as a result of the diagnostic, prognostic and therapeutic implications of specific cytogenetic abnormalities [14]. Identification of recurrent cytogenetic aberrations

* Editors' note: Analyzing cells with complex rearrangements is always a challenge. Although some markers in the photos in this chapter may appear to have potentially identifiable segments, cytogenetics is not based on guesses. Interpretation and karyogram placement in these examples have been based on the best judgment of the professionals working closely with that specimen; therefore, we editors and author have not changed that interpretation or karyogram placement in the examples provided.

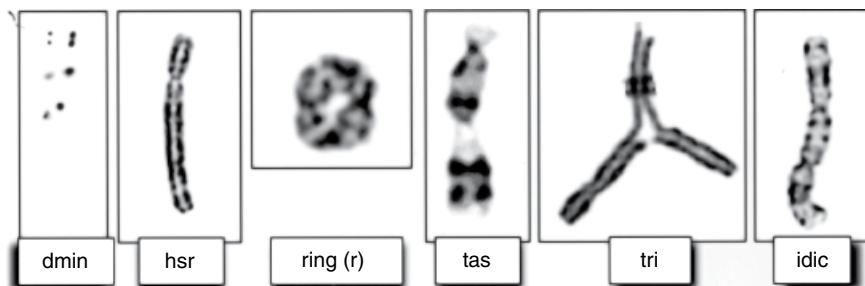


Figure 12.1 Unusual structures seen in neoplastic tissue. Shown here: dmin (double minute), hsr (homogenously staining region), r (ring), tas (telomeric association), tri (triradial), and idic (isodicentric).

is valuable because it not only provides substantial and sometimes decisive assistance with diagnostic dilemmas, but also serves as a basis for molecular approaches in determining the genes involved, with their associated consequences. It is commonly believed that gene alterations resulting from balanced structural rearrangements, especially when present as a sole abnormality, represent important early steps in the initiation of oncogenesis. Chromosome analysis is currently the primary method for identifying balanced rearrangements, such as translocations and inversions, and is therefore a valuable and critical tool for identifying pathologically relevant gene fusions.

12.1.2 Tumor classification

Human solid tumors represent a diverse group of neoplasms that can arise from various cellular components almost anywhere in the body. They include a vast array of more than 100 different diseases that may have little in common biologically except for their hyperproliferative nature, as reflected in their abnormal and uncontrolled cellular growth. In order to accomplish a successful cytogenetic study, it is important to appreciate the tumor type or diagnosis in terms of biology and growth patterns *in vivo*. Unfortunately for the cytogenetics technologist, tissues often arrive at the clinical laboratory without a diagnosis or with a differential that includes multiple diagnostic possibilities, each requiring their own optimal culture conditions. In spite of this, being knowledgeable of some basic principles will increase the chances of success.

Solid tumors are generally grouped into benign or malignant forms, based upon their biological behavior and potential for morbidity. Benign neoplasms do not invade surrounding tissues and have a low rate of local recurrence. Typically, benign neoplasms are characterized as not life-threatening; however, a few, due to size or anatomic location, can cause life-threatening complications. As a general tenet, benign solid tumors contain fewer mitotic cells and thus may take longer to proliferate in culture. Lipomas (benign adipose tumors), however, are often an exception to this principle and can grow very quickly *in vitro*.

A subset of benign neoplasms may be at risk for progression into a malignant state. The term malignant is applied to tumors that show aggressive behavior characterized by local invasion or destructive growth. They tend to have a higher risk for recurrence and are capable of metastasis. An example of the progression of a benign lesion to a malignant neoplasm is the transformation of a nevus to melanoma [15]. There are also borderline lesions in which it is difficult to ascertain malignant potential.

Neoplasms are named by their histopathological appearance and by their cell of origin or the adult tissue they resemble. The following describes the general categories of cancers:

- Leukemia and lymphoma, involving blood and bone marrow (see Chapter 11, Cytogenetic analysis of hematologic malignant diseases).
- Sarcoma, involving connective tissue; carcinoma, involving epithelial cells; mesothelioma, involving the linings of the peritoneum, pericardium and pleura; and glioma, involving glial cells.
- Germ cell tumors, involving germ cells of testicular or ovarian origin; and choriocarcinoma, involving placental tissue.

Sarcomas encompass tumors that arise from mesenchymal tissue, such as muscle, bone, fat, fibrous tissue, cartilage, and blood vessels. These are relatively rare cancers, accounting for less than 1% of all newly diagnosed cancers [16]; they occur more often

in adults than in children, and although they can appear almost anywhere, they are more likely to arise in the extremities. Cytogenetic information is available on numerous sarcomas, but since there are more than 50 soft-tissue sarcomas or sarcoma-like growths alone, and some can be extremely rare, complete cytogenetic characterization has been challenging. Because several sarcomas have shown disease-specific translocations similar to those seen in hematologic malignancies, cytogenetic studies are indicated.

Carcinomas, by contrast, are ectodermal or endodermal in origin and arise in skin or in the tissue that covers internal organs or glands. Their development is initiated by an array of stepwise genetic changes, which is reflected in complex cytogenetic abnormalities and frequent loss of specific chromosome regions. In the United States, the most common carcinomas involve the prostate, breast, lung/bronchus, and colon/rectum. Cancers of the lung and bronchus, for example, were expected to be the leading cause of cancer-related fatalities in 2013, with an estimated 87,260 male and 72,220 female deaths [16]. Pancreatic cancer was projected in 2013 as the fourth leading cause of cancer-related deaths in the United States [16].

The rules of nomenclature for solid tumor classification are not without their exceptions. Although the terms *sarcoma* and *carcinoma* are traditionally applied to malignant tumors, they do not always define a neoplasm with a destructive nature or a likelihood of metastasis, but instead these neoplasms may sometimes follow a benign clinical course. For this reason, it is essential to further qualify these terms with statements in regard to the degree of differentiation or histological grade. In general terms, low-grade lesions tend to be well differentiated, whereas high-grade tumors are often poorly differentiated. Severely anaplastic tumors may become so undifferentiated and the cells so primitive in appearance that they become difficult to classify by purely morphological means. In these instances, cytogenetic studies may provide insight into their classification.

Cytogenetic studies have historically helped classify tumors by:

1. identifying specific changes in histological subtypes, such as those seen in lipomatous tumors [17];
2. recognizing etiological connections among histologically different lesions that involve identical chromosome changes, such as those seen in dermatofibrosarcoma protuberans and giant cell fibroblastoma [18,19];
3. distinguishing subsets of clinical phenotypes within specific tumor classifications, such as alveolar rhabdomyosarcoma [20,21];
4. providing compelling evidence for its true neoplastic nature in lesions of uncertain etiology, such as aneurysmal bone cyst [22].

Further advancements in the classification of solid tumors will be aided by the continued success of cytogenetic studies.

12.2 Processing tumor specimens

12.2.1 Culture of tumor cells

Successful culture and cytogenetic analysis of solid tumors have traditionally been problematic and remain so today, even with the advances in methodology over the past years. It can be surprising and very disappointing to find that tumors that readily thrive *in vivo* fail to grow at all *in vitro*. A major obstacle for the clinical laboratory offering these tests is to establish a protocol with enough flexibility to handle the great diversity of solid tumors, while still providing a time-efficient and cost-effective service. Developing standardized methods that will produce reasonably reliable and reproducible results when performed by more than one technologist is therefore a continuous challenge to the cytogenetics laboratory.

12.2.2 Specimen requirements

Ideally, the size of a tumor specimen should be 1–2 cm³, and it should preferably be part of the same sample being submitted to pathology. However, due to the increased use of fine-needle percutaneous approaches for tumor sampling, cytogenetic laboratory management have had to adapt their protocols to include fine-needle aspirates or needle core biopsies. Samplings of this size can be successful, but the number and variety of cultures available for each given specimen is limited. Certain neoplasms, such as cartilaginous or benign fat tumors, have a low cell density per volume unit, making successful cytogenetic studies of minute samples more difficult. In large tumors of heterogeneous or varied appearance, or in instances where the tumor encroaches into multiple sites, it may be advisable to submit samples from each area. These samples should be given a unique designation and processed separately.

Mixtures of neoplastic and non-neoplastic cells are routinely found in solid tumors. The non-neoplastic component can include normal stroma or the connective supportive framework for the tissue or organ. The degree of stroma present will vary among tumors, and in certain instances, these cells may proliferate more readily in culture than the neoplastic cells.

A recurrent problem in tumor culturing is not only distinguishing between the “normal” or inflammatory tissue from the tumor, but also the presence of nonviable cells that can interfere with successful growth. Solid tumors, notably those of a malignant nature, often contain areas of necrosis and/or fibrosis; therefore, it is imperative to select the best possible tissue for cytogenetic analysis. This chapter will introduce certain culture techniques, which may help reduce the overgrowth of the non-neoplastic component and optimize cell viability.

12.2.3 Specimen transport

In order to obtain metaphases, a specimen must be viable. Fixed or frozen tissue cannot be used for cell culture and classical cytogenetics. Transport time should be kept to a minimum, preferably with receipt being on the same day, and storage during transportation should protect the tissue from extreme temperature exposure. In particular, delay of pediatric or neural tissue may be detrimental, due to the specimen’s fragility.

The tumor specimen should be transported in supplemented tissue culture medium at room temperature or stored at 4°C. Refrigeration will help control microbial contamination and reduce enzymatic damage induced by dying and necrotic cells. Several laboratories, however, avoid refrigeration of neoplastic specimens because it may be damaging to some cell types. It has also been suggested that incubation at 37°C is advantageous when the specimen cannot be processed immediately.

Media, such as RPMI 1640, MEM, and Ham’s, or sterile isotonic solutions, such as Hanks’ balanced salt solution (HBSS), may be used as transport medium for solid tumors. Supplementation with a minimal amount of FBS (~5%) and antibiotics (~1%) is often preferred (see exceptions under 12.2.4, Specimen receipt). A fungicide may also be used, if deemed necessary. If the specimen is to be transported immediately and media is not available, placement of the tissue onto sterile saline-soaked gauze may be acceptable. The tissue, however, should not be allowed to remain in saline for extended periods. Ringer’s solution may also be used if transportation time is minimal.

Because solid tumors are procured via invasive surgical procedure and are an irreplaceable source for cytogenetic information, every attempt should be made to initiate a culture as quickly as possible upon specimen receipt. Although delayed transportation is not recommended due to the critically high risk of failure, we have successfully cultured tumor specimens received 2–4 days after collection when the specimen was transported correctly. Careful monitoring of cell morphology, however, must be exercised in order to ensure that tumor cells, and not their surrounding normal stromal tissue, are being cultured.

12.2.4 Specimen receipt

Universal Precautions should be observed at all times (see Chapter 20, section 20.2, Biological hazard safety). Specimen sterility must be maintained, and all work should be performed in a laminar flow hood. The laboratory should be notified if sterility had been compromised during tissue collection so that appropriate action can be taken to minimize potential contamination.

Keep the exposure to antibiotics or fungicides to a minimum. Many laboratories include broad-spectrum antibiotics at final concentrations of 1% in the culture medium to prevent contamination. However, it is felt by some laboratories that this may inhibit the growth of some tumor cells. On the other hand, if the tissue was collected from a body region that is suspected to have a high concentration of bacteria (e.g., bowel, nasal passage), soak the specimen in a stronger antibiotic solution prior to processing. (See Chapter 4, section 4.2.8, Antimicrobial agents and monitoring for contamination, for more information on controlling microbial contamination.)

Before dissociation, the tissue should be examined, rinsed, and cleared of as much blood and debris as possible. Balanced salt solutions may be utilized as a rinse to wash tissue of myxoid material. Rinsing cell suspensions in large volumes of salt solution will also help to dilute mucus, which will, in turn, provide a tighter cell pellet after centrifugation. Normal, fatty, connective, and obviously necrotic tissue may be excised as carefully as possible; however, due to the tremendous difficulty in differentiating “normal” from tumor tissue, caution should be exercised and when preferable, the entire sample should be processed.

It may be advisable to snap freeze a small portion of the tissue whenever possible. The snap frozen tissue has proven to be valuable due to the increased number of molecular and FISH tests available for solid tumors. It may also be used for further morphological studies, if necessary (see Chapter 4, section 4.3.7, Freezing and storage of cell lines).

12.2.5 Culture media and supplementation

There are several media options available for culturing solid tumors. The one most commonly used is RPMI 1640 medium. Other suitable media include Ham's F10, Ham's F12, MEM, MF (*Mitogen Free*), McCoy's, Chang or a combination of two or more. Standard protocols require supplementing media with 10–20% fetal bovine serum (FBS), 1% penicillin/streptomycin (pen/strep) and 1% L-glutamine. HEPES may also be added at a final concentration of 1% to help maintain a balanced pH. It has been established that serum significantly inhibits the growth of some epithelial cells and stimulates fibroblasts to a high extent [23,24]. In spite of this, serum-free medium is rarely used in most cytogenetic laboratories, perhaps because serum has also been shown to improve cell attachment, even for those cells that are sensitive to its effects later in culture [25]. For specific tumors, however, special media have been developed, such as, HITES (serum-free medium supplemented with hydrocortisone, insulin, transferrin, estrogen and selenium) for small lung cell carcinoma or N3 for neuroblastoma [26,27], but due to their limitations, they are not recommended for general use.

12.2.6 Culture vessels

For tissue culture purposes, solid tumors can be placed into the following three general categories: (1) those that grow attached to a substrate as a monolayer with cell to cell connections; (2) those that are anchorage-independent and grow in suspension; and (3) those that may have both cell populations. The category you choose for a specific tumor is dependent on the tumor type or diagnosis, not the anatomical site. Open communication between the pathology and cytogenetics laboratories is therefore important in order to obtain the best possible sample and the most accurate information.

When a diagnosis is not readily available at the time the specimen is received in the laboratory, it may be useful to incubate the specimen in a collagenase/medium mixture overnight and check with the pathologist for more information the following day. Table 12.1 provides a partial list of indications and their preferred culture method. If no diagnostic information is available at culture initiation, it is best to initiate both attached and suspended cultures when possible.

Attached cultures are generally inoculated into flasks (T-12.5 or T-25) or onto 22 mm² coverslips, depending upon the laboratory's preferred harvest method (i.e., flask or *in situ*). Both methods have advantages and disadvantages. *In situ* cultures generally require less material per vessel, provide earlier harvests, and retain nuclei morphology and growth patterns following harvest. The ability to observe the morphology of fixed nuclei *in situ* cultures can be useful during the analysis stage in distinguishing tumor from normal fibroblast growth (see Figure 12.2).

Flask cultures may require a longer period of time to reach optimal confluence for harvesting, but they have the advantage of being able to capture all "free-floating" and attached cells with the same harvest. The larger fixed cell pellet from a flask harvest also affords more flexibility when cells are difficult to spread, and provides a greater quantity of material for reflex testing, if needed. Flasks with vented caps can also be kept tightly closed to help avoid contamination. Other culture vessels, such as chamber slides or flasks, may also be used. Whenever possible, it is best to initiate both *in situ* and flask cultures in order to get the benefits from both methods.

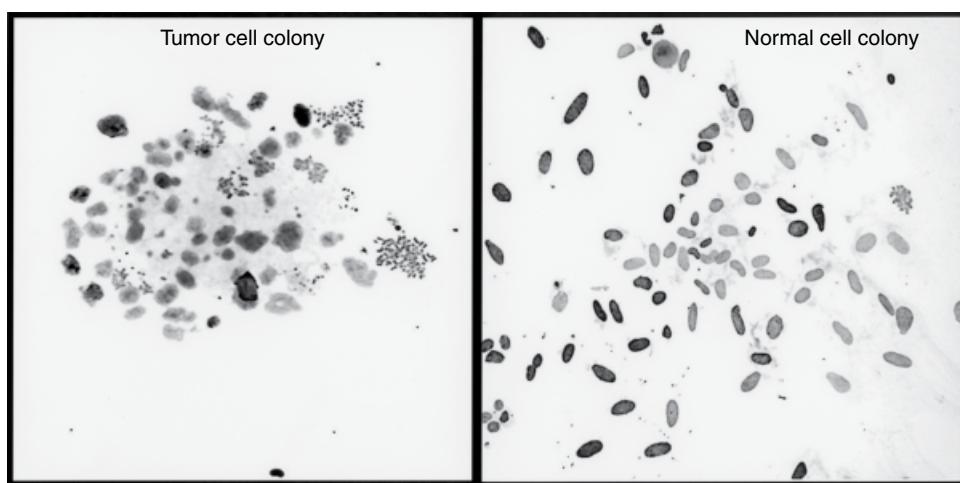
Cells propagated as a suspension culture may also be grown in a flask or Petri dish, or in a 15-mL conical tube. The type of culture vessel used will be dependent upon the amount of sample available. In lieu of a separate suspension culture, our laboratory routinely performs a "supernatant harvest" after cell attachment (typically at day 2 or 3) by collecting the media from the attached cultures, refeeding culture vessels with fresh media for continued growth, and then harvesting the removed free-floating cells. This method is useful when the amount of sample is limited, and both attached and suspension cultures are needed. A supernatant harvest, however, should not replace suspension cultures in tumors that require higher cell concentration, such as lymphomas or plasmacytomas.

12.2.7 Tissue dissociation

Dispersal of solid tissues into mixtures of single cells and small cell clusters is necessary before culture initiation. One of the first established cell culture types is the explant method, which involves disaggregating the tissue by either mincing it with sterile scalpels or curved scissors, pipetting it forcefully several times, passing it through a sterile steel mesh or sieve, pulling it apart with forceps, or "mashing" it with forceps. Following disaggregation, the fragments are placed onto a glass or plastic surface, where cells can migrate out from the tissue. Scoring the surface of the culture vessel with a scalpel after mincing the specimen also helps promote cell adhesion. If the tissue is easily dissociated or the sample is small, as in needle biopsies, these methods will reduce cell loss and may help minimize stromal contamination, since stromal cells are often locked in fibrous connective tissues. The explant method, however, is not very effective for cells with poor adhesion. Some tumors may also not readily disaggregate by mechanical means and may benefit from enzymatic dissociation. If, however, a direct harvest is

Table 12.1 Recommended culture methods for specific solid neoplasms

Tumors grown in monolayer	
Adenocarcinoma	Leiomyosarcoma
Astrocytoma	Lipoma
Basal cell carcinoma	Liposarcoma
Chondroma	Malignant fibrohistiocytoma
Chondrosarcoma	Malignant melanoma
Clear cell sarcoma	Mesothelioma
Ependymoma	Myxoma
Fibroadenoma	Myxosarcoma
Fibroma	Nevus
Fibrosarcoma	Oligodenrogloma
Glioma	Oncocytoma
Glioblastoma	Prostatic hyperplasia
Hemangiopericytoma	Renal cell carcinoma
Hemangioma	Squamous cell carcinoma
Hemanigiosarcoma	Synovial sarcoma
Infantile fibrosarcoma	Teratoma
Leiomyoma	Wilms tumor
Tumors grown in suspension	
Germ cell tumor	Neuroblastoma
Lymphoma	Plasmacytoma
Medulloblastoma	Small cell lung carcinoma
Tumors grown in monolayer/suspension	
Choriocarcinoma	Osteosarcoma
Cystadenocarcinoma	Peripheral primitive neuroectodermal tumor (PPNET)
Desmoplastic small round cell tumor	Retinoblastoma
Embryonal carcinoma of testis	Rhabdomyoma
Ewing sarcoma/ PNET/Askin	Rhabdomyosarcoma
Hepatoblastoma	Seminoma
Osteoma	

**Figure 12.2** Examples of cell morphology following in situ harvest. (a) Colony of tumor cells from malignant fibrous histiocytoma culture. Note irregular shape, grainy appearance and overlapping growth pattern. (b) Growth of normal fibroblast from same in situ coverslip.

indicated (see 12.2.12, Determination of harvest time), transfer the previously released cells to another vessel or centrifuge tube **before** proceeding with enzymatic treatment.

Owing to increased efficiency, enzymatic digestion in combination with mechanical disaggregation has proven to be preferable when processing the majority of human tumor samples. The length of incubation in a specific enzyme is, of course, dependent upon the type and concentration of enzyme used and the type of tissue being disaggregated. Crude collagenase is the enzyme preferred by many laboratories since it causes minimal cell damage, is more efficient on fibrous tissue and remains unaffected by the presence of serum. Generally, the tissue must first be minced and then incubated with the enzyme solution in a flask or Petri dish, typically at 37°C in 5% CO₂ for 20 minutes to 16 hours. Many types of collagenase are available and correspond to the specific type of collagen on which they are most effective [28]. More information on the type, concentration and length of incubation with collagenase may be found in the protocols at the end of this chapter. You may want to experiment to find an incubation time that is “preferable for your laboratory operations.”

Trypsin or pronase may be used for this purpose; however, these proteolytic enzymes have been shown to be less effective against fibrous connective tissue, and trypsin seems to cause more cell death [28]. If using trypsin for digestion, the tissue must first be rinsed in unsupplemented media or balanced salt solutions to remove all traces of serum, which will interfere with enzymatic activity. After dissociation has occurred, trypsin can be inactivated with supplemented medium.

12.2.8 Cell dilution and culture initiation

Following enzymatic digestion, the cell suspension should be examined under a phase microscope to ensure that sufficient dissociation has occurred. Small tissue clumps can typically be dispersed by pipetting the tissue several times. The dispersed cells can then be transferred to a centrifuge tube with additional medium (unsupplemented medium may be used on collagenase suspensions), centrifuged, and the supernatant discarded. Some cells may attach to the vessel during enzymatic digestion; therefore, add fresh medium to the flask or Petri dish that was used for disaggregation and retain as a primary culture.

It is important to also take note of tissue viability. To ascertain the percentage of viable cells, you can stain a small portion of the cell suspension with Trypan blue (see Protocol 12.5, Solid tumor culturing and harvesting) to help determine the most accurate dilution for culture initiation. If cell counts are performed, a typical dilution may be approximately 10⁶ cells per 5 mL. Sarcomas generally have a higher plating efficiency than most carcinomas.

For the establishment of monolayer cultures, it is important to plate the vessels with a minimal volume of cell suspension in order to allow for easier cell attachment. Traditionally, coverslips are inoculated with 0.5 mL (total volume) and T-25 flasks with 1.5 mL (total volume) of sample + medium. Solid tumors differ tremendously in proliferation capabilities; therefore, it is beneficial to use various dilutions of the cell mixture for each culture in order to obtain optimal concentration. The *in situ* method allows more opportunity to experiment with dilution, as less inoculant volume is needed per culture. After set-up, the cultures are incubated in a 37°C, 5% CO₂ humidified incubator.

12.2.9 Culture maintenance and duration

Suspension cultures typically do not require additional manipulation before harvest unless a color change to clear or yellow is observed in the medium. If there is no evidence of contamination, this color change most likely indicates depletion of media nutrients, in which case, either the culture should be split or additional medium should be added.

Flooding (see Chapter 4, section 4.3.4, Routine handling and maintenance of monolayer cultures) or feeding monolayer cultures with additional media should not be done until attachment or growth is observed using a phase microscope. This may range from a couple of hours to several days, depending on cell density and proliferation rate. Generally, you need to add 1.5 mL of additional medium to a coverslip culture and 2.5 mL to a flask culture. If no attachment is observed after several days, cell density may be too low or cells may be growing in suspension. If nonattachment is a recurring problem, it may be beneficial to explore the use of different types of coated flasks (e.g., collagen).

If growth within *in situ* cultures appears too dense when flooding, you can perform a vigorous rinse with fresh medium. Often cell attachment is still weak at this point, and you may be able to reduce culture density without the use of trypsin. If this rinse is unsuccessful, partial trypsinization or a “trypsin rinse” may be employed to thin out *in-situ* culture growth. Whenever possible, subculturing should be avoided with solid tumors, as this often stimulates the overgrowth of contaminating normal fibroblasts.

Daily monitoring of monolayer cultures is important. Changing the media and rinsing the cultures is done as needed to eliminate blood and cellular debris. If nonviable or deteriorating cells are left in the culture, they can produce proteases that will harm the remaining viable tumor cells. You will also want to take note of the patterns of cell growth. Neoplastic cells

exhibit various growth patterns and shapes in culture. Some tumors have a “spindle-shaped” appearance, grow in orderly fashion and are difficult to distinguish from a normal fibroblast culture. Others may grow in a tight cluster or “grape-like” formation, and still others in a monolayer with a somewhat chaotic pattern. Examples of tumor culture morphology can be seen in Figure 12.3.

As previously discussed, neoplastic cells contain genetic mutations that affect normal cell processes that, in turn, may result in altered cell cycles. They will thus grow at their own pace, whether it is a couple of days or several weeks. However, when there is a mixture of tumor and “normal” fibroblast cells, it is important to harvest the cultures before the fibroblast component overtakes the culture.

12.2.10 Mitotic arrest

Harvesting chromosomes is initiated by the addition of a mitotic spindle toxin that will arrest cells in metaphase (see Chapter 2, section 2.3.5, Mitotic arrest: Colcemid®). Colcemid® is the reagent of choice for many laboratories. The concentration (typically 0.01–0.05 g/mL) and length of exposure to Colcemid® (typically 45 minutes to 16 hours) can vary greatly for each laboratory and type of tissue. A concentration of 0.02 µg/mL overnight works for many tumors. Tumors with very slow growth rates may be harvested following two days of Colcemid® exposure. Remember, though, that longer Colcemid® times result in shorter chromosomes. Other examples of concentration and exposure time can be found in the protocol section at the end of this chapter (see Protocol 12.1, Solid tumor cell culture and harvest).

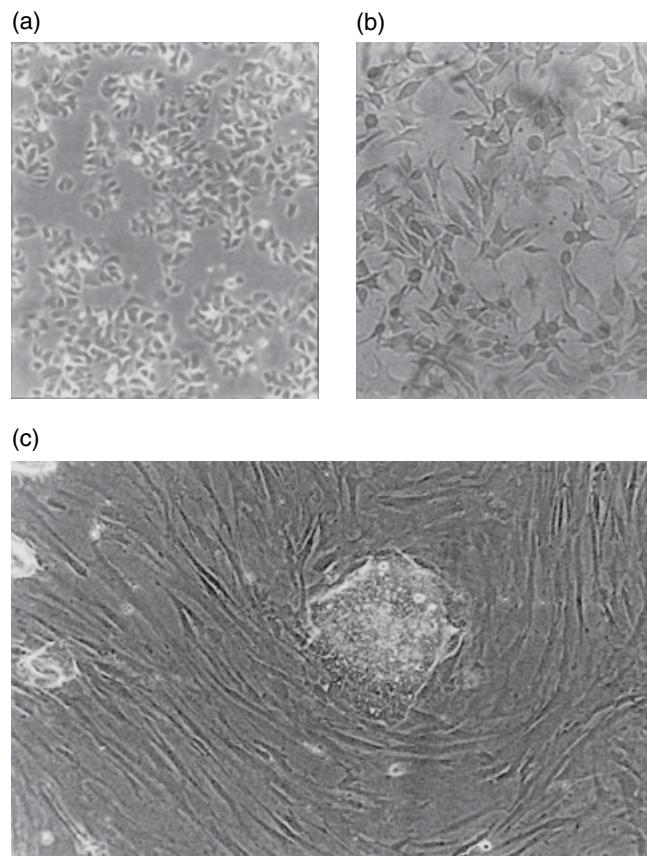


Figure 12.3 Examples of different tumor types with different growth patterns grown in vitro: (a) squamous cell carcinoma of lung, (b) a metastatic cutaneous malignant melanoma, and (c) a colony of salivary gland tumor cells surrounded by spindle-shaped normal fibroblasts interspersed with occasional tumor cells.

12.2.11 Cell synchrony

Additional methods may be utilized in an attempt to improve the quality and quantity of metaphase cells. To increase the number of metaphases, cell cycles can be synchronized by adding a methotrexate “block,” followed by a thymidine “release” prior to the addition of Colcemid® [29]. Tumors originating from patients who have received chemotherapy may exhibit resistance to methotrexate and Colcemid®. In such situations, the concentration of Colcemid® may need to be increased in order to disrupt the spindle fibers within cells undergoing mitosis [30].

Ethidium bromide (final concentration 0.01 µg/mL) may be added with Colcemid® to the cultures 1–2 hours before harvest in order to reduce the degree of chromatin condensation and produce elongated chromosomes. Some laboratories use BrdU (5-bromo-2'-deoxyuridine) or FUdR (5-fluoro-2'-deoxyuridine) overnight to yield chromosomes with higher band resolution (see Chapter 2, section 2.3.9, Chromosome anticontraction methods).

12.2.12 Determination of harvest time

As a general rule of thumb, it is best to harvest cells as soon as possible so that the tumor cells are not overgrown by stromal cells and fibroblasts. Cultures grown in suspension may be harvested at 24, 48, or 72 hours. Direct or same day harvest may also be performed on some specimens. For example, effusions (e.g., ascites and pleural fluids) and cells from fine needle aspirates from some tumors, such as Ewing sarcoma, can have a high mitotic index and are, therefore, suitable for a direct (same day) or overnight harvest. Because direct harvests will capture cells entering mitosis shortly after the sample is received in the laboratory, they circumvent chromosomal aberrations and polyploidy due to cultural artifact and thus provide a more accurate representation of the *in vivo* state of the tumor.

To perform a direct harvest, cells that have been freely liberated from the tissue after mechanical dissociation are collected **before** enzymatic digestion. Colcemid® can be added either directly to the fluid that contains the freely liberated cells or to a 1 : 1 mixture of fluid and culture medium. For the overnight culture, the sample can be centrifuged and the cells incubated overnight in supplemental medium. Colcemid® would then be added either for overnight exposure or on the following day, for a shorter period of time. If the specimen is bloody, it is advisable to add heparin before centrifugation in order to prevent clot formation [28]. Unfortunately, not all direct harvests yield metaphase cells, and when they do, chromosome morphology can be poor.

The harvest of monolayer cultures should be performed when peak mitotic activity is observed. This often occurs in 3–5 days, and is determined by the presence of numerous dividing cells that are typically round, refractile, and three-dimensional or have a “beach-ball” appearance (see Figure 12.4). Whenever possible, it is preferable to harvest cultures on multiple days for each tumor specimen in order to be capable of evaluating and modifying harvest parameters when needed, such as Colcemid® concentration, length of exposure to Colcemid®, and drying methods. Harvesting cultures on different days also provides an opportunity to capture cell populations with varying cell cycles.

Following the desired Colcemid® exposure, cells that are attached to the flask surface must be either enzymatically removed with trypsin-EDTA or mechanically removed with a cell scraper (also called a rubber policeman). Many laboratories have found the use of trypsin to be the most effective method, since cell scrapers can be too rough on fragile tumor cells. Saving the media that was withdrawn from the flask to the harvest tube will allow any free-floating tumor cells to be included in the harvest. To avoid the potential loss of unattached cells that are floating within *in situ* coverslip cultures, the medium within the dish can be collected immediately prior to the harvest and processed separately.

For epithelial tumors, some laboratories utilize a sequential harvest with partial trypsinization of the culture following Colcemid® exposure. This is thought to not only help remove fibroblasts [31], but may also boost the growth of malignant epitheloid cells.

12.2.13 Hypotonic treatment

A variety of hypotonic solutions are used successfully on solid tumor cultures, including potassium chloride (0.067–0.075 M KCl) and sodium citrate (0.7–0.8% Na₃C₆H₅O₇). The majority of laboratories use 0.075 M KCl for approximately 20 minutes.

Timing of hypotonic exposure is very important. If left too long, the hypotonic will cause the cells to burst, resulting in metaphase cells with inaccurate chromosome counts (“chromosome soup”). If the spreading and/or banding quality of metaphase cells is unsatisfactory, it may be helpful to experiment with other hypotonic solutions, concentrations and times of exposure [28,32]. (See Chapter 2, section 2.3.6, Hypotonic treatment, for an explanation of cell membrane response to hypotonic solution.)

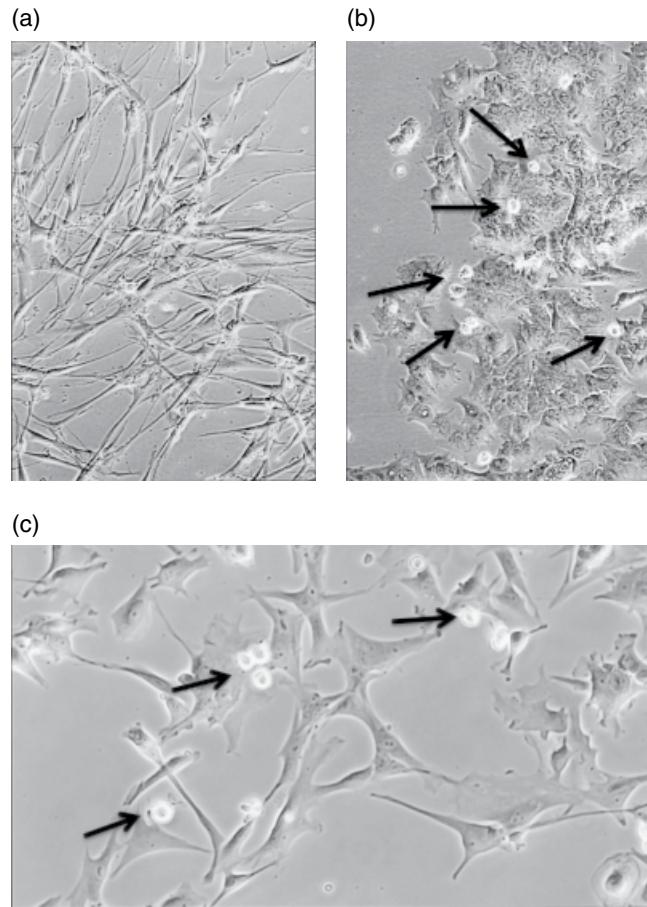


Figure 12.4 Dividing cells ready for harvest. Arrows indicating dividing cells in monolayer culture with round and three-dimensional or “beach-ball” appearance.

12.2.14 Fixation

The fixation step is used to terminate the swelling action of the hypotonic. The standard fixative is 3 : 1 methanol–glacial acetic acid; however, if chromosome spreading is a problem, changing the percentage of glacial acetic acid to 20% or 33% may help. A pre-fix step in which 2 mL of fixative is added to the hypotonic solution and allowed to equilibrate for 10 minutes before replacement with new fixative may be beneficial. Three fixative steps are adequate for most cultures; however, if the culture contains excessive debris or is unusually bloody, additional fixation should improve the quality of the metaphase cells. Extended incubation at 4°C or –20°C may also improve chromosome morphology.

12.2.15 Slide preparation and staining

Slides are prepared from the fixed cell pellet of the flask harvest for subsequent staining and analysis. Following proper dilution (see Chapter 2, section 2.4, Slide-making), cell suspension is dropped onto glass slides and dried under conditions that allow for an adequate number of well-spread metaphase cells with a minimal number of overlapped chromosomes. Many laboratories use cold, wet slides in order to achieve optimal spreading. Optimizing the drying rate for the *in situ* cultures can be difficult when seasonal changes affect ambient temperature and humidity. Controlling these parameters, along with airflow over the cells, is the best way to regulate the degree of chromosome spreading. Many laboratories use a self-contained drying chamber to provide consistency when making slides from fixed cell pellets.

After the slides are prepared, they are typically aged for one hour at 90°C or overnight at 60°C to enhance chromosome G-banding. Giemsa stain, introduced in the 1970s, was one of the first methods used to produce G-banded chromosomes and

is still utilized today; however, care must be taken not to destroy chromosome morphology with the required trypsin digestion [33,34]. GTW with Wright stain, described by Yunis in the 1980s, is now preferred by many US laboratories for G-banded preparations, due to the fact that it is easy to use, retains the morphology of chromatin material, such as double minutes that are easily lost to overdigestion, and is easily removed for subsequent studies, such as FISH [29]. Staining with Giemsa or Wright stain without the use of trypsin may also be used to more easily demonstrate the presence of double minutes. European laboratories often utilize R-banding, so that the lightly stained material with G-banding becomes darkly stained. Detailed information on the advantages of these and other staining procedures is provided in Chapter 6, Chromosome stains.

12.2.16 Chromosome analysis

Cytogenetic analysis of solid tumor samples can be very challenging, even for the most experienced cytogeneticist. Unlike studies on hematological specimens, cytogenetic studies on solid tumors are not routinely performed to monitor disease progression or treatment response, but are instead utilized for diagnostic purposes at initial diagnosis or at tumor recurrence or metastasis. Poor morphology, an inadequate number of metaphase cells, multiple random abnormalities and extremely complex karyotypes, such as those observed in highly malignant tumors, are only a few of the difficulties encountered in the analysis process. Misrepresentation of the true tumor karyotype can be influenced not only by the growth of normal stromal tissue, but it can also stem from ascertainment bias, that is, when the tissue received for cytogenetic analysis had been sampled from or includes a nontumor segment of the tissue. Careful evaluation should thus be exercised when the karyotype of a suspicious tumor is normal or mosaic normal.

Cytogenetic changes in solid tumors can range from simple to extremely complex. In cases where extensive chromosomal abnormalities are present, the use of the abbreviations "mar," indicating a marker chromosome of unknown origin, and "inc," indicating an incomplete analysis, frequently becomes necessary. Although increased cytogenetic complexity generally correlates with increasing histological grade, some decidedly aggressive tumors, such as Ewing sarcoma, can be characterized by a sole balanced translocation; thus, simple karyotypic changes might not be indicative of a less aggressive tumor. Tables 12.2 and 12.3 provide a summary of the recurrent cytogenetic abnormalities found in many mesenchymal and nonmesenchymal tumors.

A minimum of 20 banded metaphase cells should be counted and analyzed, if possible. Analysis should be attempted on cells of varying morphology and ploidy. Even cells of suboptimal quality may provide information on chromosome counts and obvious structural abnormalities. Complete characterization of multiple cell lines may also require the analysis of additional metaphase cells. When separate cultures are established in order to examine time-dependent growth pattern variability, an effort should be made to examine each culture.

Whenever possible, clonal abnormalities should be documented from two independent cultures. Two karyograms should be prepared from each clone, and one additional for each sideline and normal cell line, if present. Keep complete descriptions and/or photographs of seemingly random abnormalities, as it is not uncommon to find that a seemingly rare abnormal cytogenetic finding will actually turn into a true clone, stemline, or sideline, when enough cells have been analyzed. These seemingly rare cells, for example, may be dividing so slowly that they become significantly outnumbered in culture by normal cell growth. In some cases, however, these abnormalities are indeed random and prove to be meaningless noise. Likewise, certain specimens may present with numerous non-clonal abnormalities, including radiation-induced chromosomal breakage, rings and fragmentation. The best way to document the karyotypic instability of the tumor in this case is to make a general statement.

As a result of genetic instability, neoplastic cells undergo clonal progression, frequently causing increasingly abnormal karyotypes [35,36]. Karyotypic abnormalities in the stemline, or most basic clone, are thought to be of primary importance to initial tumor development, while those seen in subsequent sidelines may play a role in tumor progression. The distinction between primary and secondary abnormalities is important, but is often difficult to determine in tumors exhibiting multiple clones.

Compromising between the ideal and the practical is sometimes necessary in order to present a meaningful karyotype. In instances where the modal number cannot be accurately determined due to suboptimal chromosome morphology and karyotypic heterogeneity among metaphase cells, a composite (cp) karyogram, combining multiple clones into a single description, may help describe structural and numerical abnormalities that had been observed in two or more cells (chromosome gain or structural aberrations) or three or more cells (chromosome loss), but not necessarily in all cells. In addition, when abnormalities are extensive and cannot definitively be identified to be of any benefit to the patient, analysis can be completed on ten cells (see Chapter 8, section 8.7.10, Composite karyotype).

Another facet of solid tumor complexity is clonal heterogeneity among seemingly unrelated clones, an observation most likely due to the tumor being polyclonal in origin. It is important to remember that not all cells within a given tumor may proliferate or produce metaphase cells in culture; therefore, cytogenetic clones that were observed may represent only a portion of what is actually present.

General statements can be made regarding the nonrandom cytogenetic changes observed in solid tumors. As with other neoplasms, abnormalities involving chromosome 1 are common to many tumor types. Gain of chromosome 7 is one of the most

Table 12.2 Recurrent cytogenetic abnormalities of mesenchymal tumors

Tumor	Rearrangement	Chromosome gain	Chromosome loss	Loss of heterozygosity	Genes implicated
Tumors of soft tissue					
Lipoma	t(3;12)(q27;q13-15) t(12;V)(q13-15;V) t(6;V)(p21;V)	13q			<i>LPP-HMGA2</i> <i>HMGA2</i> <i>HMGA1</i>
Inter-intramuscular lipoma		Rings (1p21,12q15-21)			
Spindle cell /pleomorphic lipoma		13q 16q			
Lipoblastoma	t(8;V)(q11-13;V)				<i>PLAG1</i>
Hibernoma	t(11;V)(q13;V)		11q		<i>MEN1, GARP</i>
Chondroid lipoma	t(11;16)(q13;p13)				
Well-differentiated liposarcoma/ Atypical lipoma		Giant markers, rings (12q13-15, 12q21.3-22)			<i>MDM2, HMGA2,</i> <i>CDK4, SAS, GLI1</i>
Myxoid/round cell liposarcoma	t(12;16)(q13;p11)* t(12;22)(q13;q12)				<i>DDIT3-FUS(TLS)</i> <i>DDIT3-EWS</i>
Nodular fascitis	t(15;V)(q7;V) t(3;V)(q2 ⁻ ;V)				
Desmoplastic fibroblast (fibroma of tendon sheath, 1 case)	t(2;11)(q31-32;p13)				
Low grade fibromyxoid sarcoma	t(7;16)(q33;p11.2)				<i>BBF2H7-FUS</i>
Solitary fibrous tumor	rea(9)(q22-q31)				
Congenital/infantile fibrosarcoma	t(12;15)(p13;q25)				<i>ETV6-NTRK3</i>
Dermatofibrosarcoma protuberans	t(17;22)(q22;q13.1) or r(17;22)	8, 11, 17, 20			<i>COL1A1-PDGFB</i>
Giant cell fibrosarcoma					
Superficial fibromatoses		7, 8		γ	
Desmoid-type fibromatoses		8, 20			
Elastofibroma	t(1;V)(p32;V)		5q		
Inflammatory myofibroblastic tumor	t(1;2)(q25;p23)* t(2;2)(p23;q13) t(2;11)(p23;p15) t(2;19)(p23;p13.1) t(2;17)(p23;q23)				<i>TPM3-ALK</i> <i>ALK-RANBP2</i> <i>ALK-CARS</i> <i>ALK-TPM4</i> <i>ALK-CTLC</i>

Hemangiopericytoma	t(12;V)(q13-15;V) t(19;V)(q13;V) t(12;19)(q13;q13)	
Myxoid malignant fibrous histiocytoma (myxofibrosarcoma)	t(1;2)(p11;q35-36) t(1;V)(p11-13;V)	Ring
Giant cell tumor of tendon sheath (localized nodular tenosynovitis)	t(1;V)(p11-13;V)	
Diffuse-type giant cell tumor (pigmented villonodular tenosynovitis)	t(1;V)(p11-13;V)	5, 7
Malignant fibrous histiocytoma (undifferentiated high-grade pleomorphic sarcoma)	Complex	Hyperdiploid 1p31, 7p15-ptet, 7q32, 8p23.1, 12q13-15
Uterine leiomyoma	t(12;14)(q15;q24) t(12;V)(q15;V)	7q22-32
Leiomyosarcoma		2p24-ptet, 2q32-qter, 9p21, 11, 13, 16
Gastrointestinal stromal tumor	1q21-31	1p, 3p14.2-ptet, 8p21-ptet, 10, 13q12-13, 13q32-qter
Endometrial stromal tumor	t(7;17)(p15;q21) rea(6p21)	14,22
Rhabdoid tumor		KIT mutations <i>JAZF1-JAZ1</i>
Alveolar rhabdomyosarcoma	t(1;13)(p36;q14) t(2;13)(q35;q14)* t(2;2)(q35;p23)	22q
Embryonal rhabdomyosarcoma		PAX7-FOXO1A PAX3-FOXO1A PAX3-NCOA1
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11.2)	11p15
Alveolar soft part sarcoma	t(X;17)(p11.2;q25) unbalanced	13q21, 20
Clear cell sarcoma of soft tissue	t(12;22)(q13;q12)	
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)* t(9;15)(q22;q21) t(9;17)(q22;q11)	8,22
		WT1-EWS TEC-EWS TEC-TCF12 TEC-TAF15

(Continued)

Table 12.2 (Continued)

Tumor	Rearrangement	Chromosome gain	Chromosome loss	Loss of heterozygosity	Genes implicated
Synovial sarcoma	t(X;18)(p11.2;q11.2)				SSX1, SSX2 or SSX4-SS18
Pericytoma	t(7;12)(p22;q13)				ACTB-GLI
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11) t(12;22)(q13;q12)				ATF1-FUS ATF1-EWS
Tumors of bone and cartilage					
Aneurysmal bone cyst	t(6;17)(q22;p13)* t(17;V)(p13;V)		6p		
Chondromyxoid fibroma	rea(6) t(6;V)(q25;V)				
Ewing sarcoma/PNET	t(11;22)(q34;q12)* t(21;22)(q22;q12) t(7;22)(p22;q12) t(16;21)(p11;q22)				FLJ1-EWS ERG-EWS ETV1-EWS FUS-ERG
Giant cell tumor of bone	Telometric associations, ring(19)				
Osteochondroma	t(8;V)(q22-q24.1;V)	8q24.1			EXT1
Osteosarcoma	Complex	Hyperdiploid 1	6q, 9, 10, 13, 17		
Panosteal osteosarcoma		Ring (12q13-15)			
Neural tumors					
Neuroblastoma		Double minutes, hsr 17q	1p36, 11q123		MycN
Malignant peripheral nerve sheath tumor	Complex	Hyperdiploid			
Schwannoma		7	15, 17 22q12*		NF2

* Most common rearrangement

Table 12.3 Recurrent cytogenetic abnormalities of non-mesenchymal tumors

Tumor	Rearrangement	Chromosome Gain	Chromosome Loss	Loss of Heterozygosity	Genes Implicated
Diffuse malignant mesothelioma		5, 7, 20	1p21-22, 3p21, 4, 6q15-21, 9p21-22, 22		
Thymoma					
Type A and AB			6p		
Type B3		1q	6, 13q		
Carcinoma with t(15;19)(q13;p13.1) (midline lethal carcinoma)	t(15;19)				<i>NUT-BRD4</i>
Germ cell tumor		i(12p) adult 1q 20q children	1p, 4q, 6q	children	
Hepatoblastoma	der(1)t(1;4)	1q, 2q23-25, 20	4q		
Medulloblastoma	rea(1) i(17)(q10)*				
Meningioma			1p 22, 22q12*	1, 9q, 10q, 14q	<i>NF2</i>
Oligodendrogioma			1p, 19q		
Pleomorphic adenoma (salivary gland)	t(3;8)(p12;q12) t(5;8)(p13;q12) t(8;V)(q12;V) t(12;V)(q14-15;V)				<i>PLAG1</i>
Carcinoma ex pleomorphic adenoma			Double minutes		<i>HMGA2, MDM2</i>
Renal cell carcinoma					
Clear cell RCC	der(3)t(3;5)(p13;q22)	5, 12, 20	3p, 8, 9, 20		
Papillary RCC		7, 12, 16, 17, 20			
Adolescent papillary RCC	t(X;17)(p11.2;q25)				<i>TFE3-RCC17</i>
Mesoblastic nephroma	t(X;1)(p11.2;q21.2)				<i>TFE3-PRCC</i>
Wilms tumor	t(12;15)(p13;q25)	8, 7, 11 12	11p13-15	11p13-15	<i>ETV6-NTRK3</i>
Prostate cancer	rea(21q),t(21;21) (q22;q22) t(7;21)(p21;q22) t(17;21)(q21;q22)	8q	8p, 10q, 13q, 16, Y		<i>TMPRSS2-ERG</i>
Lung carcinoma and mesothelioma			3p		<i>TP53, RB1, P16</i>
Small cell lung carcinoma				5q, 10q	
Squamous cell carcinoma		3q			
Adenocarcinoma		1q			
Bladder cancer			9/9p		<i>CDKN2/P16</i>
Squamous cell carcinoma		7		3p, 8p, 9p, 9q, 17p	
Urothelial carcinoma	i(5p)	7	8p, 9, 11/11p, 13q, 17p		

(Continued)

Table 12.3 (Continued)

Tumor	Rearrangement	Chromosome Gain	Chromosome Loss	Loss of Heterozygosity	Genes Implicated
Breast cancer	Rearrangements of 1q, 3p, 6q, 8p i(1q), der(16)t(1;16)	7, 18, 20, dmin, hsr 1q31-q32 8q12 8q24 17q12 20q13	1p35, 3p, 16q, 17q, 6q21-22		<i>CYMC</i> <i>ERBB2</i>
Colon cancer		7, 12	1p, 15q 5q 10q 18q 17p		<i>APC</i> <i>PTEN</i> <i>DCC, SMAD4</i> <i>TP53</i>
Malignant melanoma	t(1;V)(p12-p22;V) t(1;19)(q12;p13) i(6p)	7	1p12-p22, 6q, 9p		<i>NEDD9</i>
Ovarian carcinoma	Complex rearrangements		17q		
Uterine carcinoma	Structural and numerical changes				
Uveal melanoma		3 8q 6p	3 6q		<i>TGFBR2</i> <i>CMYC</i>

*Most common rearrangement

common numerical changes; however, it has also been observed in non-neoplastic tissues, such as kidney and brain [37]. Common secondary changes in sarcomas include gain of chromosome 8 and the presence of der(16)t(1;16)(q11~12;q11~12) [38–42]. The der(16)t(1;16) is generally considered a secondary abnormality, and when present, signals that an underlying primary abnormality is likely to be present. The presence of gene amplification in the form of homogenously staining regions (hsr) or double minutes (dmin) has both prognostic and therapeutic importance. The structural aberrations known as telomeric associations (tas) (see Figure 12.1) are seldom noted in hematological malignancies, but when observed in solid tumors can be indicative of a specific diagnosis, such as giant cell tumor of bone [43,44]. Detailed descriptions and information on the proper use of ISCN nomenclature can be found in Chapter 8, The universal language of cytogenetics.

In the not too distant past, subtle differences in neoplasm classification were often considered minor since treatment was more dependent on the assigned stage and grade of the tumor. However, use of the tyrosine kinase inhibitor in gastrointestinal stromal tumors demonstrates how advancements in the understanding of oncogenesis have brought about more specialized treatment strategies for specific tumor types [45]. The WHO Classification of Tumours highlights the contributions of cytogenetics, when combined with morphology and modern immunohistochemical (IHC) techniques, in establishing reproducible and more accurate diagnostic criteria [46].

12.3 Recurrent cytogenetic abnormalities

12.3.1 Benign adipose tumors

Lipoma

Lipomas are benign tumors comprised of lobules of mature adipocytes. Though rare in children, they constitute nearly one-third of all soft tissue neoplasms in adults [47]. Most lipomas are slow-growing masses that typically do not require treatment unless they become painful or restrict movement due to size and/or location. They generally pose no risk of progression to malignancy. Subcutaneous tissue is the most common location for solitary lipomas, although the tumors may occur anywhere in the body where fat is located [48]. The majority of conventional lipomas exhibit cytogenetic abnormalities [49] and

among those, nearly 75% contain seemingly balanced rearrangements [50]. Aberrations of 12q13 ~ 15 have been noted in about two-thirds of lipomas with abnormal karyotypes [48]. The most common rearrangement is a t(3;12)(q27 ~ 28;q13 ~ 15); however, all chromosomes have been reported in either translocations or insertions with chromosome 12 [51] (see Figure 12.5). Rearrangements involving 12q frequently involve the high group mobility gene *HMGA2*, also known as *HMGIC*, at 12q15.

Among those tumors without 12q13 ~ 15 involvement, abnormalities of 13q are the next most common group (8–20%), with many seen as del(13)(q12q22) [47,51]. Translocations of 6p21 ~ 23 have also been noted in a small subset of lipomas (6–8%). *HMGA1*, also known as *HMG1Y*, at 6p21 has been proposed as a candidate gene for rearrangements involving 6p [52]. Ring chromosomes are a rare finding in ordinary lipoma; however, some most notably deep-seated, inter/intra-muscular lipomas (atypical lipomas), which lie between benign and obviously malignant lipogenic neoplasms, have been described with a variety of aberrations, including rings and giant marker chromosomes. These rings were shown to have amplifications of 12q15 ~ 21 and 1q21 [53]. No clear, consistent correlations, other than an increased incidence of abnormal karyotypes with increased age, have been identified between clinical and cytogenetic data [50].

Lipoblastoma

Lipoblastoma is a tumor of infancy, most often occurring before three years of age and primarily found in the upper and lower extremities. These are benign lesions with no incidence of metastasis or malignant transformation [54]. The histological appearance of lipoblastoma may closely resemble myxoid liposarcoma [55]; however, the latter tumor is rarely found in patients younger than 10 years of age [56]. In 2006, 24 lipoblastoma cases with cytogenetic abnormalities were reported in the NCI/NCBI Cancer Chromosomes database [57]; most of the 24 cases were near-diploid and all contain abnormalities of 8q, with breakpoints at 8q11 ~ 12 occurring in the vast majority. Preliminary data has indicated that the *PLAG1* gene at 8q12 is a central oncogenic event in lipoblastoma [58].

Hibernoma

Hibernomas are rare, benign neoplasms that comprise brown fat cells mixed with varying degrees of white adipose tissue [59]. When completely excised, these neoplasms rarely, if ever, recur. They are classically larger in size (average 9 cm) than other benign lipomas and occur primarily in the extremities or trunk of young adults [60]. Consistent cytogenetic

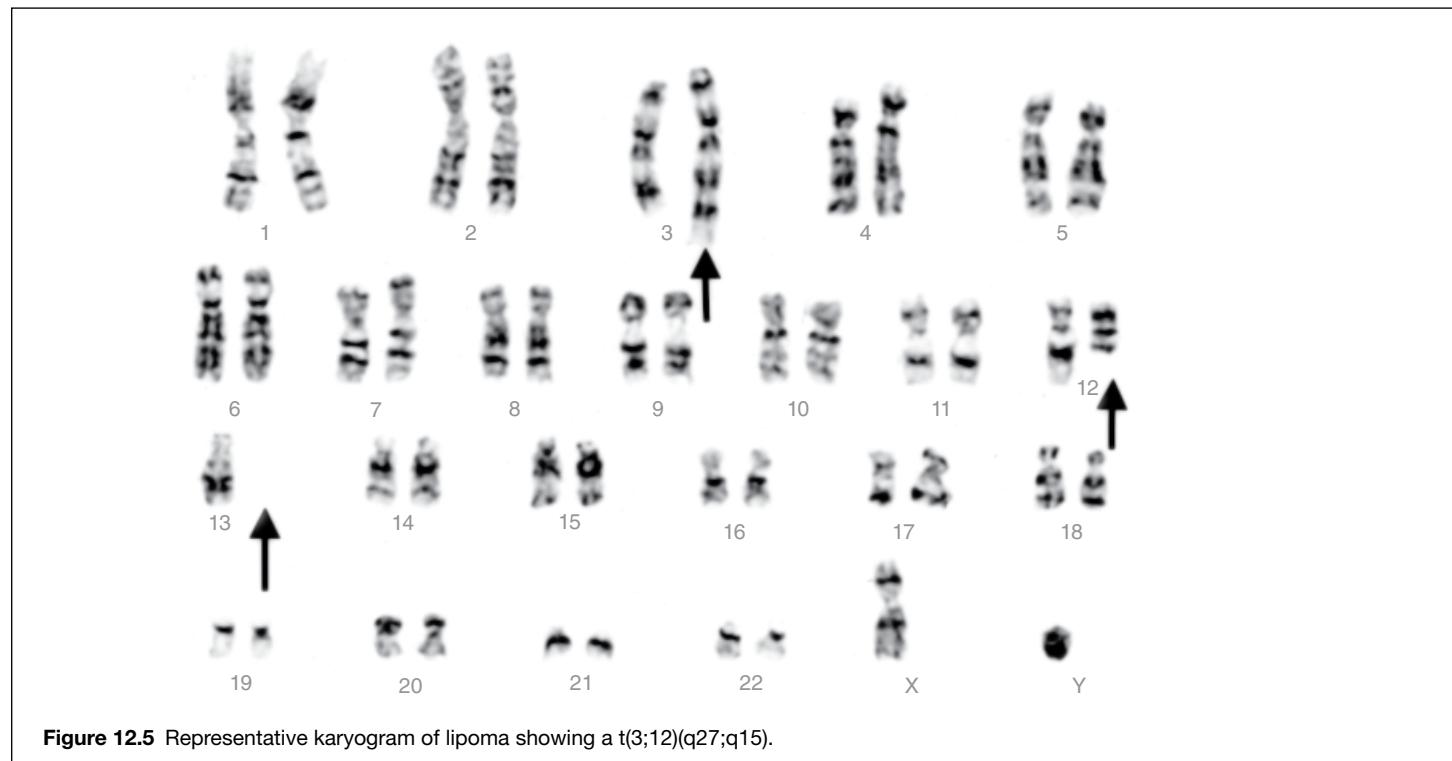


Figure 12.5 Representative karyogram of lipoma showing a t(3;12)(q27;q15).

alterations have been reported in hibernomas and include translocations and deletions of 11q [61,62]. The majority of hibernomas with chromosomal abnormalities contain structural rearrangements of 11q13 [57]. FISH analyses have shown that rearrangements of chromosome 11 are often more complex than described via conventional cytogenetics. Gisselsson et al. [63] observed homozygous loss of several loci of 11q when deletions were detected, not only in those chromosomes that were shown to be rearranged by G-banding, but also in the seemingly normal homologues, as well. The gene for multiple endocrine neoplasia type I (*MEN1*) at 11q13 was most frequently deleted. In addition to the *MEN1* deletions, heterozygous loss of a second region, approximately 3 Mb distal to *MEN1*, has been observed [63]. A later study by Maire et al. [64] reported large heterozygous deletions and common rearrangements of 11q13.5. Their observations suggested that *LRRC32* (previously known as *GARP*) at 11q13.4-q14 or another gene located nearby may have a significant role in hibernoma pathogenesis.

Chondroid lipoma

Chondroid lipoma is a rare benign adipose tumor found most commonly in proximal extremities and limb girdles. Although it resembles liposarcoma and myxoid chondrosarcoma, it exhibits nonaggressive behavior with no recurrence or metastases [65]. The three chondroid lipomas reported with cytogenetic abnormalities presented with identical rearrangements of t(11;16)(q13;p12 ~ 13). This translocation was observed as the sole abnormality in two of the three cases [66–68], suggestive of a disease-specific rearrangement. The 11q13 breakpoint was previously noted in hibernomas, raising the possibility of a common genetic deregulation.

12.3.2 Malignant adipocytic tumors

Well-differentiated liposarcoma/atypical lipomatous tumor

Liposarcoma (LPS) is the most common soft tissue sarcoma of adulthood and accounts for approximately 20% of all mesenchymal malignancies [69]. Well-differentiated liposarcoma (WDLPS), the most common subtype of LPS, is a locally aggressive, malignant adipocytic neoplasm. Based on the latest WHO classification in soft tissue tumors, WDLPS and atypical lipomatous tumor are synonyms used to describe lesions which are identical in morphology, karyotypic changes and biologic potential [70]. Four related subtypes have been described for WDLPS: adipocytic, sclerosing, inflammatory, and spindle cell [71–73]. The vast majority (~80%) of WDLPS have been characterized by supernumerary rings (see Figure 12.6), giant rod chromosomes, and double minutes, irrespective of the subtype [74,75]. These ring and giant markers contain amplifications of the 12q13–14 region, including *MDM2*, together with various other gene regions, such as *HMGAA2*, *CDK4*, *GLI1*, and *TSPAN31* (formerly known as *SAS*), but they typically lack alpha-satellite regions, making them negative for chromosome specific centromere probes by FISH [50,76,77].

A hallmark of WDLPS is the frequent observation of telomeric associations [78] (see Figure 12.7). WDLPS show a risk of local recurrence but no potential of metastases unless they undergo dedifferentiation [79]. Dedifferentiation occurs in up to 10% of WDLPS, regardless of subtype, and similar to WDLPS, most often contain ring or giant marker chromosomes [74,75].

Myxoid liposarcoma/round cell liposarcoma

Myxoid liposarcoma (MLS)/ round cell (RC) liposarcoma accounts for about 30% to 35% of all liposarcomas and share both clinical and morphologic features [80]. MLS, occurring generally in the deep soft tissues of the extremities (most often in the thigh), on average presents a decade earlier than other histological subtypes of liposarcoma and has a relatively favorable prognosis [81]. Round cell liposarcoma is regarded as a variant of MLS that has progressed to hypercellular or round-cell morphology. Most often, RC liposarcoma represents a mixed liposarcoma with both myxoid and round cell components [69]. The specific rearrangement t(12;16)(q13;p11), or a variant, is present in more than 90% of MLS cases [82,83]. The translocation leads to the fusion of the *DDIT3* (previously known as *CHOP* gene at 12q13.1-q13.2 and the *FUS* (*TLS*) gene at 16p11.2 [84]. An alternative t(12;22)(q13;q12) is present in about 5% of the MLS cases, and results in the fusion of *DDIT3* to the *EWSR1* gene at 22q12.2 [85,86]. The t(12;16) is also present in round cell liposarcoma; however, RC liposarcoma is associated with a significantly poor prognosis [80,87]. It has been proposed that retroperitoneal tumors with features of myxoid liposarcoma are part of the morphologic spectrum of well-differentiated/dedifferentiated liposarcoma rather than true MLS, since they generally do not exhibit the t(12;16)(q13;p11) [88,89].

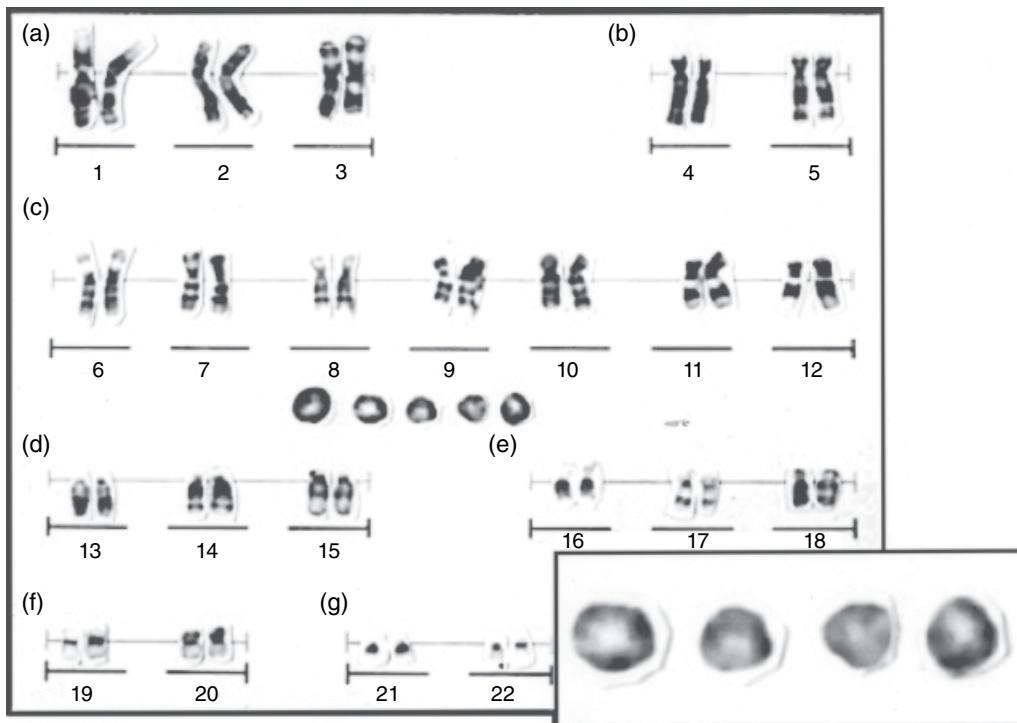


Figure 12.6 WDLPS often show supernumerary rings as represented in above karyotype and rod or giant marker chromosomes as represented in metaphase cell. Both contain amplifications of the 12q13~15 region.



Figure 12.7 A hallmark of DLPS is the frequent observation of telomeric associations.

12.3.3 Skeletal muscle tumors

Embryonal rhabdomyosarcoma

Rhabdomyosarcomas (RMS) are mesenchymal tumors belonging to the group of small round-cell tumors, and constitute the single largest category of soft tissue sarcomas in children and adolescents. Embryonal rhabdomyosarcoma (E-RMS) is the most common subtype of RMS and typically affects children less than 10 years of age [90]. By definition, E-RMS is a primitive, malignant neoplasm exhibiting the phenotypic and biologic features of embryonic skeletal muscle [91]. In spite of this, less than 9% arise within the skeletal muscle of the extremities. Nearly half occur within the head and neck region and over one-fourth are found in the genitourinary system [92]. Cytogenetic studies have shown complex structural and numerical changes in E-RMS cases. The majority are hyperdiploid and often exhibit gains of chromosomes 2, 8, 12, and 13 [93,94]. Comparative genomic hybridization (CGH) studies (also referred generically as chromosome microarray analysis) confirmed these findings, but also provided evidence of an increased copy number for chromosomes 7, 11, and 20 and loss of 1p, 6, 9q, 10, 14, 16, and 17 [95–97]. Recurrent structural rearrangements have not been shown for embryonal rhabdomyosarcoma; however, Gordon et al. [93] reported translocations involving 1p11-1q11 in 36% of the E-RMS studied.

Alveolar rhabdomyosarcoma

Alveolar rhabdomyosarcoma (A-RMS) comprises approximately 25% of all RMS cases and is characteristically observed in older children and young adults (6–21 years) as a rapidly growing mass of the extremities or trunk [98]. A-RMS tends to present as a high stage lesion and has a significantly poor prognosis compared with embryonal rhabdomyosarcoma [98,99]. Chromosomal analysis has shown that the majority of A-RMS (70–75%) are characterized by a t(2;13)(q35;q14) while a variant t(1;13)(p35;q14) can be found in approximately 10% of the cases [100,101] (see Figure 12.8). These translocations result in a fusion of the genes on chromosomes 2 (*PAX3* at 2q36.1) and 1 (*PAX7* at 1p36.13), respectively, with the

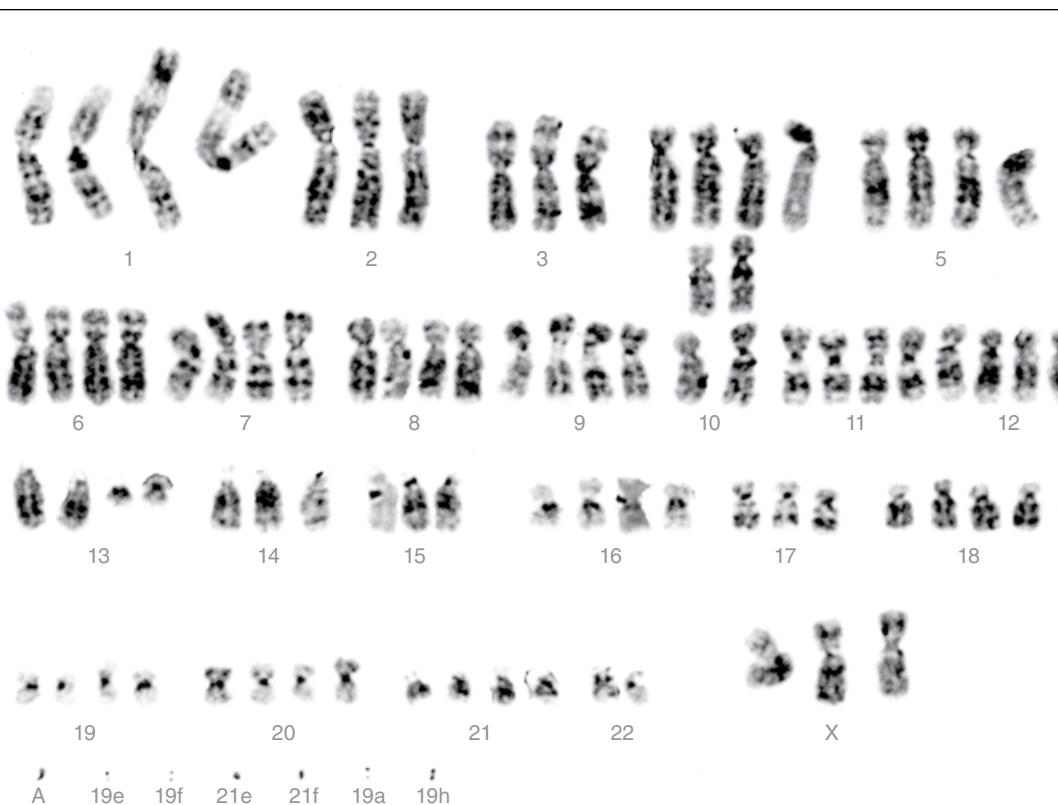


Figure 12.8 Representative karyogram of alveolar rhabdomyosarcoma showing t(1;13)(p36;q14)x2 and double minutes (dmin).

FOXO1A (FKHR) gene on chromosome 13 at q14.1 [102]. It has been reported that the *PAX3–FOXO1A* fusion transcript in patients presenting with metastatic A-RMS is indicative of a less favorable prognosis, when compared to those with a *PAX7–FOXO1A* gene fusion [103]. Approximately 10–20% of A-RMS exhibit neither a t(2;13) nor t(1;13) and are fusion negative by molecular methods [104]. Wachtel et al. [105], however, recently reported finding a novel *PAX3* translocation in an A-RMS case with a t(2;2)(q35;p23), suggesting that other *PAX3* [7] translocations may exist in alveolar rhabdomyosarcoma. Amplifications of various regions have been described in a significant number of A-RMS cases [97]. These amplifications may present themselves as double minutes and frequently represent an amplification of *MYCN* (at 2p24.3) and/or the *PAX7–FOXO1A* fusion gene [106]. Amplification of the *PAX3–FOXO1A* fusion, though possible, is a less common event [107]. Overexpression or gain of genomic copies of *MYCN* has been associated with an adverse outcome in alveolar rhabdomyosarcoma [108].

12.3.4 Tumors of uncertain differentiation

Synovial sarcoma

Unrelated to synovium, synovial sarcoma (SS) is a mesenchymal spindle cell tumor that displays varying degrees of epithelial proliferation [109]. Over 80% occur in the deep soft tissue of extremities, often adjacent to joints or tendon sheaths, and less than 5% actually originate within a joint or bursa [110]. SS accounts for 5–10% of soft tissue sarcomas and generally occurs in young adults [111]. Histologically, SS is divided into two distinct subtypes, monophasic and biphasic [112]. A unique chromosomal finding of t(X;18)(p11;q11) is found in the vast majority of synovial sarcoma cases (80–90%), irrespective of the subtype [13,113,114]. Complex rearrangements of t(X;18) involving three or more chromosomes are seen in less than 10% of reported cases [115]. The genes involved in the t(X;18) have been identified as *SYT* (*SSXT* or *SS18*) at 18q11.2 and three closely related genes at Xp11 (*SSX1* and *SSX4* at Xp11.23, and *SSX2* at Xp11.22) [116,117]. In one-third of tumors, the t(X;18) is seen as the sole abnormality; the remaining cases with secondary changes frequently exhibit gain of chromosomes 7, 8 and/or 12 or loss of chromosome 3 [109] (see Figure 12.9). Traditionally, synovial sarcoma has had a poor prognosis; however, patients with *SS18–SSX2* were shown to have a significantly better metastasis-free survival [118].

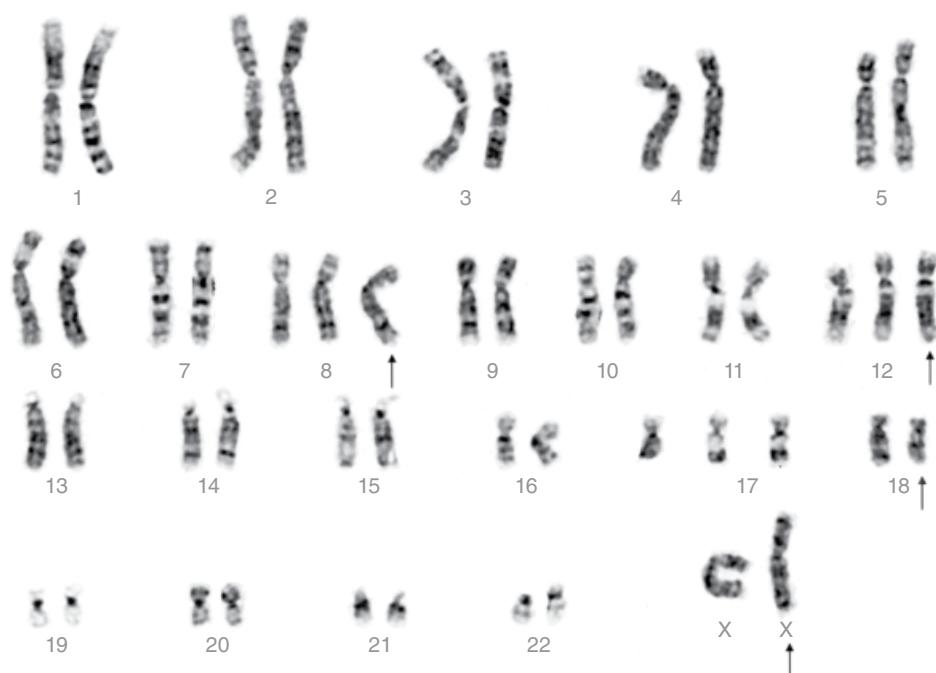


Figure 12.9 Representative karyogram of synovial sarcoma showing a t(X;18)(p11.2;q11.2) and common secondary changes of +8 and +12.

Alveolar soft part sarcoma

Alveolar soft part sarcoma (ASPS) is a rare neoplasm of uncertain histogenesis, most commonly affecting adolescents and young adults [119]. An ASPS typically presents as a slow-growing mass and commonly arises in the extremities of adults or in the head and neck region of children [120]. Cytogenetic studies revealed the presence of an add(17)(q25) as a recurring abnormality in ASPS [121]. Joyama et al. employed spectral karyotyping to further delineate the composition of this abnormality. The additional part of the long arm of chromosome 17 was found to originate from chromosome X, resulting in a final karyotype of der(17)t(X;17)(p11.2;q25) [122]. It has been demonstrated that this translocation results in the fusion of the *TFE3* gene at Xp11.22 with the *ASPL (ASPSCR1)* gene at 17q25 [123]. The balanced t(X;17) is an unusual occurrence in ASPS [124,125]. The overwhelming presence of der(17)t(X;17) suggests that the gain of Xp and/or loss of heterozygosity (LOH) of 17q25-qter sequences may have pathogenic implications. A subset of renal adenocarcinomas arising in young people is characterized by a balanced form of the t(X;17) translocation. Although they are genetically related to ASPS, they have a distinct morphology that more closely resembles that of renal cell carcinoma [126]. Patients with localized ASPS generally experience a relatively indolent clinical course with a low rate of recurrence [120].

Clear cell sarcoma of soft tissue

Clear cell sarcoma (CCS) is a rare, aggressive tumor arising primarily in the soft tissue extremities of young adults. Although not directly related to malignant melanoma, CCS is sometimes referred to as malignant melanoma of soft parts due to the presence of melanin pigment, as well as (pre) melanosomes in many cases [127]. It is also important to distinguish CCS from the unrelated pediatric lesion currently known as clear cell sarcoma of kidney [128,129]. A specific translocation, t(12;22)(q13;q12), has been associated with CCS [130]. This translocation, present in the majority of cases, results in the fusion of the *ATF1* and *EWSR1* genes at 12q13 and 22q, respectively [57,131]. Cytogenetically, t(12;22) found in CCS appears identical to the t(12;22) seen in a small number of cases of myxoid liposarcoma (MLS); however, molecularly, they differ due to the involvement of separate genes at 12q13 [86]. Many cases contain structural and numerical abnormalities in addition to the t(12;22), including +7, +8, and both structural and numerical aberrations of chromosome 22 [132]. Generally, clear cell sarcoma has a poor prognosis; many patients develop metastases and recurrences [133,134]. Recently, it has been reported that angiomyomatoid (malignant) fibrous histiocytoma (AFMH), a rare soft tissue tumor of low malignant potential and uncertain differentiation, is also characterized by a t(12;22)(q13;q12) with an *EWSR1-ATF1* fusion similar to clear cell sarcoma or by a t(12;16)(q13;p11) with *FUS-ATF1* fusion [135,136].

Extraskeletal myxoid chondrosarcoma

Extraskeletal myxoid chondrosarcoma (EMC) is a malignant soft tissue neoplasm arising most often in the deep soft tissues of the lower extremities, specifically the thigh, popliteal fossa, and buttock [137]. EMC is typically considered a tumor of adulthood and is rarely present in children or adolescents [138]. The first and most common characteristic abnormality described for EMC was the t(9;22)(q22;q12) translocation and results in the fusion of the *NP4A3 (CHN)* gene at 9q22 to the *EWS* gene at 22q12 [139–141]. The following variant translocations specific for EMC have subsequently been identified: t(9;17)(q22;q11), fusing genes *NP4A3* and *TAF15*, and t(9;15)(q22;q21), fusing genes *NP4A3* and *TCF12* [142,143]. A fourth gene, *TFG* at 3q12.2, has also been described as a fusion partner for *NP4A3* in EMC [144]. Extraskeletal myxoid chondrosarcoma has a high potential for recurrence and metastasis, and even though it is associated with a long survival in some cases, it eventually has a high rate of tumor-associated death [145].

Desmoplastic small round cell tumor

Desmoplastic small round cell tumor (DSRCT) is a rare, aggressive neoplasm composed of small round cells of indeterminate histogenesis [146] and is located predominantly in the peritoneal surfaces of the abdomen with involvement of many abdominal organs [147]. Nearly 80% of DSRCT tumors arise in boys or young men [148]. The t(11;22)(p13;q12), found in 75–90% of the cases, is a primary chromosomal abnormality unique to DSRCT, and results in the fusion of the Ewing sarcoma gene (*EWS*) localized to 22q12 and the Wilms tumor suppressor gene (*WT1*) localized to 11p13 [149–151]. A small number of DSRCT cases with chromosomal abnormalities have been reported to lack cytogenetically a t(11;22) (e.g., t(11;17)(p13;q11.2) or t(2;21;22)(p23;q22;q13)); however, the *EWS-WT1* fusion status was not examined molecularly in these rare instances [152–154]. Although it has been reported that patients with localized extra-abdominal disease have a better prognosis, most likely due to increased feasibility of resection, the overall prognosis for DSRCT is very poor with the majority dying within two to three years, despite aggressive treatment [155,156].

12.3.5 Bone tumors

Aneurysmal bone cysts and giant cell tumor

Aneurysmal bone cysts (ABC) are enigmatic, benign lesions of indeterminate cause. The lesion is histopathologically characterized by blood-filled spaces separated by fibrous septa [157]. ABC generally occurs in the first two decades of life and is principally located in the tibia, femur, pelvis, humerus, and spine [158]. Although ABC was initially described as a non-neoplastic lesion [159], its association with recurrent chromosome abnormalities has provided compelling evidence to the contrary [22]. The translocation $t(16;17)(q22;p13)$ was observed as a recurrent anomaly in ABC in 1999 [160,161] and subsequent molecular studies have identified the genes involved as being *CDH11* at 16q21 and *USP6 (TRE17)* at 17p13 [162]. Other less common translocations involving 17p13 have been described in ABC, including $t(1;17)$, $t(3;17)$, $t(9;17)$ and $t(17;17)$. Each of the variant 17p translocations was shown to be associated with a novel *USP6* fusion oncogene [163]. FISH and molecular studies have demonstrated that aberrations of 17p13 occur in 63–69% of ABC cases [164,165]. A second benign and locally aggressive neoplasm, known as giant cell tumor of bone (GCTOB), has been shown to coexist with ABC in some instances. GCTOB, in contrast, is cytogenetically characterized by telomeric associations in about three-fourths of cases; tumors with such associations or fusions have exhibited reduction in telomere length. Rings and dicentric chromosomes have frequently been observed in these tumors, as well [166]. In combined giant cell tumor/aneurysmal bone cyst lesions, each component seems to retain its own karyotypic abnormality [22]. Recurrences of ABC, following curettage, occur in a substantial number of cases (20–70%) and rare cases of malignant transformation have been reported [167].

Osteosarcoma

Osteosarcoma (OS) is the most common nonhematologic malignant tumor of bone in both children and adults [168]. Conventional osteosarcoma typically arises in the intramedullary cavity of the long bones, in particular, the distal femur, the proximal tibia and the proximal humerus [169]. Unlike other sarcomas, conventional OS is not associated with a disease-specific rearrangement; however, involvement of certain chromosomal regions is recurrent. The majority of OS cases contain clonal karyotypic aberrations that tend to be complex with numerous structural and numerical changes [170]. Cytogenetic studies have identified the most common numeric abnormalities to involve gain of chromosome 1 and loss of chromosomes 9, 10, 13, and 17. Partial or complete loss of the long arm of chromosome 6 is seen in a significant number of cases. The most frequently rearranged segments include 1p11–13, 1q10–12, 1q21–22, 11p15, 12p13, 14p, 15p, 17p12–13, 19q13, and 22q11–13 [171,172] (see Figure 12.10). Comparative genomic hybridization (CGH) studies have identified additional regions of loss at 10q, 5q, and 13q and gain at 6p and 8q (listed in order of frequency) [173,174]. Conventional OS when left untreated is fatal, or when treated with ablative surgery alone, is associated with limited survival. With the utilization of multi-disciplinary therapy incorporating surgery and chemotherapy, prognosis has significantly improved, with long-term survival being achieved in two-thirds of the cases [175]. Parosteal osteosarcoma, a rare low-grade tumor found on the surface of the bone, is frequently characterized by ring chromosomes comprised of amplified material from chromosome 12q13–15 [176,177] (see Figure 12.11).

Ewing sarcoma/primitive neuroectodermal tumor

Ewing sarcoma (ES)/primitive neuroectodermal tumor (PNET) is a small round cell tumor that shows varying degrees of neuroectodermal differentiation and arises most often in the bones of children or adolescents [178]. Nearly all ES cases contain rearrangements involving the *EWSR1* gene at 22q12.2. The most common translocation is $t(11;22)(q24;q12)$ (>90%) and results in the oncogenic fusion of the *FLI1* gene on 11q24.1-q24.3 to the *EWS* gene [9,10,179] (see Figure 12.12). A second Ewing sarcoma translocation, $t(21;22)(q22;q12)$, fuses the *ERG* gene located at band 21q22.3 to the *EWS* gene and is found in 5–10% of ES cases [179,180]. Additional rare variants of *EWS* fusion with other *ETS* family genes have been described, including *ETV1* at 7p22, *EIAF* at 17q21 and *FEV* at 2q36 [181–183]. In 2003, four cases of Ewing sarcoma were reported with translocations involving 16p11 and 21q22. The $t(16;21)(p11q22)$, observed also in rare cases of acute myeloid leukemia, juxtaposes genes *FUS* (16p11.2) to *ERG* (21q22.3) [184]. With recent advancements in treatment protocols, the five-year overall survival rate in localized ES/PNET has reportedly improved to over 40% [185].

12.3.6 Tumors of the nervous system

Peripheral nerve sheath tumors

Peripheral nerve sheath tumors (PNST), arising generally from Schwann cells of the peripheral nerve, are categorized as benign and malignant variants and are often linked with several hereditary disorders, including neurofibromatosis type 1 (NF1) and

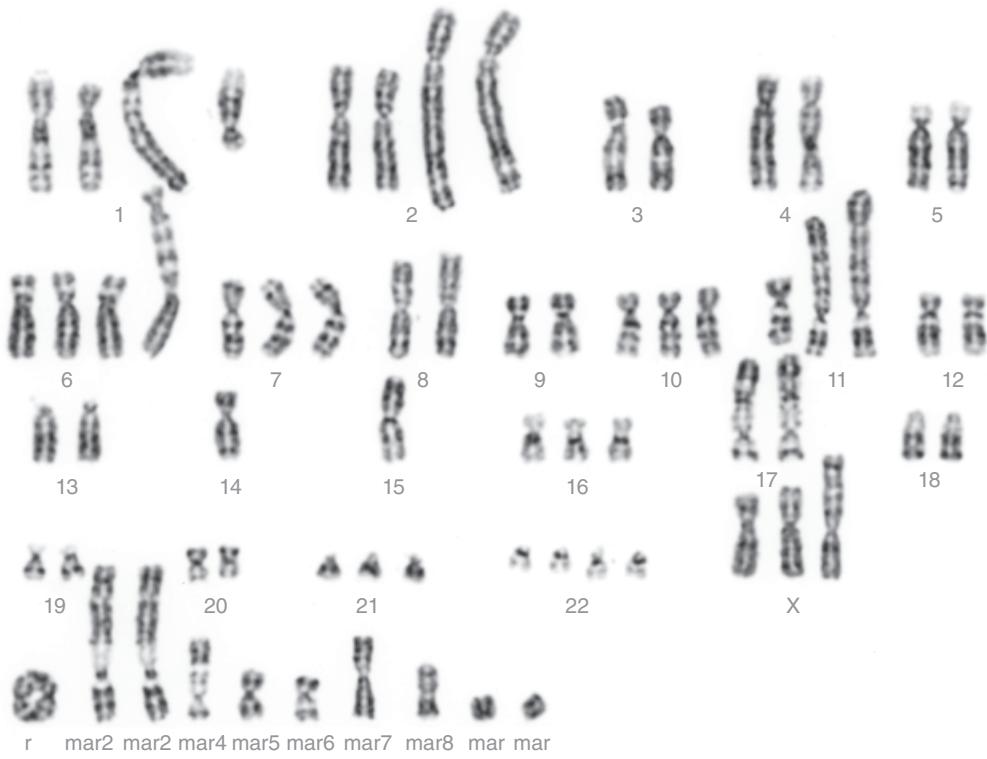


Figure 12.10 Representative karyogram of osteosarcoma showing multiple numerical and structural abnormalities. Marker chromosomes of unknown origin are designated by the “mar” abbreviation.

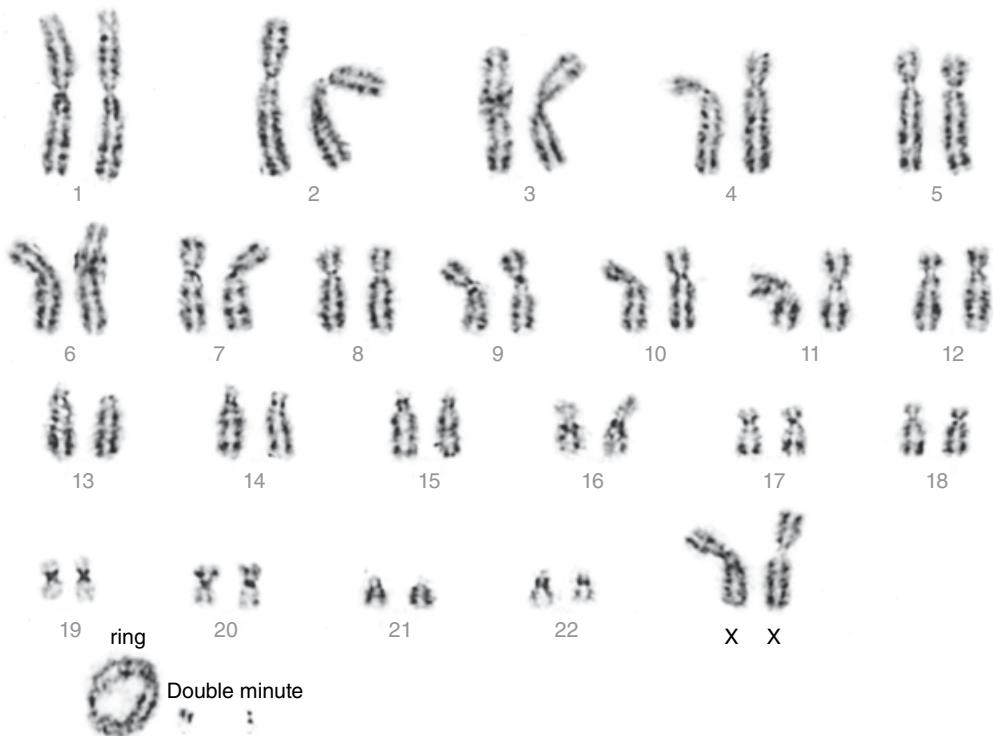


Figure 12.11 Representative karyogram of parosteal osteosarcoma showing amplification in the form of double minutes and a ring chromosome.

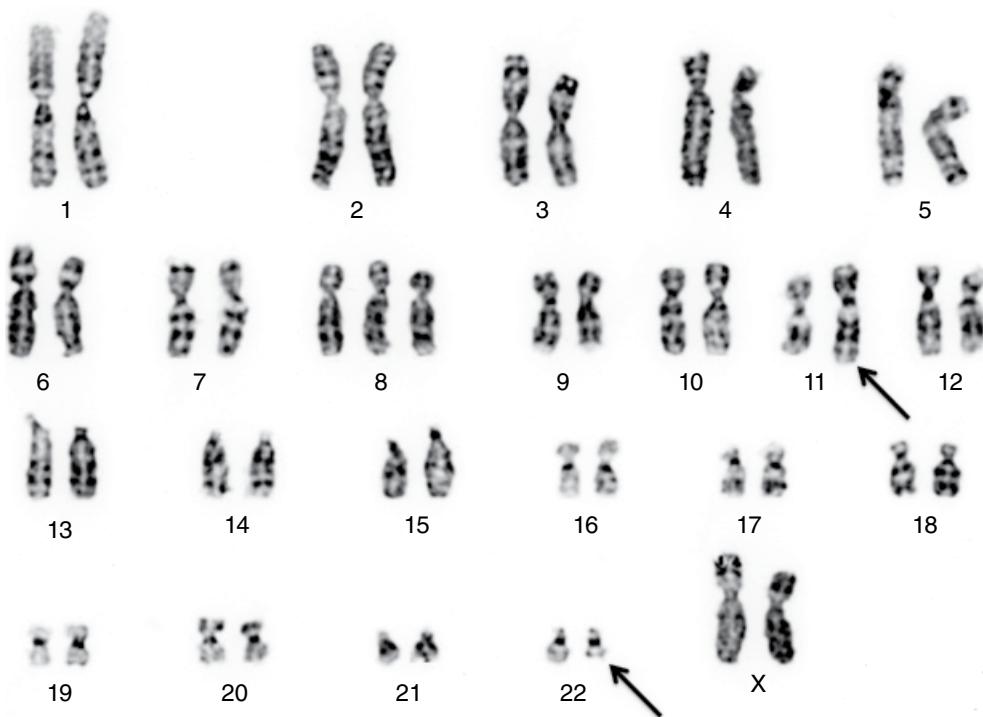


Figure 12.12 Representative karyogram of Ewing sarcoma showing a $t(11;22)(q24;q12)$ and a common secondary change of $+8$.

neurofibromatosis type 2 (NF2) [186]. Schwannomas, benign PNST, are associated with sensory nerves of the spine or intracranial region [187]. They may occur sporadically or in association with NF2 and are characterized by near-diploid karyotypes with partial or complete loss of 22q in 50–80% of the cases [188,189]. NF2 is caused by mutations in the *NF2* gene, a tumor suppressor, located at 22q12.2; however, studies of schwannoma cases with partial deletions of 22q have shown loss of other regions outside of *NF2*. These studies indicate that several regions, in addition to the *NF2* locus, harbor genes involved with the development of schwannoma [190]. Other recurrent abnormalities of schwannoma include loss of a sex chromosome or gain of 7 [189].

Highly aggressive tumors, known as malignant peripheral nerve sheath tumors (MPNST), usually occur in young and middle-aged adults and commonly arise in deep soft tissues of the proximal extremities. About half of MPNST cases are diagnosed in patients with NF1, frequently arising from plexiform neurofibroma [191,192]. The majority of MPNST display complex karyotypes, often with chromosome numbers in the triploid or tetraploid range [188,189]. The risk of recurrence following surgery is low for benign PNST, but relatively high for MPNST with a greater than 60% incidence of tumor related death [186].

Neuroblastoma

Neuroblastoma (NB) belongs to the group of small blue round cell tumors and is the third most common childhood malignancy with approximately one-fourth being present at birth, implicating a prenatal etiology in some cases. NB most commonly occurs within the retroperitoneum or mediastinum [187]. Extrachromosomal double minutes (dmin) and homogeneously staining regions (hsr) of metaphase chromosomes (see Figure 12.1) have been reported in half of all cases with chromosomal abnormalities and result from the amplification of the oncogene *MYCN* at 2p24.3 [5,6,193]; dmin are three times more common than hsr in this disease.

Deletions of the short arm of chromosome 1 are the most common chromosomal abnormality in NB and occur in 70% to 80% of all cases [194,195], usually accompanied by other complex aberrations. LOH analysis has defined the smallest region of overlap for the deletion to be within 1p36.3. It has been proposed that inactivation of a tumor suppressor gene

within 1p36.3 is associated with an increased risk for disease relapse [196,197]. A strong correlation of 1p loss with *MYCN* amplification has been reported as an indication of an advanced disease stage and decreased overall survival [196].

Another frequent genetic alteration in NB is gain of chromosome arm 17q, which arises from unbalanced translocations. Both 1p and 11q have been shown to be frequently involved in unbalanced translocations, resulting in loss of 1p or 11q and gain of 17q [198,199]; 5% of NBs in advanced sporadic tumors have also shown amplification of the ALK gene at 2p23, providing an alternate mechanism for treating the high-risk tumor [200].

Note: cases with near-triploid karyotypes and lacking deletions of 1p or *MYCN* amplification constitute a subgroup of patients with a more favorable prognosis [201]. LOH studies of NB have also identified regions of loss on 11q; however, unlike 1p36 LOH, 11q23 LOH is not thought to be associated with *MYCN* amplification [202].

Medulloblastoma

Medulloblastomas (MB) are highly malignant embryonal neoplasms of the central nervous system which belong to the group of primitive neuroectodermal tumors (cPNET). This tumor represents the most common malignant brain tumor in children, representing nearly 25% of all pediatric brain tumors [203,204]. Loss of a portion of 17p, typically through the formation of an isochromosome 17q [i(17q)], occurs in 30–60% of cases by cytogenetic analysis, making it the most consistent chromosomal abnormality observed in MB [205–207]. Some studies have shown a correlation between i(17q) and a poor overall survival in MB [208]. The i(17q), found in a variety of tumors, is the most common isochromosome in human cancer with a frequency of 3–4% [209] and an adverse overall prognosis. Cytogenetic studies have also noted abnormalities of chromosome 1 in MB, including unbalanced translocations, deletions and duplications [210].

Meningioma

Meningioma is a common neoplasm arising in the meningeal covering of the brain and spinal cord. Although meningiomas occur most often in the sixth and seventh decade of life, it can be seen as a rare entity in children [211]. The vast majority of meningiomas are benign, slow growing tumors; however, approximately 10% can be classified as atypical or anaplastic malignant tumors that exhibit a more aggressive clinical course [212]. Loss or deletions of chromosome 22 in meningioma was one of the earliest recurrent cytogenetic abnormalities described in solid tumors, being demonstrated prior to banding in 1972 [7,8]. Cytogenetic and molecular studies have confirmed loss of chromosome 22q in nearly 70% of typical meningiomas [213]. Allelic loss of 22q, along with inactivating mutations of *NF2* at 22q12.2, have been identified in both sporadic and neurofibromatosis type 2-associated tumor [214]. The second most common cytogenetic aberration observed in meningiomas is the 1p deletion [215,216]. LOH of 1p and additional chromosomal loci such as 9q, 10q, and 14q is more common in atypical and anaplastic variants and is associated with tumor progression [217,218]. Increasing hypodiploidy from losses of chromosomes 8, 14, X, and Y is also associated with karyotypic evolution [219].

Gliomas

Gliomas encompass a diverse group of tumors primarily of the central nervous system including astrocytomas, glioblastomas and oligodendrogiomas. Cytogenetic studies of low-grade astrocytomas (LGA) frequently exhibit normal karyotypes that may be associated with a more favorable prognosis in adult patients (cancer-related deaths in the United States) [220]; however, it appears that for children, normal cytogenetics may not predict a better prognosis [221]. Among the LGA cases with abnormal karyotypes, the most frequent abnormalities include the loss of sex chromosomes and 22q, and a gain of chromosome 7 [222,223]. Diffuse astrocytomas and glioblastomas are often characterized by gain of 7, loss of 10, 13 and 17, and structural abnormalities involving 1p, 1q, 5p and 9p [222–224]. LOH and cytogenetic studies often show concordant results; however, while trisomy 19 was reported by cytogenetic and comparative genomic hybridization (CGH) analysis [224,225], the loss of 19q13.2-qter was detected by LOH studies in secondary glioblastomas [226]. Double minutes have been reported as frequent findings in anaplastic astrocytomas and glioblastomas [222]. The most common cytogenetic findings of oligodendrogiomas include loss of sex chromosomes, gain of 7, deletions of 22q, and rearrangements of 1p [222,223]. Clinical studies of low-grade astrocytomas have demonstrated an unpredictable clinical course with 5-year survival ranges from 27% to 85% in pediatric patients [227]; however, progress has been shown in the overall median survival of adult patients [228]. In glioblastoma, prognosis is generally poor (approximately 1 year); however, patients 35 years or younger and with a good initial Karnofsky performance score (KPS) have a significantly better prognosis than older patients [229,230]. Molecular cytogenetic studies and their prognostic implications in astrocytic tumors are further discussed in the fluorescence in situ hybridization (FISH) section of this chapter.

12.3.7 Tumors of the lung

Lung cancer is the most common and most deadly neoplasm worldwide [231]. Nearly all lung tumors are carcinomas and can be grouped into two broad categories: small cell lung carcinoma (SCLC), which comprises 20–25% of cases, and non-small-cell lung carcinoma (NSCLC), which comprises 70–80% of cases. The NSCLC group can be further divided into three main subtypes including adenocarcinoma, large cell carcinoma and squamous cell carcinoma [232]. Lung carcinomas exhibit complex karyotypes that result from the accumulation of multiple genetic alterations; however, there are recurrent alterations common to all subtypes. The three most frequent aberrations detected thus far include mutations of gene *p53* (*TP53*) at 17p13.1; inactivation of the Rb pathway including *p16^{INK4}* and *RB1* (13q14.2) mutations; and LOH of chromosome 3p [233]. The frequency of mutations of *p53* is positively correlated with exposure to tobacco smoke, and carriers of *p53* germline mutations demonstrate an increased risk for the development of lung cancer [234,235]. LOH of chromosome 3p accompanied by chromosome 3p deletions are detected in nearly all SCLC [236] and up to 80% of NSCLC cell lung cancers [237–239] (Figure 12.13). Deletion of 3p led to LOH studies in both lung cancer and renal carcinomas. Several potential tumor suppressor genes are within this region, including the *FHIT* gene at 3p14.2. This gene region encompasses the common fragile site *FRA3B*, which is particularly prone to carcinogen-induced DNA damage [240]. Genetic changes that appear to be more frequent in specific histological types include LOH of chromosome 5q and 10q in SCLC [241,242], amplification of telomeric 3q in squamous cell carcinoma [243], and over-representation of 1q in adenocarcinoma [244]. Specific loci associated with frequent amplifications in lung carcinoma such as 5p, 7p12 (*EGFR*), and 8q24 (*MYC*) have been targeted for analysis by commercially available multi-color DNA probe panels [245]. *ALK* (anaplastic lymphoma kinase) is a tyrosine kinase that was originally discovered because of its involvement in a translocation t(2;5) (*ALK/NPM* [nucleophosmin] fusion) in anaplastic large cell lymphoma. The *ALK* gene is rearranged, mutated or amplified in several types of tumors, including lymphoma, NB and NSCLC.

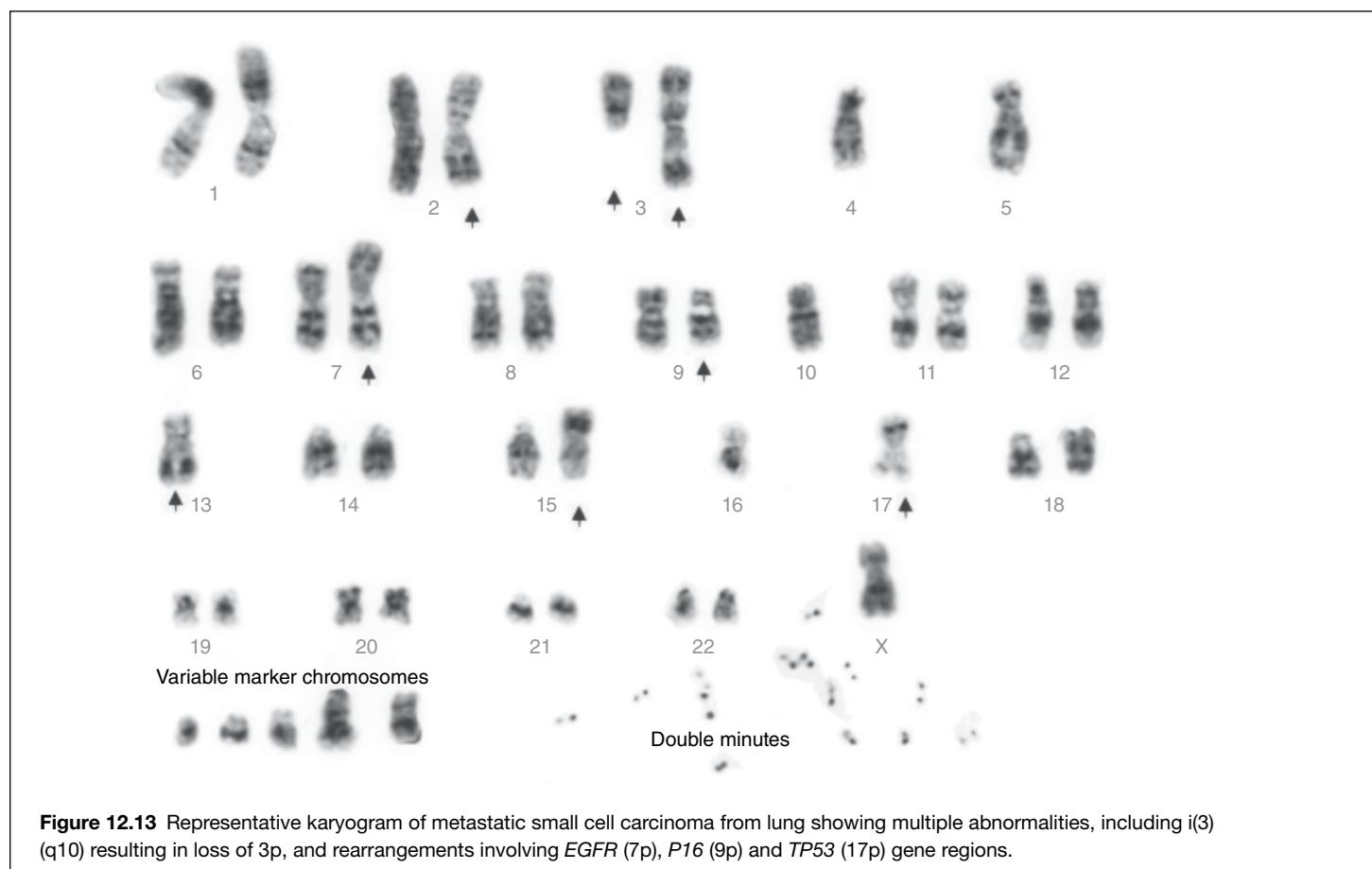


Figure 12.13 Representative karyogram of metastatic small cell carcinoma from lung showing multiple abnormalities, including i(3) (q10) resulting in loss of 3p, and rearrangements involving *EGFR* (7p), *P16* (9p) and *TP53* (17p) gene regions.

In 2007, Soda et al. [246] reported that an inversion on chromosome 2 short arm caused a fusion of *ALK* with *EML4* (echinoderm microtubule-associated protein-like 4) inhibiting apoptosis and inducing cell proliferation. Recent studies have indicated that the *ALK*-*EML4* gene fusion is present in 2–7% of all NSCLC in the United States, with a higher frequency in nonsmoking patients. Patients with this gene fusion are typically younger nonsmokers who do not have mutations in either the *EGFR* gene or in the *K-Ras* gene. Lung cancer patients whose tumors carry the *ALK* rearrangement respond well to the TKI crizotinib (Xalkori).

The recently published College of American Pathologists guidelines [247] recommend FISH testing for *ALK* rearrangements in adenocarcinomas and mixed lung cancers with an adenocarcinoma component, but not for other types of lung cancer such as squamous, small cell, or large cell carcinoma with no evidence for adenocarcinoma differentiation. The requirement for this new assay to determine treatment has translated into a growing need for FISH testing. Often, *EGFR* mutation testing is being done at the same time or before FISH testing, so CAP guidelines cover both tests.

Other interphase FISH tests used for determining treatment of carcinoma of lung include *ROS1*, *RET* (both for rearrangements), *MET*, and *FGFR1* for amplifications.

12.3.8 Tumors of the liver

Hepatoblastoma (HB) is the most common pediatric liver tumor occurring typically in the first 5 years of life [248]. While most cases occur sporadically, it has been shown that patients with Beckwith-Wiedemann syndrome or familial adenomatous polyposis are at an increased risk for developing hepatoblastomas [249,250]. Hepatoblastomas are classified histologically as epithelial or mixed, containing admixtures of epithelial and mesenchymal elements [251]. Cytogenetic studies have identified recurrent abnormalities involving gain of chromosomes 2q and 20 [252]. A small number of cases exhibited a recurrent translocation involving chromosomes 1 and 4, resulting in partial trisomy 1q and partial monosomy 4q [253]. CGH studies of HB have confirmed the most common genetic imbalances to be gains of 1q, 2q (specifically 2q24) and 20 and, to a lesser extent, loss of 4q [254,255]. Hepatoblastoma historically was associated with a very poor prognosis, but with recent advances in surgical techniques and chemotherapy, patients now have over a 70% 5-year survival rate [256].

12.3.9 Tumors of the thymus

Thymoma is a rare neoplasm originating from the epithelial cells of the thymus, which may present in either a benign or malignant form. Thymomas can frequently cause autoimmune disorders, in particular myasthenia gravis [257]. There are two major types of thymoma, type A and type B, with further subdivisions of B1, B2, and B3. Additional classifications include thymomas with both type A and B features, designated as type AB, and thymic squamous carcinoma [258]. Deletions of 6p have been reported as recurrent abnormalities in type A and AB thymomas [259]. Type B3 has been characterized by gain of chromosome 1q and loss of chromosomes 6 and 13q. Thymic squamous cell carcinomas exhibit recurrent gains of chromosomes 1q, 17q, and 18 and losses of chromosomes 3p, 6, 16q, and 17p [260].

Carcinoma with t(15;19) translocation, also known as midline lethal carcinoma or NUT midline carcinoma, is a very rare, aggressive thymic carcinoma of unknown etiology. Eight cases have been reported to date, with all cases arising in the mediastinum, most often adjacent to the thymus, of children or young adults [261–263]. The cytogenetic hallmark of this neoplasm is a t(15;19)(q14;p13.1) [264] with few, if any, other structural abnormalities. This rearrangement causes a fusion of the *BRD4* gene at 19p13.12 with the *NUT* gene at 15q14 [265]. A complex three-way translocation t(11;15;19) (p15;q12;p13.3) has been reported in one case [263]. All cases reported thus far have demonstrated a rapidly fatal course with an average survival of 18 weeks [261].

12.3.10 Tumors of the salivary glands

Pleomorphic adenoma is a benign tumor originating in the major and minor salivary glands. It is the most common salivary neoplasm, with 70–80% arising in the parotid gland [266,267]. Two-thirds of pleomorphic adenomas have clonal structural abnormalities, and can be classified into the following three groups according to their cytogenetic abnormalities: (1) tumors with 8q12 rearrangements (nearly one-half of all cases), (2) tumors with 12q13–15 rearrangements, and (3) tumors with clonal changes other than 8q12 or 12q13–15 [268,269]. Various translocations involving 8q12 have been reported, the most common being t(3;8)(p21;q12) and t(5;8)(p13;q12) (see Figure 12.14). These translocations result in the rearrangement and overexpression of *PLAG1* at 8q12 [270,271]. Several different chromosomes have been involved with 12q rearrangements in pleomorphic adenoma [57]. The target gene in the chromosome 12 abnormalities is *HMG2A2* at 12q15 [272].

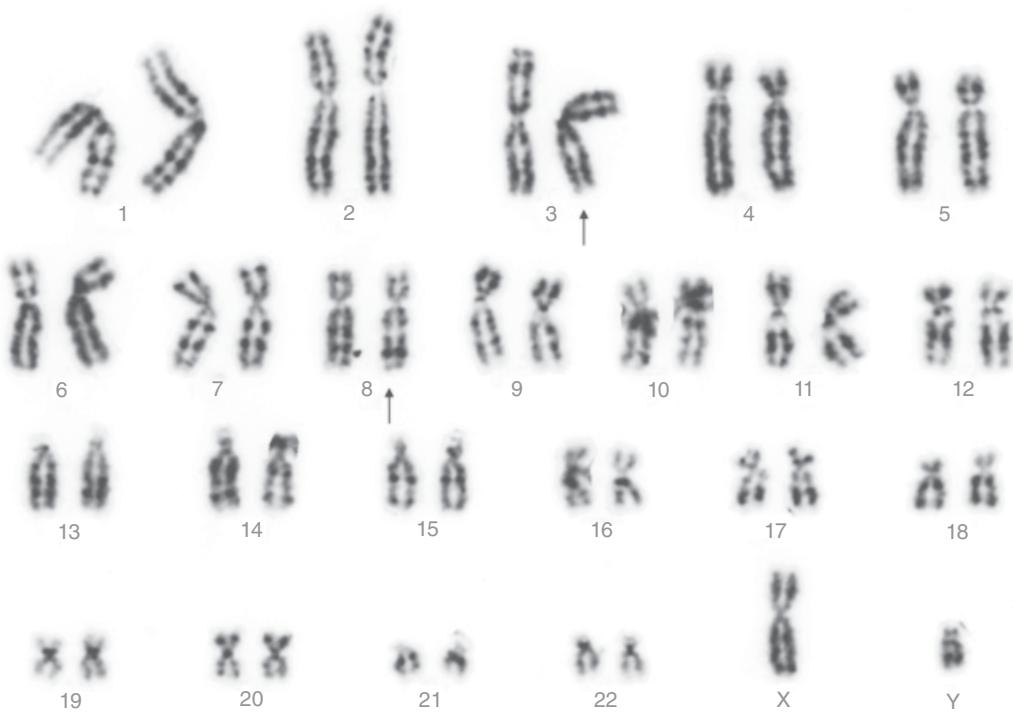


Figure 12.14 Representative karyogram of pleomorphic adenoma showing a translocation involving 3p and 8q [t(3;8)(p13;q12)]. The breakpoint at 3p13 is proximal to the more commonly reported 3p21 breakpoint.

Carcinoma ex pleomorphic adenoma is a carcinoma (CexPA) arising from a pre-existing benign pleomorphic adenoma. Röijer et al. [273] described a CexPA case containing a 12q14–15 translocation proximal to *HMG2A* and double minutes comprised of the *HMG2A* and *MDM2* (12q13-q14) gene regions, suggesting the contribution of their amplification to malignant transformation.

12.3.11 Tumors of the prostate

There has been a significant increase in the incidence of prostate cancer since the 1980s. In 2002 it ranked as the fifth most common cancer in the world in terms of the number of new cases, and in 2010 has jumped to the second most common form of cancer [16]. Three-quarters of all cases are in men aged 65 or more [274]. Most cases studied cytogenetically had normal karyotypes. Clonal abnormalities have been observed more commonly in poorly differentiated cancers that confer a shorter survival; +7 appears to be related to progressive disease [218]. Various recurrent chromosomal aberrations have been described in prostate cancer, including a gain of 8q, and loss of Y, 13q, 10q, 16p, and 16q (listed in order of frequency) [275,276]. Deletions of 8p and 16q have been suggested as markers for prediction of pathological staging and disease progression [277,278]. The use of a bioinformatics approach recently has identified recurrent gene fusions between *TMPRSS2* at 21q22.3 and *ETS* family members including *ERG* at 21q22.3, *ETV1* at 7p21.2, and *ETV4* at 17q21 [279,280]. Tomlins et al. developed a novel bioinformatic approach, termed cancer outlier profile analysis (COPA), to identify genes markedly overexpressed in a subset of cancers from DNA microarray data, and subsequently applied a variety of molecular techniques to characterize the oncogenic fusions [278]. *TMPRSS2/ERG* and *TMPRSS2/ETV1* gene fusions, of which *TMPRSS2/ERG* is the most common, occur in approximately 80% of prostate cancer cases and are generated through cryptic inter- or intra-chromosomal rearrangements [281].

12.3.12 Tumors of the kidney

Renal cell carcinoma (RCC) is a group of malignant tumors derived from the epithelium of the renal tubules. There are several distinct subtypes of malignant renal cell neoplasms as described by the major morphological classification systems, all of which demonstrate unique morphological, clinical and genetic characteristics [282]. Because of the distinctive

chromosomal aberrations associated with the different histologies, cytogenetic and molecular cytogenetic analyses have contributed greatly to the diagnostic evaluation of renal cell carcinoma.

Clear cell renal cell carcinoma

Clear cell, the most common form of RCC, is characterized by complete or partial loss of the short arm of chromosome 3 in the majority of cases [283]. LOH studies have identified at least three different regions as being relevant for sporadic clear cell RCC. These regions include 3p14.2, the location of the *FHIT* tumor suppressor gene; 3p21; and 3p25.3, the von Hippel–Lindau disease (*VHL*) gene locus [284,285]. Von Hippel–Lindau syndrome, caused by mutations of the *VHL* gene, predisposes the patient to the development of various benign and malignant neoplasms, including renal cell carcinoma. Other cytogenetic aberrations frequently found in clear cell RCC include gain of i(5p), 5q, and 7, and loss of 6q, 17p, and Y [219]. The recurrent der(3)t(3;5)(p11 ~ 22;q13 ~ 31), resulting in loss of 3p and gain of 5q, is a common finding; data has suggested that its presence correlates with a significantly better outcome [286] (see Figure 12.15). Allelic losses on chromosome 17p have been associated with disease progression [287], but the significance of +7 and –Y is probably not relevant, since these same abnormalities have been observed in non-neoplastic kidney tissue [288,289].

Papillary renal cell carcinoma

Papillary RCC is also frequently characterized by gain of chromosomes 7 and 17 and loss of Y; however, in contrast to clear cell RCC, deletions of 3p are infrequent [290,291]. LOH studies demonstrated additional genetic imbalances, including allelic duplications at regions 7q, 12q, 16q, 17q, and 20q [292]. Gain of chromosome 20 has been associated with the progression of papillary RCC [293]. Germ-line mutations of the *MET* oncogene at 7q31 have been detected in patients with familial papillary RCC [294].

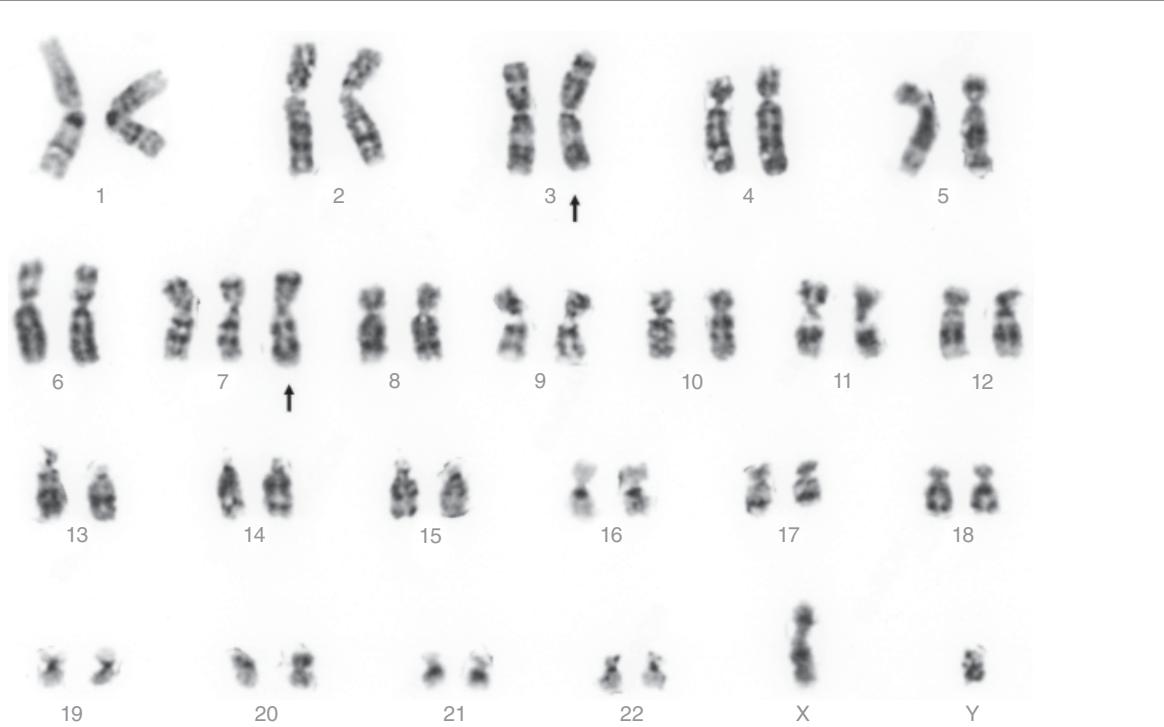


Figure 12.15 Representative karyogram of clear cell renal cell carcinoma showing a der(3)t(3;5)(p12;q13) resulting in loss of 3p and gain of 5q, and trisomy 7.

Renal carcinomas associated with Xp11.2 translocations

A group of renal carcinomas, predominantly affecting children and young adults, are characterized by several different translocations which result in fusions involving the *TFE3* gene at chromosome Xp11.22. The most common of these translocations is t(X;1)(p11.2;q21.2) and results in a fusion of the *TFE3* gene to a papillary RCC (*PRCC*) gene located at 1q21.11 [295,296]. Another subset of RCCs displaying *TFE3* rearrangements contain a t(X;17)(p11;q25) as the sole cytogenetic abnormality and involve fusions with the gene *RCC17* at 17q25 [297]. Very rare cases of papillary RCC exhibit t(X;1)(p11.2;p34), resulting in the fusion of the *PSF* (*SFPQ* at 1p34.3) and *TFE3* genes, or inv(X)(p11;q12), resulting in *TFE3/NONO* fusions [298].

Mesoblastic nephroma

Congenital mesoblastic nephroma (CMN), a benign condition, is the most common renal tumor in neonates. CMN is divided into various subtypes based on histological appearance, including classical, cellular and mixed [299]. Trisomies of chromosome 8, 11, and 17 are common in cellular and mixed CMN [300]. Mesoblastic nephromas characterized by +11 are also typically characterized by a t(12;15)(p13;q25) associated with oncogenic fusion of the *ETV6* gene region at 12p13 with the *NTRK3* gene region at 15q24-25 [301]. This abnormality has also been described in congenital fibrosarcoma, which has a histological appearance very similar to CMN. This suggests that they are pathologically related and possibly represent a single neoplastic entity [301–303]. The t(12;15) is difficult to detect by conventional cytogenetic techniques and may require additional studies, such as FISH, for confirmation [304].

Nephroblastoma (Wilms tumor)

Wilms tumor (WT) or nephroblastoma is the most common tumor of renal origin found in children and is frequently associated with various congenital syndromes, including WARG (Wilms tumor, aniridia, mental retardation, and genitourinary abnormalities), Denys–Drash syndrome, Beckwith–Wiedeman, and Simpson Golabi Behmel syndrome. Nearly 2% of WT cases appear to be inherited as an autosomal dominant trait [305]. Familial WT susceptibility genes have been proposed at 11p13 (*WT1*), 17q12 ~ q21 (*FWT1*) and 19q13 (*FWT2*) [306–308]. Recurrent cytogenetic abnormalities detected in Wilms tumor include rearrangements of 1p/1q and 11p13 ~ 15, deletions of 16q and gains of 6, 7, 8, 12, 13, and 18 [219,309–311]. LOH of 1p and 16q has been associated with a subset of Wilms tumor patients who have a significantly increased risk of relapse and death [312].

12.3.13 Tumors involving germ cells of testicular or ovarian origin

Germ cell tumors (GCT) are a heterogeneous group of neoplasms, which can be found at different, although limited, anatomical locations. More than half of the testicular germ cell tumors are mixed neoplasms containing more than one tumor type including seminoma and nonseminoma subtypes (including embryonal carcinoma, teratoma, yolk sac tumor, and choriocarcinoma) [313]. Gain of 12p sequences is the only consistent structural chromosomal anomaly observed in invasive seminomas and nonseminomas from adult patients. Nearly 80% of the invasive tumors have extra copies of 12p due to the formation of an isochromosome (i(12p)) [314–316]. In addition, the i(12p) negative tumors also show gain of 12p, due to other chromosomal changes [317]. Carcinoma in situ (CIS), the precursor of seminomas and nonseminomatous germ cell tumors, do not typically exhibit i(12p) when in the “dormant” state but do show gain of 12p when adjacent to overt tumors [318,319]. Isochromosome 12p is also a recurrent structural rearrangement in several subtypes of ovarian GCT [320,321]. The presence of this anomaly in both testicular and ovarian GCT suggests that they may arise from a similar pathogenesis process. The majority of childhood GCT are diagnosed at nongonadal sites, whereas, in adults, testicular tumors predominate. The cytogenetic aberrations observed in GCT in children also differ from that in adult. Most malignant GCT in children do not show chromosomal gain of 12p, but are instead often characterized by imbalances of chromosome 1 (1p loss, 1q gain) [322]. Deletions of 4q and 6q and gain of 20q were also reported at high frequency in prepubertal GCT [323]. In testicular GCT of adult patients, a high frequency of biallelic expression of both *H19* and *IGF2* at 11p15 has been found. The role of genomic imprinting in the development of germ cell tumors is further discussed in Chapter 10, section 10.3, Germ cell tumors – UPD and imprinting.

12.3.14 Tumors of the pleura

Diffuse malignant mesothelioma is a highly lethal tumor derived from the mesothelial cells of the pleura. It is largely seen in patients over 60 years of age, and in most industrialized countries 80–90% of cases in male patients are related to prior asbestos exposure. In contrast, only about 20% of mesothelioma tumors in women from North America are caused by asbestos [324].

Cytogenetic analysis of mesothelioma generally is associated with complex numerical and structural chromosomal changes [325]. Though no specific anomaly has been described, a number of recurrent abnormalities have been observed. Common numerical changes in mesothelioma include loss of 4 and 22, and gain of 5, 7, and 20 [326]. Deletion hot spots have been observed by several investigators at 1p21-22, 3p21, 6q15-21, 9p21-22, and 22q12, suggesting that tumor suppressor genes critical to mesothelioma development may reside at these loci [327–329].

12.4 Molecular genetic and cytogenetic techniques

12.4.1 FISH applications

Classical cytogenetics depends upon the presence of metaphase cells for the identification of numerical and structural chromosomal anomalies. It is specifically limited by minimal resolution and reduced efficacy in the highly rearranged karyotypes common to neoplasms. The advent of molecular cytogenetic techniques, in particular FISH, has circumvented these limitations and provided a rapid and straightforward procedure for the detection of specific chromosomal abnormalities.

FISH may be employed as a primary or secondary means of investigation, but its practicality is dependent upon the differential diagnosis and the availability of DNA probes specific for the loci in question. Unlike conventional cytogenetics, FISH analysis is not confined to the availability of fresh tissue and allows for the detection of low-level mosaicism when performed on interphase cells. FISH analysis can be performed on a variety of samples such as touch preparations, cytocentrifuge preparations, unstained paraffin-embedded tissue sections, or cultured cells. A cytocentrifuge preparation concentrates the density of the cell pellet and can be advantageous when fluid samples containing a paucity of cells (e.g., cerebral spinal fluid) need to be studied for FISH analysis. One substantial advantage to performing FISH on paraffin sections is that tissue and cell morphology is preserved and can be used as a guide to the detection of relevant cell populations. Before FISH is performed on a paraffin-embedded section, it is important to identify the location of the malignant cells of interest. This can best be accomplished by an initial review of the corresponding hematoxylin and eosin (H&E) stained slide by a pathologist. One drawback for FISH analysis on paraffin sections is that it is traditionally performed on sections 4–5 µm thick. This results in numerous incomplete nuclei and truncated probe signals. Extraction of the nuclei from 50- to 60-µm-thick paraffin blocks can alleviate some of these problems; however, it also results in the loss of tissue architecture. Decalcified or B5 fixed tissues are often less likely to yield successful FISH results. Detailed information on the principles and methodology of FISH analysis can be found in Chapter 16, Fluorescence in situ hybridization (FISH).

Several cytogenetic laboratories have capabilities that allow them to develop “homebrew” probe systems, but for the purpose of this chapter we will limit ourselves to the discussion of commercially available FISH probes. See Table 12.4 for FISH probes used on solid tumors. Cytogenetic laboratories, unfortunately, often find themselves limited by the number of commercial FISH probes available due to the scarceness of disease-specific cytogenetic abnormalities in solid tumors. This has limited the utility of FISH technology as a diagnostic tool in the clinical setting. The lack of specific targets has been circumvented partially by the introduction of multicolor FISH probes. Multicolor DNA probe panels have been developed to allow for the analysis of solid malignancies characterized by the gain or loss of multiple chromosomes or loci. This strategy can be utilized for the early detection of bladder cancer recurrence by assessing the presence of aneusomic cells in bladder washings or voided urine [330] (see Figure 12.16). Multicolor probe systems are also used on bronchial washings to assess the chromosomal regions or genes commonly gained in lung carcinoma [331,332] and on tissue samples to determine the status of unique loci on chromosome 8 associated with prostate cancer [275,333].

Additional examples are emerging regarding the applicability of FISH assays to determine the presence of gene amplification or loss for prognostic and/or therapeutic purposes. The evaluation of HER2/neu gene amplification in breast carcinoma is a well-known example of the use of a FISH assay as an effective prognostic marker and predictor of therapy response in solid neoplasms. Amplification of *ERBB2* (formerly referred as HER2/neu) as detected by FISH, is an important criterion in the selection of patients eligible for treatment with trastuzumab (Herceptin®) [334,335]. FISH has also proven its diagnostic utility with the detection of *MYCN* amplification, an indication of poor prognosis in NB patients [336,337]. Recently, FISH probes have been developed for regions 20q13 and 17p13.1 that overlap the aurora kinase A and B (*AURKA* and *AURKB*) genes, respectively. Aurora kinases, critical for the most important steps of mitosis including centromere duplication, are abnormally expressed in several solid malignancies including breast, ovary, prostate, pancreas, and colorectal cancer and their inhibition is being targeted for the development of novel anti-cancer agents [338,339]. DNA probes are also available for the gene regions of *TERT* (5p15.33) and *TERC* (3q26.2) associated with telomerase activity. High levels of telomerase, the enzyme responsible for the maintenance of telomere length, has been found in solid tumors and has been linked to cellular immortality. Amplification of the *TERT* and *TERC* gene regions has been detected by FISH in various tumors, such as non-small lung carcinoma, squamous cell carcinoma of head and neck, and uterine cervix cancer [340,341].

Table 12.4 DNA fluorescence in situ hybridization probes available for the study of human solid malignancies

Probe Description	Type(s) of Deregulation	Cancer Types
D1Z2 (1p36)	Deletion	Neuroblastoma: incidence ~40% and associated with <i>MYCN</i> amplification
1p36/19q13	Deletion	Diffuse glioma: associated with favorable chemotherapeutic response and overall survival
MYCN (2p24)	Amplification	Neuroblastoma: >25%, associated with poor prognosis
hTERC (3q26)	Low level amplification	Various Ca: positive pap smear associated with development of cervical Ca
hTERT (5p15)	Amplification	Various Ca: associated with poor prognosis in non-small cell lung Ca
EGFR (7p12)	Amplification	Glioblastoma and various Ca: possible prognostic indicator
MYC (8q24)	Amplification	Various Ca: part of multi-probe system for prostate Ca
p16 (9p21)	Deletion	Glioma, various Ca: part of multi-probe system for bladder Ca
PTEN (10p23)	Deletion	Glioblastoma: associated with a poor prognosis
GLI1 (12q13)	Amplification	Glioma, liposarcoma, osteosarcoma
RB1 (13q14)	Deletion	Retinoblastoma: 20% deletions >1Kb, osteosarcoma, melanoma, brain tumor
p53 (17p13)	Deletion	Various Ca and sarcomas
AURKB (17p13)	Deletion	Various neoplasms
HER2 (17q12)	Amplification	Breast Ca: associated with chemotherapeutic response
TOP2A (17q21)	Amplification/Deletion	Breast Ca: possible predictor for therapy response
AURKA (20q13)	Amplification	Prostate Ca, colorectal Ca, breast Ca: possible prognostic indicator
Bladder Ca probe set 3, 7, 17 centromere p16	Aneuploidy Deletion	Recurrent urothelial Ca
Prostate Ca probe set 8 centromere LPL (8p22) MYC (8q24)	Deletion Gain	Prostate Ca
Lung Ca probe set 6 centromere EGFR (7p12) MYC (8q24) 5p15.2	Gain Amplification Amplification Amplification	Non-small cell lung Ca
EWSR1 (22q12)	Translocation	Ewing sarcoma: t(11;22) >80% t(21;22) 5-10% Other variants <5% Desmoplastic small round cell tumor: t(11;22) >75% Clear cell sarcoma: t(12;22) >75% Extraskeletal myxoid chondrosarcoma: t(9;22) >75% Myxoid/round cell liposarcoma: t(12;22) <5%
SS18 (18q11)	Translocation	Synovial sarcoma: t(X;18) >90%
FKHR (13q14)	Translocation	Alveolar rhabdomyosarcoma: t(1;13) 10-20% t(2;13) >75%
DDIT3 (12q13)	Translocation	Myxoid/round cell liposarcoma: t(12;16) >75% t(12;22) <5%
FUS (16p11)	Translocation	Myxoid/round cell liposarcoma: t(12;16) >75% Angiomatoid fibrous histiocytoma: t(12;16) Low grade fibromyxoid sarcoma: t(7;16)

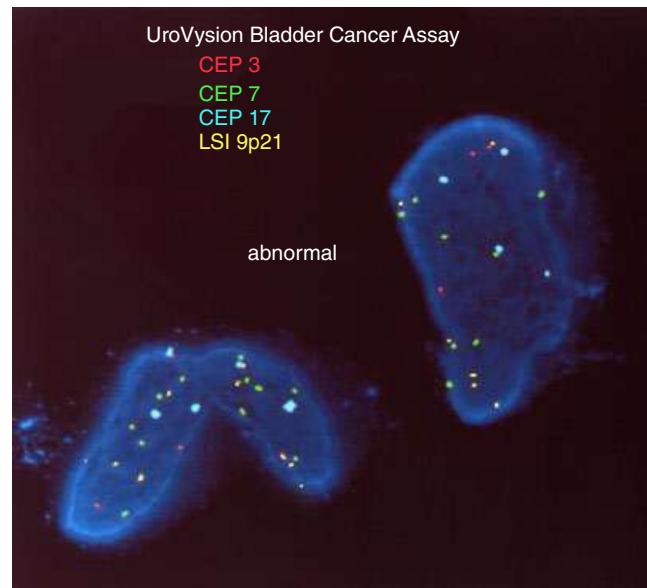


Figure 12.16 Interphase cell from urothelial carcinoma showing copy number gain for all probes of the UroVysis™ assay. (Vysis, Abbott Molecular Inc., Des Plaines, IL). See insert for color representation of this figure.

A variety of DNA probes are available for the evaluation of neurological cancers. DNA probes specific for regions 1p36 and 19q13 are used to detect deletions often associated with diffuse glioma. Loss of these regions has been associated with favorable chemotherapeutic response and overall survival [342]. FISH probes are also available to determine copy number for the *EGFR* gene at 7p12. The true prognostic value is under debate, but recent studies have shown amplification of *EGFR* to be a favorable indicator in glioblastoma multiforme (GMB) when correlated with patient age [343,344]. Detection of *PTEN* (10q23) loss by FISH has been associated with a poor prognosis for patients with GMB, but is a rare finding in other glioma subtypes such as anaplastic astrocytoma or oligodendroglioma [344–346].

The locus-specific probe for the *EWSR1* gene region at 22q12.2 was one of the first probes commercially developed for the study of translocations exclusive to mesenchymal neoplasms. The current configuration of this probe set is a dual color, break-apart system designed to detect disruptions of the *EWS* gene region like those present in the t(11;22)(q34;q12) of Ewing sarcoma. Disruption of the *EWS* gene region is also observed in several other sarcomas, including desmoplastic small round cell tumor, clear cell sarcoma, angiomyoid fibrous histiocytoma, myxoid liposarcoma, and myxoid chondrosarcoma. This can be problematic, since positive interphase FISH results for *EWS* rearrangements do not allow for distinction between these tumors.

Like the *EWS* probe, other dual-color, break-apart FISH probes have been designed to detect chromosomal translocations found in solid tumors, include the following: (1) *SS18* (formerly *SYT*) gene region at 18q11.2 for synovial sarcoma, (2) *DDIT3* gene region at 12q13.1-q13.2 for myxoid liposarcoma, (3) *FUS* gene region at 16p11.2 for myxoid liposarcoma, angiomyoid fibrous histiocytoma and low grade fibromyxoid sarcoma, and (4) the *FOXO1* (previously *FKHR*) gene region at 13q14.1 for alveolar rhabdomyosarcoma (see Figure 12.17). As it stands now, the *FOXO1* probe set cannot be used to distinguish between the t(1;13) and t(2;13) of alveolar rhabdomyosarcoma in interphase cells and thus will not provide this important prognostic information.

Recently, chromogenic in situ hybridization (CISH) has emerged as a potential alternative to FISH. This technology allows for the use of DNA probes to evaluate relevant chromosomal regions in interphase cells without the use of a fluorescent microscope or specialized imaging software. In CISH, the probes are detected by a peroxidase reaction, and visualization of the red, green or brown enzyme precipitates is carried out on an ordinary brightfield microscope [347,348]. The CISH assay is extremely stable and can be archived for several years; however, many laboratories find the FISH methodology simpler to perform and the number of probes available in the CISH format is comparatively few. The most well-known example of the utility of CISH probes is the evaluation of HER2/neu oncogene in breast carcinoma. Several studies have shown the results between FISH and CISH assays to be concordant in the determination of HER2/neu amplification [349–351].

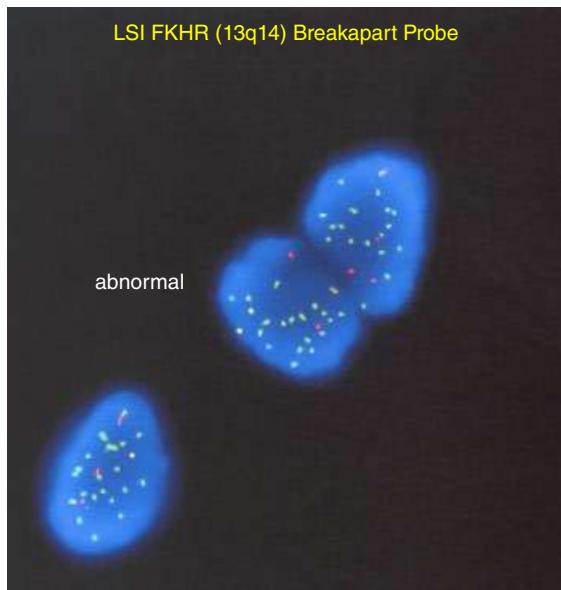


Figure 12.17 Disruption of *FKHR* (13q14) gene in alveolar rhabdomyosarcoma. Interphase cell from alveolar rhabdomyosarcoma specimen showing disruption of LSI® *FKHR* (13q14) dual-color, break-apart rearrangement probe (split red and green signal) with amplification of 3' region (green signal) (Vysis, Abbott Molecular Inc., Des Plaines, IL). See insert for color representation of this figure.

12.4.2 RT-PCR

Other molecular techniques, such as RT-PCR (reverse transcriptase polymerase chain reaction) can be employed to detect several of the chimeric or fusion genes created by the disease specific translocations found in human malignancies. RT-PCR can be performed on fresh, frozen or fixed tissue and is more sensitive than both FISH and classical cytogenetics. It utilizes specific synthetic oligonucleotides to amplify sections of genes, and when positive, ultimately confirms the identity of both chromosomes involved in a given translocation. RT-PCR analysis may also provide additional prognostic information by identifying a more detailed classification of the fusion product within a specific disease entity. In Ewing sarcoma, the most common *EWSR1/FLI1* gene fusion, type 1 (resulting in the fusion of exon 7 of *EWSR1* to exon 6 of *FLI1*), has been reported to be associated with a better prognosis than larger fusion types [352].

As with all methodologies, RT-PCR has its limitations. Specifically, it requires the presence of RNA of sufficient quality and is unable to detect chromosomal anomalies other than those for which the test was designed.

12.4.3 Chromosome microarray analysis and multicolor karyotyping

Two major variations on FISH technology, comparative genomic hybridization (CGH) and multicolor karyotyping, have contributed significantly to our knowledge of cancer cytogenetics in the past decade. CGH technology has been utilized for the study of solid malignancies since its inception in 1992 by Kallioniemi et al. [353] and allows for the detection of copy number changes of the entire genome in a single step. Since CGH analysis is performed on extracted DNA, cell culture is not necessary. However, it is limited by its low resolution (5–10 Mb for the detection of copy-number loss and gain, and 2 Mb for amplification) and its inability to detect balanced rearrangements. CGH is also limited by its ability to detect an aberrant karyotype within a mosaic sample. It has been shown that mosaicism is usually not detected if there is greater than a 30–50% dilution of aberrant DNA by normal DNA [354].

Early contributions from chromosome microarray technologies include the detection of chromosomal regions potentially harboring tumor suppressor genes and oncogenes, including regions of amplification that previously had not been identified by any other means [355,356]. Array-based CGH or matrix CGH [357,358] technology replaced the chromosomal targets of standard CGH with well-defined genomic clones such as BACs, PACs or cosmids and oligonucleotides, thus significantly increasing the ability to detect small segments of genomic imbalance. High-density CGH arrays designed for specific neoplasms have likewise offered the potential to identify disease-related genetic aberrations not yet detected and may prove to be

informative in the diagnosis/prognosis of malignancies. Additional information on chromosome microarray analysis can be found in Chapter 18, Genomic microarray technology for the cytogenetics laboratory.

Multicolor karyotyping allows for the painting of every human chromosome in a unique color. This is accomplished through the labeling of specific probes with one or more combinations of five spectrally distinct fluorochromes to produce 24 differentially labeled whole chromosome paints. The two main types of multicolor karyotyping techniques are spectral karyotyping (SKY) and multiplex-FISH (M-FISH). The central difference between the two techniques is in the method of image acquisition. SKY relies on the capture of a single image acquired through a customized multi-band optical filter [359], while M-FISH technology captures the five spectrally distinct fluorochromes separately through single-band filters and combines them into a single image [360]. These methodologies are limited by their inability to detect intrachromosomal aberrations, their low resolution and their need for mitotic cells. Nevertheless, both multicolor karyotyping techniques have been very useful in the identification of marker chromosomes, the recognition of subtle translocations and the characterization of complex chromosomal rearrangements found in solid tumors. Additional information on chromosomal CGH and multicolor karyotyping (SKY and M-FISH) can be found in Chapter 17, Multicolor FISH (SKY and M-FISH) and CGH.

12.5 Conclusion

The identification of specific chromosomal abnormalities in solid neoplasms has provided valuable insight into the molecular changes underlying tumorigenesis. Cytogenetic analysis has proven to be a powerful ancillary tool in the formulation of an accurate diagnosis, particularly in the realm of sarcoma studies. Our present understanding of the recurrent abnormalities is but the tip of the iceberg and we will continue to modify and expand our current perceptions as further knowledge is gained. Even the highly complex and seemingly uninformative changes observed in carcinomas have, through the application of molecular approaches, highlighted critical regions that harbor key oncogenes and tumor suppressor genes. By combining classical and molecular genetic techniques, scientists have bridged basic research to clinical practice. The application of these techniques will continue to increase and as a result, feasibly lead to improved early detection, more accurate tumor profiling, selection of targeted therapeutic approaches and the monitoring of clinical outcome in patients afflicted with solid tissue malignancies.

Glossary

Artifact: a structure or substance not normally present but produced by an external agent or action, such as a structure seen in a microscopic specimen after fixation that is not present in the living tissue. *Cultural artifact* would thus indicate that the error has occurred within any cultural aspect of the process, such as the presence of an isolated and random translocation rearrangement. *Technical artifact* would indicate that the error has occurred within a technical aspect of the process, such as the random loss of chromosomes due to overspreading. Neither example would represent the true karyotype of the source tissue.
 artifact. (n.d.). *The American Heritage® Stedman's Medical Dictionary*. Retrieved August 08, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/artifact>

Ascertainment bias: systematic failure to represent equally all classes of cases or people supposed to be represented in a sample. In cytogenetics laboratory practice, ascertainment bias refers to situations when the tissue received for cytogenetic analysis has been sampled from or includes a non-neoplastic, or non-representative, segment of the intended tissue, thus creating either a full or mosaic misrepresentation of the true karyotype.
<http://www.medilexicon.com/medicaldictionary.php?t=1008> on 8/8/11.

Ascites: see Chapter 4, Glossary.

Core needle biopsy: a procedure that removes small but solid samples of tissue using a hollow "core" needle. The needle is larger than that used in fine-needle aspirates and thus removes a larger sample for evaluation.
 Retrieved July 24, 2011 from <http://en.wikipedia.org/wiki/Biopsy>

Cultural artifact: see artifact.

Dedifferentiation: A process by which structures or behaviors that were specialized for a specific function *lose* their specialization and become simplified or generalized. dedifferentiation.(n.d.).
 Dictionary.com Unabridged. Retrieved July 24, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/dedifferentiation>

Fine needle aspiration (FNA): a percutaneous ("through the skin") procedure that uses a fine needle and a syringe to sample fluid from a breast cyst or remove clusters of cells from a solid mass.

Retrieved July 24, 2011, from <http://en.wikipedia.org/wiki/Biopsy>

Heterozygosity: zygosity refers to the similarity of genes for a trait (inherited characteristic) in an organism. If both genes are the same, the organism is homozygous for the trait. If the genes are different, the organism is heterozygous for that trait. If one gene is missing, it is hemizygous, and if both genes are missing, it is nullizygous.

Retrieved July 24, 2011, from <http://en.wikipedia.org/wiki/Zygosity>

Histogenesis: embryologic formation of tissues from undifferentiated cells from the three primary germ layers – endoderm, mesoderm, and ectoderm.

Retrieved July 24, 2011 from <http://en.wikipedia.org/wiki/Histogenesis>

Karnofsky performance score: a measure given by a physician to rate a patient's ability to perform certain ordinary tasks: for example, 100 – normal, no complaints; 70 – unable to carry on normal activity; 50 – requires considerable assistance; 40 – disabled; 30 – hospitalization recommended.

Retrieved July 20, 2011 from <http://wwwENCYCLO.co.uk/>

Metastasis: transmission of pathogenic microorganisms or cancerous cells from an original site to one or more sites elsewhere in the body, usually by way of the blood vessels or lymphatics; a secondary cancerous growth formed by transmission of cancerous cells from a primary growth located elsewhere in the body.

metastasis.(n.d.). *The American Heritage® Stedman's Medical Dictionary*. Retrieved July 26, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/metastasis>

Nevus: a congenital circumscribed growth or mark on the skin, such as a mole or a birthmark, colored by hyperpigmentation or increased vascularity. It can also be a benign localized overgrowth of melanin-forming cells arising in the skin early in life. nevus. (n.d.). *The American Heritage® Stedman's Medical Dictionary*. Retrieved July 26, 2011 from Dictionary.com website: <http://dictionary.reference.com/browse/nevus>

Pleural effusions: see Chapter 4, Glossary.

References

1. Hardy PA, Zacharias H. Reappraisal of the Hansemann-Boveri hypothesis on the origin of tumors. *Cell Biol Int* 2005; 29(12):983–992.
2. Wunderlich V. JMM—past and present. Chromosomes and cancer. *J Mol Med* 2002; 80(9):545–548.
3. Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. *Science* 1960;132–197.
4. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and giemsa staining. *Nature* 1973; 243(5405):290–293.
5. Levan A, Manolov G, Clifford P. Chromosomes of a human neuroblastoma: a new case with accessory minute chromosomes. *J Natl Cancer Inst* 1968; 41(6):1377–1387.
6. Biedler JL, Spengler BA. Metaphase chromosome anomaly: association with drug resistance and cell-specific products. *Science* 1976; 191(4223):185–187.
7. Mark J, Levan G, Mitelman F. Identification by fluorescence of the G chromosome lost in human meningomas. *Hereditas* 1972; 71(1):163–168.
8. Zankl H, Zang KD. Cytological and cytogenetical studies on brain tumors. 4. Identification of the missing G chromosome in human meningiomas as no. 22 by fluorescence technique. *Humangenetik* 1972; 14(2):167–169.
9. Aurias A, Rimbaut C, Buffe D, Dubouset J, Mazabraud A. Translocation of chromosome 22 in Ewing's sarcoma. *N Engl J Med* 1983; 309:496–497.
10. Turc-Carel C, Philip I, Berger MP, Philip T, Lenoir G. Chromosomal translocation (11; 22) in cell lines of Ewing's sarcoma. *N Engl J Med* 1983; 309:497–498.
11. Sandberg AA. *The Chromosomes in Human Cancer and Leukemia*, 2nd ed. New York: Elsevier Science Publishing, 1990.
12. Dal Cin P, Sandberg AA. Karyotypic analysis of solid tumors. *Curr Top Pathol* 1990; 82:257–288.
13. *Mitelman Database of Chromosome Aberrations in Cancer* 2006. Mitelman F, Johansson B, Mertens F, eds. <http://cgap.nci.nih.gov/library1.unmc.edu:2048/Chromosomes/Mitelman>
14. *ACMG Technical Standards and Guidelines for Clinical Genetics Laboratories; Section E6.5: Chromosome Studies for Solid Tumor Abnormalities*, 2009 Edition, Revised 01/2010. <http://www.acmg.net/AM/PrinterTemplate.cfm?Section=Publications1>
15. Hussein MR. Genetic pathways to melanoma tumorigenesis. *J Clin Pathol* 2004; 57(8):797–801.
16. American Cancer Society: Cancer Facts & Figures 2013 (pages 10, 25). <http://www.cancer.org/acs/groups/content/@epidemiologysurveilance/documents/document/acspc-036845.pdf>
17. Weiss SW. Lipomatous tumors. *Monogr Pathol* 1996; 38:207–239.

18. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors. Dermatofibrosarcoma protuberans and giant cell fibroblastoma. *Cancer Genet Cytogenet* 2003; 140(1):1–12.
19. Cin PD, Sciot R, de Wever I, Brock P, Casteels-Van Daele M, Van Damme B, Van Den Berghe H. Cytogenetic and immunohistochemical evidence that giant cell fibroblastoma is related to dermatofibrosarcoma protuberans. *Genes Chromosomes Cancer* 1996; 15(1):73–75.
20. Kelly KM, Womer RB, Sorensen PH, Xiong QB, Barr FG. Common and variant gene fusions predict distinct clinical phenotypes in rhabdomyosarcoma. *J Clin Oncol* 1997; 15(5):1831–1836.
21. Barr FG. Molecular genetics and pathogenesis of rhabdomyosarcoma. *J Pediatr Hematol Oncol* 1997; 19(6):483–491.
22. Sciot R, Dorfman H, Brys P, Dal Cin P, De Wever I, Fletcher CD, Jonson K, Mandahl N, Mertens F, Mitelman F, Rosai J, Rydhholm A, Samson I, Tallini G, Van den Berghe H, Vanni R, Willén H. Cytogenetic-morphologic correlations in aneurysmal bone cyst, giant cell tumor of bone and combined lesions. A report from the CHAMP study group. *Mod Pathol* 2000; 13(11):1206–1210.
23. Lechner JF, Haugen A, McClendon IA, Pettis EW. Clonal growth of normal adult human bronchial epithelial cells in a serum-free medium. *In Vitro* 1982; 18(7):633–642.
24. Hammond SL, Ham RG, Stampfer MR. Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. *Proc Natl Acad Sci USA* 1984; 81(17):5435–5439.
25. Pandis N, Heim S, Bardi G, Limon J, Mandahl N, Mitelman F. Improved technique for short-term culture and cytogenetic analysis of human breast cancer. *Genes Chromosomes Cancer* 1992; 5(1):14–20.
26. Carney DN, Bunn PA Jr, Gazdar AF, Pagan JA, Minna JD. Selective growth in serum-free hormone-supplemented medium of tumor cells obtained by biopsy from patients with small cell carcinoma of the lung. *Proc Natl Acad Sci USA* 1981; 78(5):3185–3189.
27. Freshney RI. *Culture of Animal Cells: A Manual of Basic Techniques*, 5th ed. Hoboken, New Jersey: John Wiley & Sons; 2005:129–421.
28. Mandahl N. Methods in solid tumour cytogenetics. In: Rooney DE, Czepulkowski BH, eds. *Human Cytogenetics. A Practical Approach. Volume II. Malignancy and Acquired Abnormalities*, 2nd ed. Oxford: Oxford University Press, 1992:155–87.
29. Yunis JJ. New chromosome techniques in the study of human neoplasia. *Hum Pathol* 1981; 12(6):540–549.
30. Thompson FH. Cytogenetic methods and findings in human solid tumors. In: Barch MJ, Knutsen T, Spurbeck J, eds. *The AGT Cytogenetics Laboratory Manual*, 3rd ed. Philadelphia: Lippincott-Raven; 1997:375–430.
31. Band V, Sager R. Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *Proc Natl Acad Sci USA* 1989; 86(4):1249–1253.
32. Trent JM, Crickard K, Gibas A, Goodacre A, Pathak S, Sandberg AA, Thompson F, Whang-Peng J, Wolman S. Methodological advances in the cytogenetic analysis of human solid tumors. *Cancer Genet Cytogenet* 1986; 19:57–66.
33. Wang HC, Federoff S. Banding in human chromosomes treated with trypsin. *Nature New Biol* 1972; 235:52–53.
34. Seabright M. A rapid banding technique for human chromosomes. *Lancet* 1971; 2:971–972.
35. Nowell PC. Tumors as clonal proliferation. *Virchows Arch B Cell Pathol* 1978; 29(1–2):145–150.
36. Heim S, Mitelman F. Cytogenetically unrelated clones in hematological neoplasms. *Leukemia* 1989; 3(1):6–8.
37. Johansson B, Heim S, Mandahl N, Mertens F, Mitelman F. Trisomy 7 in nonneoplastic cells. *Genes Chromosomes Cancer* 1993; 6(4):199–205.
38. Stark B, Mor C, Jeison M, Gobuzov R, Cohen IJ, Goshen Y, Stein J, Fisher S, Ash S, Yaniv I, Zaizov R. Additional chromosome 1q aberrations and der(16)t(1;16), correlation to the phenotypic expression and clinical behavior of the Ewing family of tumors. *J Neurooncol* 1997; 31(1–2):3–8.
39. Mugneret F, Lizard S, Aurias A, Ture-Carel C. Chromosomes in Ewing's sarcoma. II. Nonrandom additional changes, trisomy 8 and der(16)t(1;16). *Cancer Genet Cytogenet* 1988; 32(2):239–245.
40. Day SJ, Nelson M, Rosenthal H, Vergara GG, Bridge JA. Der(16)t(1;16)(q21;q13) as a secondary structural aberration in yet a third sarcoma, extraskeletal myxoid chondrosarcoma. *Genes Chromosomes Cancer* 1997; 20(4):425–427.
41. Birch NC, Antonescu CR, Nelson M, Sarra L, Neff JR, Seemayer T, Bridge JA. Inconspicuous insertion 22;12 in myxoid/round cell liposarcoma accompanied by the secondary structural abnormality der(16)t(1;16). *J Mol Diagn* 2003; 5(3):191–194.

42. McManus AP, Min T, Swansbury GJ, Gusterson BA, Pinkerton CR, Shipley JM. Der(16)t(1;16)(q21;q13) as a secondary change in alveolar rhabdomyosarcoma. A case report and review of the literature. *Cancer Genet Cytogenet* 1996; 87(2):179–181.
43. Zheng MH, Siu P, Papadimitriou JM, Wood DJ, Murch AR. Telomeric fusion is a major cytogenetic aberration of giant cell tumors of bone. *Pathology* 1999; 31(4):373–378.
44. Bridge JA, Mouron BJ, Neff JR, Bhatia PS. Significance of chromosomal abnormalities in a malignant giant cell tumor of bone. *Cancer Genet Cytogenet* 1991; 57(1):87–92.
45. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors. Gastrointestinal stromal tumors. *Cancer Genet Cytogenet* 2002; 135(1):1–22.
46. Swerdlow S, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein HJ, Thiele J, Vardiman JW, eds. The International Agency for Research on Cancer: *WHO Classification of Tumors of Haematopoietic and Lymphoid Tissue (IARC WHO classification of tumours)*, 4th ed. World Health Organization. Lyon, France: IARC Press; 2008.
47. Mandahl N. Soft tissue tumors: lipoma/benign lipomatous tumors. *Atlas Genet Cytogenet Oncol Haematol*. May 2000. <http://AtlasGeneticsOncology.org/Tumors/lipoma5050.html>.
48. Sandberg AA, Bridge JA. Tumors of fat. In: Sandberg AA, Bridge JA, eds. *The Cytogenetics of Bone and Soft Tissue Tumors*. Austin, TX: R.G. Landes; 1994:147–216.
49. Willén H, Akerman M, Dal Cin P, De Wever I, Fletcher CD, Mandahl N, Mertens F, Mitelman F, Rosai J, Rydholm A, Sciot R, Tallini G, Van den Berghe H, Vanni R. Comparison of chromosomal patterns with clinical features in 165 lipomas: a report of the CHAMP study group. *Cancer Genet Cytogenet* 1998; 102(1):46–49.
50. Nielsen GP, Manhahl N. Lipoma. In: Fletcher CDM, Unni KK, Mertens F, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone*. Lyon: IARC Press; 2002:20–22.
51. Sandberg AA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: Lipoma. *Cancer Genet Cytogenet* 2004; 150(2):93–115.
52. Kazmierczak B, Dal Cin P, Wanschura S, Borrmann L, Fusco A, Van den Berghe H, Bullerdiek J. HMGIY is the target of 6p21.3 rearrangements in various benign mesenchymal tumors. *Genes Chromosomes Cancer* 1998; 23(4):279–285.
53. Pedeutour F, Forus A, Coindre JM, Berner JM, Nicolo G, Michiels JF, Terrier P, Ranchere-Vince D, Collin F, Myklebost O, Turc-Carel C. Structure of the supernumerary ring and giant rod chromosomes in adipose tissue tumors. *Genes Chromosomes Cancer* 1999; 24(1):30–41.
54. Collins MH, Chatten J. Lipoblastoma/lipoblastomatosis: a clinicopathologic study of 25 tumors. *Am J Surg Pathol* 1997; 21(10):1131–1137.
55. Bolen JW, Thorning D. Benign lipoblastoma and myxoid liposarcoma: a comparative light- and electron-microscopic study. *Am J Surg Pathol* 1980; 4(2):163–174.
56. Miller GG, Yanchar NL, Magee JF, Blair GK. Lipoblastoma and liposarcoma in children: An analysis of 9 cases and a review of the literature. *Can J Surg* 1998; 41(6):455–458.
57. NCI/NCBI Cancer Chromosomes database July 2006 <http://www.ncbi.nlm.nih.gov.library1.unmc.edu:2048/entrez/query.fcgi?db=CancerChromosomes>.
58. Hibbard MK, Kozakewich HP, Dal Cin P, Sciot R, Tan X, Xiao S, Fletcher JA. PLAG1 fusion oncogenes in lipoblastoma. *Cancer Res* 2000; 60(17):4869–4872.
59. Gaffney EF, Hargreaves HK, Semple E, Vellios F. Hibernoma: distinctive light and electron microscopic features and relationship to brown adipose tissue. *Hum Pathol* 1983; 14(8):677–687.
60. Furlong MA, Fanburg-Smith JC, Miettinen M. The morphologic spectrum of hibernoma: a clinicopathologic study of 170 cases. *Am J Surg Pathol* 2001; 25(6):809–814.
61. Mrozek K, Karakousis CP, Bloomfield CD. Band 11q13 is nonrandomly rearranged in hibernomas. *Genes Chromosomes Cancer* 1994; 9(2):145–147.
62. Mertens F, Rydholm A, Brosjo O, Willen H, Mitelman F, Mandahl N. Hibernomas are characterized by rearrangements of chromosome bands 11q13–21. *Int J Cancer* 1994; 58(4):503–505.
63. Gisselsson D, Hoglund M, Mertens F, Dal Cin P, Mandahl N. Hibernomas are characterized by homozygous deletions in the multiple endocrine neoplasia type I region. Metaphase fluorescence in situ hybridization reveals complex rearrangements not detected by conventional cytogenetics. *Am J Pathol* 1999; 155(1):61–66.

64. Maire G, Forus A, Foa C, Bjerkehagen B, Mainguené C, Kresse SH, Myklebost O, Pedeutour F. 11q13 alterations in two cases of hibernoma: large heterozygous deletions and rearrangement breakpoints near GARP in 11q13.5. *Genes Chromosomes Cancer* 2003; 37(4):389–395.
65. Meis JM, Enzinger FM. Chondroid lipoma. A unique tumor simulating liposarcoma and myxoid chondrosarcoma. *Am J Surg Pathol* 1993; 17(11):1103–1112.
66. Ballaux F, Debiec-Rychter M, De Wever I, Sciot R. Chondroid lipoma is characterized by t(11;16)(q13;p12–13). *Virchows Arch* 2004; 444(2):208–210.
67. Gisselsson D, Domanski HA, Höglund M, Carlén B, Mertens F, Willén H, Mandahl N. Unique cytological features and chromosome aberrations in chondroid lipoma: a case report based on fine-needle aspiration cytology, histopathology, electron microscopy, chromosome banding, and molecular cytogenetics. *Am J Surg Pathol* 1999; 23(10):1300–1304.
68. Thomson TA, Horsman D, Bainbridge TC. Cytogenetic and cytologic features of chondroid lipoma of soft tissue. *Mod Pathol* 1999; 12(1):88–91.
69. Dei Tos AP. Liposarcoma: new entities and evolving concepts. *Ann Diagn Pathol* 2000; 4(4):252–266.
70. Dei Tos, A. P., Pedeutour F. Atypical lipomatous tumor/well differentiated liposarcoma. In: Fletcher CDM, Unni KK, Mertens F, eds. *World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Soft Tissue and Bone*. Lyon: IARC Press; 2002:35–37.
71. Weiss SW. *Histologic typing of soft tissue tumours. World Health Organization Histological Classification of Tumours*. Berlin: Springer; 1994.
72. Dei Tos AP, Mentzel T, Newman PL, Fletcher CD. Spindle cell liposarcoma, a hitherto unrecognized variant of liposarcoma. Analysis of six cases. *Am J Surg Pathol* 1994; 18(9):913–921.
73. Kraus MD, Guillou L, Fletcher CD. Well-differentiated inflammatory liposarcoma: an uncommon and easily overlooked variant of a common sarcoma. *Am J Surg Pathol* 1997; 21(5):518–527.
74. Fletcher CD, Akerman M, Dal Cin P, de Wever I, Mandahl N, Mertens F, Mitelman F, Rosai J, Rydholm A, Sciot R, Tallini G, van den Berghe H, van de Ven W, Vanni R, Willen H. Correlation between clinicopathological features and karyotype in lipomatous tumors. A report of 178 cases from the chromosomes and morphology (CHAMP) collaborative study group. *Am J Pathol* 1996; 148(2):623–630.
75. Rosai J, Akerman M, Dal Cin P, DeWever I, Fletcher CD, Mandahl N, Mertens F, Mitelman F, Rydholm A, Sciot R, Tallini G, Van den Berghe H, Van de Ven W, Vanni R, Willen H. Combined morphologic and karyotypic study of 59 atypical lipomatous tumors. Evaluation of their relationship and differential diagnosis with other adipose tissue tumors (a report of the CHAMP study group). *Am J Surg Pathol* 1996; 20(10):1182–1189.
76. Dei Tos AP, Doglioni C, Piccinin S, et al. Coordinated expression and amplification of the MDM2, CDK4, and HMGI-C genes in atypical lipomatous tumours. *J Pathol* 2000; 190(5):531–536.
77. Elkahloun AG, Bittner M, Hoskins K, Gemmill R, Meltzer PS. Molecular cytogenetic characterization and physical mapping of 12q13–15 amplification in human cancers. *Genes Chromosomes Cancer* 1996; 17(4):205–214.
78. Mandahl N, Mertens F, Willen H, Rydholm A, Kreicbergs A, Mitelman F. Nonrandom pattern of telomeric associations in atypical lipomatous tumors with ring and giant marker chromosomes. *Cancer Genet Cytogenet* 1998; 103(1):25–34.
79. Sandberg AA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: liposarcoma. *Cancer Genet Cytogenet* 2004; 155(1):1–24.
80. Orvieto E, Furlanetto A, Laurino L, Del Tos AP. Myxoid and round cell liposarcoma: a spectrum of myxoid adipocytic neoplasia. *Semin Diagn Pathol* 2001; 18(4):267–273.
81. Antonescu CR, Ladanyi M. Myxoid liposarcoma. In: Fletcher CDM, Unni KK, Mertens F, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone*. Lyon: IARC Press; 2002:40–43.
82. Turc-Carel C, Limon J, Dal Cin P, Rao U, Karakousis C, Sandberg AA. Cytogenetic studies of adipose tissue tumors. II. Recurrent reciprocal translocation t(12;16)(q13;p11) in myxoid liposarcomas. *Cancer Genet Cytogenet* 1986; 23(4):291–299.
83. Rubin BP, Dal Cin P. The genetics of lipomatous tumors. *Semin Diagn Pathol* 2001; 18(4):286–293.

84. Rabitts TH, Forster A, Larson R, Nathan P. Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t(12;16) in malignant liposarcoma. *Nat Genet* 1993; 4(2):175–180.
85. Mandahl N. Soft tissue tumors: liposarcoma/malignant lipomatous tumors. *Atlas Genet Cytogenet Oncol Haematol*. May 2000 <http://AtlasGeneticsOncology.org/Tumors/liposarc5029.html>
86. Dal Cin P, Sciot R, Panagopoulos I, Aman P, Samson I, Mandahl N, Mitelman F, Van den Berghe H, Fletcher CD. Additional evidence of a variant translocation t(12;22) with EWS/CHOP fusion in myxoid liposarcoma: clinicopathological features. *J Pathol* 1997; 182(4):437–441.
87. Kuroda M, Ishida T, Horiuchi H, Kida N, Uozaki H, Takeuchi H, Tsuji K, Imamura T, Mori S, Machinami R, Watanabe T. Chimeric TLS/FUS-CHOP gene expression and the heterogeneity of its junction in human myxoid and round cell liposarcoma. *Am J Pathol* 1995; 147(5):1221–1227.
88. Antonescu CR, Elahi A, Humphrey M, Lui MY, Healey JH, Brennan MF, Woodruff JM, Jhanwar SC, Ladanyi M. Specificity of TLS-CHOP rearrangement for classic myxoid/round cell liposarcoma: absence in predominantly myxoid well-differentiated liposarcomas. *J Mol Diagn* 2000; 2(3):132–138.
89. Meis-Kindblom JM, Sjogren H, Kindblom LG, Peydró-Mellquist A, Röijer E, Aman P, Stenman G. Cytogenetic and molecular genetic analyses of liposarcoma and its soft tissue simulators: recognition of new variants and differential diagnosis. *Virchows Arch* 2001; 439(2):141–151.
90. Gurney JG, Davis S, Severson RK, Fang JY, Ross JA, Robison LL. Trends in cancer incidence among children in the U.S. *Cancer* 1996 Aug 1; 78(3):532–541.
91. Parham DM, Barr FG. Embryonal rhabdomyosarcoma. In: Fletcher CDM, Unni KK, Mertens F, editors. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone*. Lyon: IARC Press; 2002:146–149.
92. Newton WA, Jr, Soule EH, Hamoudi AB, Reiman HM, Shimada H, Beltangady M, Maurer H. Histopathology of childhood sarcomas, intergroup rhabdomyosarcoma studies I and II: clinicopathologic correlation. *J Clin Oncol* 1988; 6(1):67–75.
93. Gordon T, McManus A, Anderson J, Min T, Swansbury J, Pritchard-Jones K, Shipley J; United Kingdom Children's Cancer Study Group; United Kingdom Cancer Cytogenetics Group. Cytogenetic abnormalities in 42 rhabdomyosarcoma: a United Kingdom cancer cytogenetics group study. *Med Pediatr Oncol* 2001; 36(2):259–267.
94. Whang-Peng J, Knutson T, Theil K, Horowitz ME, Triche T. Cytogenetic studies in subgroups of rhabdomyosarcoma. *Genes Chromosomes Cancer* 1992; 5:299–310.
95. Weber-Hall S, Anderson J, McManus A, Abe S, Nojima T, Pinkerton R, Pritchard-Jones K, Shipley J. Gains, losses, and amplification of genomic material in rhabdomyosarcoma analyzed by comparative genomic hybridization. *Cancer Res* 1996; 56(14):3220–3224.
96. Bridge JA, Liu J, Weibold V, Baker KS, Perry D, Kruger R, Qualman S, Barr F, Sorensen P, Triche T, Suijkerbuijk R. Novel genomic imbalances in embryonal rhabdomyosarcoma revealed by comparative genomic hybridization and fluorescence in situ hybridization: an intergroup rhabdomyosarcoma study. *Genes Chromosomes Cancer* 2000; 27(4):337–344.
97. Bridge JA, Liu J, Qualman SJ, Suijkerbuijk R, Wenger G, Zhang J, Wan X, Baker KS, Sorensen P, Barr FG. Genomic gains and losses are similar in genetic and histologic subsets of rhabdomyosarcoma, whereas amplification predominates in embryonal with anaplasia and alveolar subtypes. *Genes Chromosomes Cancer* 2002; 33(3):310–321.
98. Harms D. Alveolar rhabdomyosarcoma: A prognostically unfavorable rhabdomyosarcoma type and its necessary distinction from embryonal rhabdomyosarcoma. *Curr Top Pathol* 1995; 89:273–296.
99. Raney RB, Anderson JR, Barr FG, Donaldson SS, Pappo AS, Qualman SJ, Wiener ES, Maurer HM, Crist WM. Rhabdomyosarcoma and undifferentiated sarcoma in the first two decades of life: a selective review of intergroup rhabdomyosarcoma study group experience and rationale for intergroup rhabdomyosarcoma study V. *J Pediatr Hematol Oncol* 2001; 23(4):215–220.
100. Kullendorff CM, Donner M, Mertens F, Mandahl N. Chromosomal aberrations in a consecutive series of childhood rhabdomyosarcoma. *Med Pediatr Oncol* 1998; 30(3):156–159.
101. Biegel JA, Meek RS, Parmiter AH, Conard K, Emanuel BS. Chromosomal translocation t(1;13)(p36;q14) in a case of rhabdomyosarcoma. *Genes Chromosomes Cancer* 1991; 3(6):483–484.
102. Barr FG. Molecular genetics and pathogenesis of rhabdomyosarcoma. *J Pediatr Hematol Oncol* 1997; 19(6):483–491.

103. Sorensen PH, Lynch JC, Qualman SJ, Tirabosco R, Lim JF, Maurer HM, Bridge JA, Crist WM, Triche TJ, Barr FG. PAX3-FKHR and PAX7-FKHR gene fusions are prognostic indicators in alveolar rhabdomyosarcoma: a report from the children's oncology group. *J Clin Oncol* 2002; 20(11):2672–2679.
104. Barr FG, Qualman SJ, Macris MH, Melnyk N, Lawlor ER, Strzelecki DM, Triche TJ, Bridge JA, Sorensen PH. Genetic heterogeneity in the alveolar rhabdomyosarcoma subset without typical gene fusions. *Cancer Res* 2002; 62(16):4704–4710.
105. Wachtel M, Dettling M, Koscielniak E, Stegmaier S, Treuner J, Simon-Klingenstein K, Buehlmann P, Niggli F, Schaefer B. Gene expression signatures identify rhabdomyosarcoma subtypes and detect a novel t(2;2)(q35;p23) translocation fusing PAX3 to NCOA1. *Cancer Res* 2004; 64(16):5539–5545.
106. Frascella E, Lenzini E, Schafer BW, Brecevic L, Dorigo E, Toffolatti L, Nanni P, De Giovanni C, Rosolen A. Concomitant amplification and expression of PAX7-FKHR and MYCN in a human rhabdomyosarcoma cell line carrying a cryptic t(1;13)(p36;q14). *Cancer Genet Cytogenet* 2000; 121(2):139–145.
107. Barr FG, Nauta LE, Davis RJ, Schafer BW, Nycum LM, Biegel JA. In vivo amplification of the PAX3-FKHR and PAX7-FKHR fusion genes in alveolar rhabdomyosarcoma. *Hum Mol Genet* 1996; 5(1):15–21.
108. Williamson D, Lu YJ, Gordon T, Sciot R, Kelsey A, Fisher C, Poremba C, Anderson J, Pritchard-Jones K, Shipley J. Relationship between MYCN copy number and expression in rhabdomyosarcomas and correlation with adverse prognosis in the alveolar subtype. *J Clin Oncol* 2005; 23(4):880–888.
109. Fisher C, de Bruijn DRH, Geurts van Kessel A. Synovial sarcoma. In: Fletcher CDM, Unni KK, Mertens F, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone*. Lyon: IARC Press; 2002:200–204.
110. Fisher C. Synovial sarcoma. *Ann Diagn Pathol* 1998; 2(6):401–421.
111. Kransdorf MJ. Malignant soft-tissue tumors in a large referral population: distribution of diagnoses by age, sex, and location. *Am J Roentgenol* 1995; 164(1):129–134.
112. Krall RA, Kostianovsky M, Patchefsky AS. Synovial sarcoma: a clinical, pathological, and ultrastructural study of 26 cases supporting the recognition of a monophasic variant. *Am J Surg Pathol* 1981; 5(2):137–151.
113. Pérot C. Soft tissue tumors: synovial sarcoma. *Atlas Genet Cytogenet Oncol Haematol*. February 2000. <http://AtlasGeneticsOncology.org/Tumors/SynovSarcID5044.html>.
114. Limon J, Dal Cin P, Sandberg AA. Translocations involving the X chromosome in solid tumors: presentation of two sarcomas with t(X;18)(q13;p11). *Cancer Genet Cytogenet* 1986; 23:87–91.
115. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors. Synovial sarcoma. *Cancer Genet Cytogenet* 2002; 133(1):1–23.
116. Skytting B, Nilsson G, Brodin B, Xie Y, Lundeberg J, Uhlén M, Larsson O. A novel fusion gene, SYT-SSX4, in synovial sarcoma. *J Natl Cancer Inst* 1999; 91(11):974–975.
117. Crew AJ, Clark J, Fisher C, Gill S, Grimer R, Chand A, Shipley J, Gusterson BA, Cooper CS. Fusion of SYT to two genes, SSX1 and SSX2, encoding proteins with homology to the Kruppel-associated box in human synovial sarcoma. *EMBO J* 1995; 14(10):2333–2340.
118. Kawai A, Woodruff J, Healey JH, Brennan MF, Antonescu CR, Ladanyi M. SYT-SSX gene fusion as a determinant of morphology and prognosis in synovial sarcoma. *N Engl J Med* 1998; 338(3):153–160.
119. Ordonez N, Ladanyi M. Alveolar soft part sarcoma. In: Fletcher CDM, Unni KK, Mertens F, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone*. Lyon: IARC Press; 2002:208–210.
120. Portera CA Jr, Ho V, Patel SR, Hunt KK, Feig BW, Respondek PM, Yasko AW, Benjamin RS, Pollock RE, Pisters PW. Alveolar soft part sarcoma: Clinical course and patterns of metastasis in 70 patients treated at a single institution. *Cancer* 2001; 91(3):585–591.
121. van Echten J, van den Berg E, van Baarlen J, van Noort G, Vermey A, Dam A, Molenaar WM. An important role for chromosome 17, band q25, in the histogenesis of alveolar soft part sarcoma. *Cancer Genet Cytogenet* 1995; 82(1):57–61.
122. Joyama S, Ueda T, Shimizu K, Kudawara I, Mano M, Funai H, Takemura K, Yoshikawa H. Chromosome rearrangement at 17q25 and Xp11.2 in alveolar soft-part sarcoma: A case report and review of the literature. *Cancer* 1999; 86(7):1246–1250.

123. Ladanyi M, Lui MY, Antonescu CR, Krause-Boehm A, Meindl A, Argani P, Healey JH, Ueda T, Yoshikawa H, Meloni-Ehrig A, Sorensen PH, Mertens F, Mandahl N, van den Berghe H, Sciot R, Dal Cin P, Bridge J. The der(17)t(X;17)(p11;q25) of human alveolar soft part sarcoma fuses the TFE3 transcription factor gene to ASPL, a novel gene at 17q25. *Oncogene* 2001; 20(1):48–57.
124. Heimann P, Devalck C, Debusscher C, Sariban E, Vamos E. Alveolar soft-part sarcoma: Further evidence by FISH for the involvement of chromosome band 17q25. *Genes Chromosomes Cancer* 1998; 23(2):194–197.
125. Uppal S, Aviv H, Patterson F, Cohen S, Benevenia J, Aisner S, Hameed M. Alveolar soft part sarcoma—reciprocal translocation between chromosome 17q25 and Xp11. Report of a case with metastases at presentation and review of the literature. *Acta Orthop Belg* 2003; 69(2):182–187.
126. Argani P, Antonescu CR, Illei PB, Lui MY, Timmons CF, Newbury R, Reuter VE, Garvin AJ, Perez-Atayde AR, Fletcher JA, Beckwith JB, Bridge JA, Ladanyi M. Primary renal neoplasms with the ASPL-TFE3 gene fusion of alveolar soft part sarcoma: a distinctive tumor entity previously included among renal cell carcinomas of children and adolescents. *Am J Pathol* 2001; 159(1):179–192.
127. Chung EB, Enzinger FM. Malignant melanoma of soft parts. A reassessment of clear cell sarcoma. *Am J Surg Pathol* 1983; 7(5):405–413.
128. Rubin BP, Fletcher JA, Renshaw AA. Clear cell sarcoma of soft parts: report of a case primary in the kidney with cytogenetic confirmation. *Am J Surg Pathol* 1999; 23(5):589–594.
129. Haas JE, Bonadio JF, Beckwith JB. Clear cell sarcoma of the kidney with emphasis on ultrastructural studies. *Cancer* 1984; 54(12):2978–2987.
130. Bridge JA, Sreekantaiah C, Neff JR, Sandberg AA. Cytogenetic findings in clear cell sarcoma of tendons and aponeuroses. Malignant melanoma of soft parts. *Cancer Genet Cytogenet* 1991; 52:101–106.
131. Zucman J, Delattre O, Desmaze C, Epstein AL, Stenman G, Speleman F, Fletchers CDM, Aurias A, Thomas G. EWS and ATF-1 gene fusion induced by t(12;22) translocation in malignant melanoma of soft parts. *Nat Genet* 1993; 4(4):341–345.
132. Travis JA, Bridge JA. Significance of both numerical and structural chromosomal abnormalities in clear cell sarcoma. *Cancer Genet Cytogenet* 1992; 64(2):104–6.
133. Montgomery EA, Meis JM, Ramos AG, Frisman DM, Martz KL. Clear cell sarcoma of tendons and aponeurosis. a clinicopathologic study of 58 cases with analysis of prognostic factors. *Int J Surg Pathol* 1993; 1:89–100.
134. Deenik W, Mooi WJ, Rutgers EJ, Peterse JL, Hart AA, Kroon BB. Clear cell sarcoma (malignant melanoma) of soft parts: a clinicopathologic study of 30 cases. *Cancer* 1999; 86(6):969–975.
135. Raddaoui E, Donner LR, Panagopoulos I. Fusion of the FUS and ATF1 genes in a large, deep-seated angiomyomatoid fibrous histiocytoma. *Diagn Mol Pathol* 2002; 11(3):157–162.
136. Hallor KH, Mertens F, Jin Y, Meis-Kindblom JM, Kindblom LG, Behrendtz M, Kalén A, Mandahl N, Panagopoulos I. Fusion of the EWSR1 and ATF1 genes without expression of the MITF-M transcript in angiomyomatoid fibrous histiocytoma. *Genes Chromosomes Cancer* 2005; 44(1):97–102.
137. Antonescu CR, Argani P, Erlandson RA, Healey JH, Ladanyi M, Huvos AG. Skeletal and extraskeletal myxoid chondrosarcoma: a comparative clinicopathologic, ultrastructural, and molecular study. *Cancer* 1998; 83(8):1504–1521.
138. Hachitanda Y, Tsuneyoshi M, Daimaru Y, Enjoji M, Nakagawara A, Ikeda K, Sueishi K. Extraskeletal myxoid chondrosarcoma in young children. *Cancer* 1988; 61(12):2521–2526.
139. Hinrichs SH, Jaramillo MA, Gumerlock PH, Gardner MB, Lewis JP, Freeman AE. Myxoid chondrosarcoma with a translocation involving chromosomes 9 and 22. *Cancer Genet Cytogenet* 1985; 14(3–4):219–226.
140. Orndal C, Carlén B, Akerman M, Willén H, Mandahl N, Heim S, Rydholm A, Mitelman F. Chromosomal abnormality t(9;22)(q22;q12) in an extraskeletal myxoid chondrosarcoma characterized by fine needle aspiration cytology, electron microscopy, immunohistochemistry and DNA flow cytometry. *Cytopathology* 1991; 2(5):261–270.
141. Clark J, Benjamin H, Gill S, Sidhar S, Goodwin G, Crew J, Gusterson BA, Shipley J, Cooper CS. Fusion of the EWS gene to CHN, a member of the steroid/thyroid receptor gene superfamily, in a human myxoid chondrosarcoma. *Oncogene* 1996; 12(2):229–235.
142. Sjogren H, Meis-Kindblom J, Kindblom LG, Aman P, Stenman G. Fusion of the EWS-related gene TAF2N to TEC in extraskeletal myxoid chondrosarcoma. *Cancer Res* 1999; 59(20):5064–5067.

143. Sjogren H, Wedell B, Meis-Kindblom JM, Kindblom LG, Stenman G. Fusion of the NH₂-terminal domain of the basic helix-loop-helix protein TCF12 to TEC in extraskeletal myxoid chondrosarcoma with translocation t(9;15) (q22;q21). *Cancer Res* 2000; 60(24):6832–6835.
144. Hisaoka M, Ishida T, Imamura T, Hashimoto H. TFG is a novel fusion partner of NOR1 in extraskeletal myxoid chondrosarcoma. *Genes Chromosomes Cancer* 2004; 40(4):325–328.
145. Meis-Kindblom JM, Bergh P, Gunterberg B, Kindblom LG. Extraskeletal myxoid chondrosarcoma: a reappraisal of its morphologic spectrum and prognostic factors based on 117 cases. *Am J Surg Pathol* 1999; 23(6):636–650.
146. Antonescu CR, Gerald W. Desmoplastic small round cell tumour. In: Fletcher CDM, Unni KK, Mertens F, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone*. Lyon: IARC Press; 2002:216–218.
147. Gerald WL, Miller HK, Battifora H, Miettinen M, Silva EG, Rosai J. Intra-abdominal desmoplastic small round-cell tumor. Report of 19 cases of a distinctive type of high-grade polyphenotypic malignancy affecting young individuals. *Am J Surg Pathol* 1991; 15(6):499–513.
148. Ordonez NG. Desmoplastic small round cell tumor: I: a histopathologic study of 39 cases with emphasis on unusual histological patterns. *Am J Surg Pathol* 1998; 22(11):1303–1313.
149. Sawyer JR, Tryka AF, Lewis JM. A novel reciprocal chromosome translocation t(11;22)(p13;q12) in an intraabdominal desmoplastic small round-cell tumor. *Am J Surg Pathol* 1992; 16(4):411–416.
150. Gerald WL, Ladanyi M, de Alava E, Cuatrecasas M, Kushner BH, LaQuaglia MP, Rosai J. Clinical, pathologic, and molecular spectrum of tumors associated with t(11;22)(p13;q12): Desmoplastic small round-cell tumor and its variants. *J Clin Oncol* 1998; 16(9):3028–3036.
151. Ladanyi M, Gerald W. Fusion of the EWS and WT1 genes in the desmoplastic small round cell tumor. *Cancer Res* 1994; 54(11):2837–2840.
152. el-Kattan I, Redline RW, el-Naggar AK, Grimes MC, Abdul-Karim FW. Cytologic features of intraabdominal desmoplastic small round cell tumor. A case report. *Acta Cytol* 1995; 39(3):514–520.
153. Shen WP, Towne B, Zadeh TM. Cytogenetic abnormalities in an intraabdominal desmoplastic small cell tumor. *Cancer Genet Cytogenet* 1992; 64(2):189–191.
154. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors. Desmoplastic small round-cell tumors. *Cancer Genet Cytogenet* 2002; 138(1):1–10.
155. Saab R, Khouri JD, Krasin M, Davidoff AM, Navid F. Desmoplastic small round cell tumor in childhood: the St. Jude children's research hospital experience. *Pediatr Blood Cancer* 2007; 49(3):274–279.
156. Kushner BH, LaQuaglia MP, Wollner N, Meyers PA, Lindsley KL, Ghavimi F, Merchant TE, Boulad F, Cheung NK, Bonilla MA, Crouch G, Kelleher JF Jr, Steinherz PG, Gerald WL. Desmoplastic small round-cell tumor: Prolonged progression-free survival with aggressive multimodality therapy. *J Clin Oncol* 1996; 14(5):1526–1531.
157. Rosenberg AE, Nielsen GP, Fletcher JA. Aneurysmal bone cyst. In: Fletcher CDM, Unni KK, Mertens F, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone*. Lyon: IARC Press; 2002:338–339.
158. Mankin HJ, Hor nicek FJ, Ortiz-Cruz E, Villafuerte J, Gebhardt MC. Aneurysmal bone cyst: a review of 150 patients. *J Clin Oncol* 2005; 23(27):6756–6762.
159. Vergel De Dios AM, Bond JR, Shives TC, McLeod RA, Unni KK. Aneurysmal bone cyst. A clinicopathologic study of 238 cases. *Cancer* 1992; 69(12):2921–2931.
160. Panoutsakopoulos G, Pandis N, Kyriazoglou I, Gustafson P, Mertens F, Mandahl N. Recurrent t(16;17)(q22;p13) in aneurysmal bone cysts. *Genes Chromosomes Cancer* 1999; 26(3):265–266.
161. Herens C, Thiry A, Dresse MF, Born J, Flagothier C, Vanstraeten G, Allington N, Bex V. Translocation (16;17) (q22;p13) is a recurrent anomaly of aneurysmal bone cysts. *Cancer Genet Cytogenet* 2001; 127(1):83–84.
162. Oliveira AM, Hsi BL, Weremowicz S, Rosenberg AE, Dal Cin P, Joseph N, Bridge JA, Perez-Atayde AR, Fletcher JA. USP6 (Tre2) fusion oncogenes in aneurysmal bone cyst. *Cancer Res* 2004; 64(6):1920–1923.
163. Oliveira AM, Perez-Atayde AR, Dal Cin P, Gebhardt MC, Chen CJ, Neff JR, Demetri GD, Rosenberg AE, Bridge JA, Fletcher JA. Aneurysmal bone cyst variant translocations upregulate USP6 transcription by promoter swapping with the ZNF9, COL1A1, TRAP150, and OMD genes. *Oncogene* 2005; 24(21):3419–3426.

164. Oliveira AM, Perez-Atayde AR, Inwards CY, Medeiros F, Derr V, Hsi BL, Gebhardt MC, Rosenberg AE, Fletcher JA. USP6 and CDH11 oncogenes identify the neoplastic cell in primary aneurysmal bone cysts and are absent in so-called secondary aneurysmal bone cysts. *Am J Pathol* 2004; 165(5):1773–1780.
165. Althof PA, Ohmori K, Zhou M, Bailey JM, Bridge RS, Nelson M, Neff JR, Bridge JA. Cytogenetic and molecular cytogenetic findings in 43 aneurysmal bone cysts: aberrations of 17p mapped to 17p13.2 by fluorescence in situ hybridization. *Mod Pathol* 2004; 17(5):518–525.
166. Bridge JA, Neff JR, Mouron BJ. Giant cell tumor of bone. Chromosomal analysis of 48 specimens and review of the literature. *Cancer Genet Cytogenet* 1992; 58:2–13.
167. Kyriakos M, Hardy D. Malignant transformation of aneurysmal bone cyst, with an analysis of the literature. *Cancer* 1991; 68(8):1770–1780.
168. Bell W, Siegal GP. Bone tumours. In: Cullinane C, Burchill S, Squire J, O’Leary J, Lewis I, eds. *Molecular Biology and Pathology of Paediatric Cancer*. London: Oxford University Press, 2003: Chapter 13.
169. Campanacci M. *Bone and Soft Tissue Tumors*, 2nd ed. New York: Springer-Verlag; 1999.
170. Biegel JA, Womer RB, Emanuel BS. Complex karyotypes in a series of pediatric osteosarcomas. *Cancer Genet Cytogenet* 1989; 38(1):89–100.
171. Bridge JA, Nelson M, McComb E, McGuire MH, Rosenthal H, Vergara G, Maale GE, Spanier S, Neff JR. Cytogenetic findings in 73 osteosarcoma specimens and a review of the literature. *Cancer Genet Cytogenet* 1997; 95(1):74–87.
172. Boehm AK, Bayani J, Nelson M, Bridge JA. Cytogenetic finding in 36 osteosarcoma specimens and a review of the literature. *Ped Pathol Mol Med* 2000; 19:359–376.
173. Ozaki T, Schaefer KL, Wai D, Buerger H, Flege S, Lindner N, Kevric M, Diallo R, Bankfalvi A, Brinkschmidt C, Juergens H, Winkelmann W, Dockhorn-Dworniczak B, Bielack SS, Poremba C. Genetic imbalances revealed by comparative genomic hybridization in osteosarcomas. *Int J Cancer* 2002; 102(4):355–365.
174. Man TK, Lu XY, Jaeweon K, Perlaky L, Harris CP, Shah S, Ladanyi M, Gorlick R, Lau CC, Rao PH. Genome-wide array comparative genomic hybridization analysis reveals distinct amplifications in osteosarcoma. *BMC Cancer* 2004; 4:45.
175. Carrle D, Bielack SS. Current strategies of chemotherapy in osteosarcoma. *Int Orthop* 2006; 30(6):445–451.
176. Sinovic JF, Bridge JA, Neff JR. Ring chromosome in parosteal osteosarcoma. Clinical and diagnostic significance. *Cancer Genet Cytogenet* 1992; 62(1):50–52.
177. Szymanska J, Mandahl N, Mertens F, Tarkkanen M, Karaharju E, Knuutila S. Ring chromosomes in parosteal osteosarcoma contain sequences from 12q13–15: A combined cytogenetic and comparative genomic hybridization study. *Genes Chromosomes Cancer* 1996; 16(1):31–34.
178. Ushigome S, Machinami R, Sorensen PH. Ewing sarcoma/Primitive neuroectodermal tumour (PNET). In: Fletcher CDM, Unni KK, Mertens F, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone*. Lyon: IARC Press; 2002:298–300.
179. Delattre O, Zucman J, Melot T, Garau X, Zucker J, Lenoir G, Ambros P, Sheer D, Turc-Carel C, Triche T, Aurias T, Aurias A, Thomas G. The Ewing family of tumors--a subgroup of small-round-cell tumors defined by specific chimeric transcripts. *N Engl J Med* 1994; 331(5):294–299.
180. Sorensen PH, Lessnick SL, Lopez-Terrada D, Liu XF, Triche TJ, Denny CT. A second Ewing’s sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, *ERG*. *Nat Genet* 1994; 6(2):146–151.
181. Peter M, Couturier J, Pacquement H, Michon J, Thomas G, Magdelenat H, Delattre O. A new member of the ETS family fused to EWS in Ewing tumors. *Oncogene* 1997; 14(10):1159–1164.
182. Jeon IS, Davis JN, Braun BS, Sublett JE, Roussel MF, Denny CT, Shapiro DN. A variant Ewing’s sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1. *Oncogene* 1995; 10(6):1229–1234.
183. Kaneko Y, Yoshida K, Handa M, Toyoda Y, Nishihira H, Tanaka Y, Sasaki Y, Ishida S, Higashino F, Fujinaga K. Fusion of an ETS-family gene, EIAF, to EWS by t(17;22)(q12;q12) chromosome translocation in an undifferentiated sarcoma of infancy. *Genes Chromosomes Cancer* 1996; 15(2):115–121.
184. Shing DC, McMullan DJ, Roberts P, Smith K, Chin SF, Nicholson J, Tillman RM, Ramani P, Cullinane C, Coleman N. FUS/ERG gene fusions in Ewing’s tumors. *Cancer Res* 2003; 63(15):4568–4576.

185. Kutluk MT, Yalcin B, Akyuz C, Varan A, Ruacan S, Buyukpamukcu M. Treatment results and prognostic factors in Ewing sarcoma. *Pediatr Hematol Oncol* 2004; 21(7):597–610.
186. Mertens F, Lothe RA. Nervous system: peripheral nerve sheath tumors. *Atlas Genet Cytogenet Oncol Haematol*. May 2001. <http://AtlasGeneticsOncology.org/Tumors/PeriphNervSheatID5094.html>,
187. Cotran RS, Kumar V, Robbins SL. *Pathologic Basis of Disease*, 5th ed. Philadelphia: WB Sanders; 1994:1352.
188. Letson GD, Muro-Cacho CA. Genetic and molecular abnormalities in tumors of the bone and soft tissues. *Cancer Control* 2001; 8(3):239–251.
189. Mertens F, Dal Cin P, De Wever I, Fletcher CD, Mandahl N, Mitelman F, Rosai J, Rydholm A, Sciot R, Tallini G, van Den Berghe H, Vanni R, Willén H. Cytogenetic characterization of peripheral nerve sheath tumours: a report of the CHAMP study group. *J Pathol* 2000; 190(1):31–38.
190. Bruder CE, Ichimura K, Tingby O, Hirakawa K, Komatsuzaki A, Tamura A, Yuasa Y, Collins VP, Dumanski JP. A group of schwannomas with interstitial deletions on 22q located outside the NF2 locus shows no detectable mutations in the NF2 gene. *Hum Genet* 1999; 104(5):418–424.
191. Evans DG, Baser ME, McGaughran J, Sharif S, Howard E, Moran A. Malignant peripheral nerve sheath tumours in neurofibromatosis 1. *J Med Genet* 2002; 39(5):311–314.
192. Friedrich RE, Kluwe L, Funsterer C, Mautner VF. Malignant peripheral nerve sheath tumors (MPNST) in neurofibromatosis type 1 (NF1): diagnostic findings on magnetic resonance images and mutation analysis of the NF1 gene. *Anticancer Res* 2005; 25(3A):1699–1702.
193. Brodeur GM, Seeger RC. Gene amplification in human neuroblastomas: Basic mechanisms and clinical implications. *Cancer Genet Cytogenet* 1986; 19(1–2):101–111.
194. Gilbert F, Balaban G, Moorhead P, Bianchi D, Schlesinger H. Abnormalities of chromosome 1p in human neuroblastoma tumors and cell lines. *Cancer Genet Cytogenet* 1982; 7(1):33–42.
195. Brodeur GM, Sekhon G, Goldstein MN. Chromosomal aberrations in human neuroblastomas. *Cancer* 1977; 40(5):2256–2263.
196. Maris JM, Guo C, Blake D, White PS, Hogarty MD, Thompson PM, Rajalingam V, Gerbing R, Stram DO, Matthay KK, Seeger RC, Brodeur GM. Comprehensive analysis of chromosome 1p deletions in neuroblastoma. *Med Pediatr Oncol* 2001; 36(1):32–36.
197. White PS, Thompson PM, Gotoh E, Okawa ER, Igarashi J, Kok M, Winter Gregory SG, Hogarty MD, Maris JM, Brodeur GM. Definition and characterization of a region of 1p36.3 consistently deleted in neuroblastoma. *Oncogene* 2005; 24(16):2684–2694.
198. Stallings RL, Carty P, McArdle L, Mullarkey M, McDermott M, Breathnach F, O'Meara A. Molecular cytogenetic analysis of recurrent unbalanced t(11;17) in neuroblastoma. *Cancer Genet Cytogenet* 2004; 154(1):44–51.
199. Van Roy N, Laureys G, Van Gele M, Opdenakker G, Miura R, van der Drift P, Chan A, Versteeg R, Speleman F. Analysis of 1;17 translocation breakpoints in neuroblastoma: implications for mapping of neuroblastoma genes. *Eur J Cancer* 1997; 33(12):1974–1978.
200. Carén H, Abel F, Kognér R, Martinsson T. High incidence of NA mutations and gene amplifications of the ALK gene in advanced sporadic neuroblastoma tumours. *Biochem J* 2008; 416(2):153–159.
201. Hayashi Y, Inaba T, Hanada R, Yamamoto K. Chromosome findings and prognosis in 15 patients with neuroblastoma found by VMA mass screening. *J Pediatr* 1988; 112(4):567–571.
202. Attiyeh EF, London WB, Mosse YP, Wang Q, Winter C, Khazi D, McGrady PW, Seeger RC, Look AT, Shimada H, Brodeur GM, Cohn SL, Matthay KK, Maris JM; Children's Oncology Group. Chromosome 1p and 11q deletions and outcome in neuroblastoma. *N Engl J Med* 2005; 353(21):2243–2253.
203. Farwell JR, Dohrmann GJ. Brain and spinal cord tumors of infancy. *Trans Am Neurol Assoc* 1977; 102:130–133.
204. Doolittle ND. State of the science in brain tumor classification. *Semin Oncol Nurs* 2004; 20(4):224–230.
205. Vagner-Capodano AM, Zattara-Cannoni H, Gambarelli D, Gentet JC, Genitori L, Lena G, Graziani N, Raybaud C, Choux M, Grisoli F. Detection of i(17q) chromosome by fluorescent in situ hybridization (FISH) with interphase nuclei in medulloblastoma. *Cancer Genet Cytogenet* 1994; 78(1):1–6.
206. Biegel JA, Rorke LB, Packer RJ, Sutton LN, Schut L, Bonner K, Emanuel BS. Isochromosome 17q in primitive neuroectodermal tumors of the central nervous system. *Genes Chromosomes Cancer* 1989; 1(2):139–147.

207. Giordana MT, Migheli A, Pavanelli E. Isochromosome 17q is a constant finding in medulloblastoma. An interphase cytogenetic study on tissue sections. *Neuropathol Appl Neurobiol* 1998; 24(3):233–238.
208. Pan E, Pellarin M, Holmes E, Smirnov I, Misra A, Eberhart CG, Burger PC, Biegel JA, Feuerstein BG. Isochromosome 17q is a negative prognostic factor in poor-risk childhood medulloblastoma patients. *Clin Cancer Res* 2005; 11(13):4733–4740.
209. Scheurlen WG, Schwabe GC, Seranski P, Joos S, Harbott J, Metzke S, Döhner H, Poustka A, Wilgenbus K, Haas OA. Mapping of the breakpoints on the short arm of chromosome 17 in neoplasms with an i(17q). *Genes Chromosomes Cancer* 1999; 25(3):230–240.
210. Bigner SH, Mark J, Friedman HS, Biegel JA, Bigner DD. Structural chromosomal abnormalities in human medulloblastoma. *Cancer Genet Cytogenet* 1988; 30(1):91–101.
211. Perry A, Dehner LP. Meningeal tumors of childhood and infancy. An update and literature review. *Brain Pathol* 2003; 13(3):386–408.
212. Capodano AM. Nervous system tumors: meningioma. *Atlas Genet Cytogenet Oncol Haematol*. July 2000: <http://AtlasGeneticsOncology.org/Tumors/MeningiomaID5014.html>
213. Lekanne Deprez RH, Riegman PH, van Drunen E, Warringa UL, Groen NA, Stefanko SZ, Koper JW, Avezaat CJJ, Zwarthoff EC, Hagemeizer A. Cytogenetic, molecular genetic and pathological analyses in 126 meningiomas. *J Neuropathol Exp Neurol* 1995; 54(2):224–235.
214. Ng HK, Lau KM, Tse JY, Lo KW, Wong JH, Poon WS, Huang DP. Combined molecular genetic studies of chromosome 22q and the neurofibromatosis type 2 gene in central nervous system tumors. *Neurosurgery* 1995; 37(4):764–773.
215. Sulman EP, Dumanski JP, White PS, Zhao H, Maris JM, Mathiesen T, Bruder C, Cnaan A, Brodeur GM. Identification of a consistent region of allelic loss on 1p32 in meningiomas: correlation with increased morbidity. *Cancer Res* 1998; 58(15):3226–3230.
216. Maillo A, Orfao A, Sayagues JM, Diaz P, Gómez-Moreta JA, Caballero M, Santamarta D, Santos-Briz A, Morales F, Tabernero MD. New classification scheme for the prognostic stratification of meningioma on the basis of chromosome 14 abnormalities, patient age, and tumor histopathology. *J Clin Oncol* 2003; 21(17):3285–3295.
217. Lee JY, Finkelstein S, Hamilton RL, Rekha R, King JT, Jr, Omalu B. Loss of heterozygosity analysis of benign, atypical, and anaplastic meningiomas. *Neurosurgery* 2004; 55(5):1163–1173.
218. Lamszus K, Kluwe L, Matschke J, Meissner H, Laas R, Westphal M. Allelic losses at 1p, 9q, 10q, 14q, and 22q in the progression of aggressive meningiomas and undifferentiated meningeal sarcomas. *Cancer Genet Cytogenet* 1999; 110(2):103–110.
219. Heim S, Mitelman F. *Cancer Cytogenetics*, 2nd ed. New York: John Wiley & Sons; 1995:442.
220. Kimmel DW, O'Fallon JR, Scheithauer BW, Kelly PJ, Dewald GW, Jenkins RB. Prognostic value of cytogenetic analysis in human cerebral astrocytomas. *Ann Neurol* 1992; 31:534–542.
221. Orr LC, Fleitz J, McGavran L, Wyatt-Ashmead J, Handler M, Foreman NK. Cytogenetics in pediatric low-grade astrocytomas. *Med Pediatr Oncol* 2002; 38:173–177.
222. Thiel G, Losanowa T, Kintzel D, Nisch G, Martin H, Vorpahl K, Witkowski R. Karyotypes in 90 human gliomas. *Cancer Genet Cytogenet* 1992; 58:109–120.
223. Jenkins RB, Kimmel DW, Moertel CA, Schultz CG, Scheithauer BW, Kelly PJ, Dewald GW. A cytogenetic study of 53 human gliomas. *Cancer Genet Cytogenet* 1989; 39:253–279.
224. Ransom DT, Ritland SR, Moertel CA, Dahl RJ, O'Fallon JR, Bernd W, Scheithauer BW, Kimmel DW, Kelly PJ, Olopade OI, Diaz MO, Jenkins RB. Correlation of cytogenetic analysis and loss of heterozygosity studies in human diffuse astrocytomas and mixed oligoastrocytomas. *Genes Chromosomes Cancer* 1992; 5:357–374.
225. Schröck E, Thiel G, Lozanova T, du Manoir S, Meffert MC, Jauch A, Speicher MR, Nürnberg P, Vogel S, Janisch W, Donis-Keller H, Ried T, Witkowski T, Cremer T. Comparative genomic hybridization of human malignant gliomas reveals multiple amplification sites and nonrandom chromosomal gains and losses. *Am J Pathol* 1994; 144:1203–1218.
226. Nakamura M, Yang F, Fujisawa H, Yonekawa Y, Kleihues P, Ohgaki H. Loss of heterozygosity on chromosome 19 in secondary glioblastomas. *J Neuropathol Exp Neurol* 2000; 59:539–543.

227. Wessels PH, Weber WE, Raven G, Ramaikers FC, Hopman AH, Twijnstra A. Supratentorial grade II astrocytoma; biological features and clinical course. *Lancet Neurol* 2003; 2:395–403.
228. Johannesen TB, Langmark F, Lote K. Progress in long-term survival in adult patients with supratentorial low-grade gliomas: a population-based study of 993 patients in whom tumors were diagnosed between 1970–1993. *J Neurosurg* 2003; 99:854–862.
229. Ulutin C, Fayda M, Aksu G, Cetinayak O, Kuzhan O, Ors F, Beyzadeoglu M. Primary glioblastomas multiforme in younger patients: a single-institution experience. *Tumori* 2006; 92:407–411.
230. Krex D, Klink B, Hartmann C, Deimling AV, Pietsch T, Simon M, Sabel M, Steinbach JP, Heese O, Reifenberger G, Weller M, Schackert G, German Glioma Network. Long term survival with glioblastoma multiforme. *Brain* 2007; 130(Pt 10):2596–2606.
231. Edwards BK, Brown ML, Wingo PA, Howe HL, Ward E, Ries LA, Schrag D, Jamison PM, Jemal A, Wu XC, Friedman C, Harlan L, Warren J, Anderson RN, Pickle LW. Annual report to the nation on the status of cancer, 1975–2002, featuring population-based trends in cancer treatment. *J Natl Cancer Inst* 2005; 97(19):1407–1427.
232. Thurlbeck WM, Churg AM, eds. *Pathology of the Lung*, 2nd ed. New York: Atlas Thieme Medical Publishers; 1995:437–455.
233. Gazdar A, Franklin WA, Brambilla E, Hainaut P, Yokota J, Harris CC. Genetic and molecular alterations. Tumours of the lung. In: Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart*. Lyon: IARC Press; 2004:21–23.
234. Vähäkangas KH, Bennett WP, Castrén K, Welsh JA, Khan MA, Blömeke B, Alavanja MC, Harris CC. p53 and K-ras mutations in lung cancers from former and never-smoking women. *Cancer Res* 2001; 61(11):4350–4356.
235. Hwang SJ, Cheng LS, Lozano G, Amos CI, Gu X, Strong LC. Lung cancer risk in germline p53 mutation carriers: association between an inherited cancer predisposition, cigarette smoking, and cancer risk. *Hum Genet* 2003; 113(3):238–243.
236. Whang-Peng J, Kao-Shan CS, Lee EC, Bunn PA, Carney DN, Gazdar AF, Minna JD. A specific chromosome defect associated with human small cell lung cancer, deletion 3p(14–23). *Science* 1982; 215:181–182.
237. Whang-Peng J, Knutsen T, Gazdar A, Steinberg SM, Oie H, Linnoila I, Mulshine J, Nau M, Minna JD. Nonrandom structural and numerical chromosome changes in non-small cell lung cancer. *Genes Chromosomes Cancer* 1991; 3:168–188.
238. Petersen I, Langreck H, Wolf G, Schwendel A, Psille R, Vogt P, Reichel MB, Ried T, Dietel M. Small-cell lung cancer is characterized by a high incidence of deletions on chromosomes 3p, 4q, 5q, 10q, 13q and 17p. *Br J Cancer* 1997; 75(1):79–86.
239. Braga E, Senchenko V, Bazov I, Loginov W, Liu J, Ermilova V, Kazubskaya T, Garkavtseva R, Mazurenko N, Kisseljov F, Lerman MI, Klein G, Kisilev L, Zabarovsky ER. Critical tumor-suppressor gene regions on chromosome 3p in major human epithelial malignancies: allelotyping and quantitative real-time PCR. *Int J Cancer* 2002; 100(5):534–541.
240. Wali A, Srinivasan R, Shabnam MS, Majumdar S, Joshi K, Behera D. Loss of fragile histidine triad gene expression in advanced lung cancer is consequent to allelic loss at 3p14 locus and promoter methylation. *Mol Cancer Res* 2006; 4(2):93–99.
241. Girard L, Zochbauer-Muller S, Virmani AK, Gazdar AF, Minna JD. Genome-wide allelotyping of lung cancer identifies new regions of allelic loss, differences between small cell lung cancer and non-small cell lung cancer, and loci clustering. *Cancer Res* 2000; 60(17):4894–4906.
242. Virmani AK, Fong KM, Kodagoda D, McIntire D, Hung J, Tonk V, Minna JD, Gazdar AF. Allelotyping demonstrates common and distinct patterns of chromosomal loss in human lung cancer types. *Genes Chromosomes Cancer* 1998; 21(4):308–319.
243. Pei J, Balsara BR, Li W, Litwin S, Gabrielson E, Feder M, Jen J, Testa JR. Genomic imbalances in human lung adenocarcinomas and squamous cell carcinomas. *Genes Chromosomes Cancer* 2001; 31(3):282–287.
244. Petersen I, Bujard M, Petersen S, Wolf G, Goeze A, Schwendel A, Langreck H, Gellert K, Reichel M, Just K, du Manoir S, Cremer T, Dietel M, Ried T. Patterns of chromosomal imbalances in adenocarcinoma and squamous cell carcinoma of the lung. *Cancer Res* 1997; 57(12):2331–2335.

245. Romeo MS, Sokolova IA, Morrison LE, Zeng C, Barón AE, Hirsch FR, Miller YE, Franklin WA, Varella-Garcia M. Chromosomal abnormalities in non-small cell lung carcinomas and in bronchial epithelia of high-risk smokers detected by multi-target interphase fluorescence in situ hybridization. *J Mol Diagn* 2003; 5(2):103–112.
246. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, Sugiyama Y, Mano H. Identification of the transforming *EML4-ALK* fusion gene in non-small-cell lung cancer. *Nature* 2007;448(7153): 561–566.
247. Lindeman, N, Cagle, P, Beasley, M, Dhananjay, A, Dacic, S, Giaccone, G, Jenkins, R, Kwaitkowski, D, Saldivar, J, Squire, J, Thunnissen, E, Ladanyi, M. Molecular testing guideline for selection of lung cancer patients for *EGFR* and *ALK* tyrosine kinase inhibitors. Guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. *Arch Pathol Lab Med*, 2013; 8(7):823–859.
248. Exelby PR, Filler RM, Grosfeld JL. Liver tumors in children in the particular reference to hepatoblastoma and hepatocellular carcinoma: American academy of pediatrics surgical section survey—1974. *J Pediatr Surg* 1975; 10(3):329–337.
249. DeBaun MR, Tucker MA. Risk of cancer during the first four years of life in children from the Beckwith-Wiedemann syndrome registry. *J Pediatr* 1998; 132(3 Pt 1):398–400.
250. Hughes LJ, Michels VV. Risk of hepatoblastoma in familial adenomatous polyposis. *Am J Med Genet* 1992; 43(6):1023–1025.
251. Ishak KG, Glunz PR. Hepatoblastoma and hepatocarcinoma in infancy and childhood. report of 47 cases. *Cancer* 1967; 20(3):396–422.
252. Swarts S, Wisecarver J, Bridge JA. Significance of extra copies of chromosome 20 and the long arm of chromosome 2 in hepatoblastoma. *Cancer Genet Cytogenet* 1996; 91(1):65–67.
253. Schneider NR, Cooley LD, Finegold MJ, Douglass EC, Tomlinson GE. The first recurring chromosome translocation in hepatoblastoma: der(4)t(1;4)(q12;q34). *Genes Chromosomes Cancer* 1997; 19(4):291–294.
254. Hu J, Wills M, Baker BA, Perlman EJ. Comparative genomic hybridization analysis of hepatoblastomas. *Genes Chromosomes Cancer* 2000; 27(2):196–201.
255. Weber RG, Pietsch T, von Schweinitz D, Lichter P. Characterization of genomic alterations in hepatoblastomas. A role for gains on chromosomes 8q and 20 as predictors of poor outcome. *Am J Pathol* 2000; 157(2):571–578.
256. Roebuck DJ, Perilongo G. Hepatoblastoma: an oncological review. *Pediatr Radiol* 2006; 36(3):183–186.
257. Thomas CR, Wright CD, Loehrer PJ. Thymoma: state of the art. *J Clin Oncol* 1999; 17(7):2280–2289.
258. Marx A, Strobel P, Zettl A, Thymomas. In: Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart*. Lyon: IARC Press; 2004:152–153.
259. Herens C, Radermecker M, Servais A, Quatresooz P, Jardon-Jeghers C, Bours V, de Leval L. Deletion (6)(p22p25) is a recurrent anomaly of thymoma: report of a second case and review of the literature. *Cancer Genet Cytogenet* 2003; 146(1):66–69.
260. Zettl A, Strobel P, Wagner K, Katzenberger T, Ott G, Rosenwald A, Peters K, Krein A, Semik M, Müller-Hermelink HK, Marx A. Recurrent genetic aberrations in thymoma and thymic carcinoma. *Am J Pathol* 2000; 157(1):257–266.
261. Marx A, French CA, Fletcher JA. Carcinoma with t(15;19) translocation. In: Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart*. Lyon: IARC Press; 2004:185–187.
262. Engleson J, Soller M, Panagopoulos I, Dahlen A, Dictor M, Jerkeman M. Midline carcinoma with t(15;19) and BRD4-NUT fusion oncogene in a 30-year-old female with response to docetaxel and radiotherapy. *BMC Cancer* 2006; 6:69.
263. Toretsky JA, Jenson J, Sun CC, Eskenazi AE, Campbell A, Hunger SP, Caires A, Frantz C, Hill JL, Stambberg J. Translocation (11;15;19): A highly specific chromosome rearrangement associated with poorly differentiated thymic carcinoma in young patients. *Am J Clin Oncol* 2003; 26(3):300–306.
264. Kubonishi E, Takehara N, Iwata J, Sonobe H, Ohtsuki Y, Abe T, Miyoshi I. Novel t(15;19)(q15;p13) chromosome abnormality in a thymic carcinoma. *Cancer Res* 1991; 51:3327–3328.
265. French CA, Miyoshi I, Kubonishi I, Grier HE, Perez-Atayde AR, Fletcher JA. BRD4-NUT fusion oncogene: a novel mechanism in aggressive carcinoma. *Cancer Res* 2003; 63(2):304–307.

266. Spiro RH. Salivary neoplasms: Overview of a 35-year experience with 2,807 patients. *Head Neck Surg* 1986; 8(3):177–184.
267. Eveson JW, Cawson RA. Salivary gland tumours. A review of 2410 cases with particular reference to histological types, site, age and sex distribution. *J Pathol* 1985; 146(1):51–58.
268. Bullerdiek J, Wobst G, Meyer-Bolte K, Chilla R, Haubrich J, Thode B, Bartnitzke S. Cytogenetic subtyping of 220 salivary gland pleomorphic adenomas: correlation to occurrence, histological subtype, and in vitro cellular behavior. *Cancer Genet Cytogenet* 1993; 65(1):27–31.
269. Mark J, Dahlenfors R, Ekedahl C, Stenman G. The mixed salivary gland tumor—a normally benign human neoplasm frequently showing specific chromosomal abnormalities. *Cancer Genet Cytogenet* 1980; 2:231–241.
270. Kas K, Voz ML, Röijer E, Aström AK, Meyen E, Stenman G, Van de Ven WJ. Promoter swapping between the genes for a novel zinc finger protein and beta-catenin in pleiomorphic adenomas with t(3;8)(p21;q12) translocations. *Nat Genet* 1997; 15(2):170–174.
271. Voz ML, Astrom AK, Kas K, Mark J, Stenman G, Van de Ven WJ. The recurrent translocation t(5;8)(p13;q12) in pleiomorphic adenomas results in upregulation of PLAG1 gene expression under control of the LIFR promoter. *Oncogene* 1998; 16(11):1409–1416.
272. Geurts JM, Schoenmakers EF, Roijer E, Stenman G, Van de Ven WJ. Expression of reciprocal hybrid transcripts of HMGIC and FHIT in a pleiomorphic adenoma of the parotid gland. *Cancer Res* 1997; 57(1):13–17.
273. Röijer E, Nordkvist A, Ström AK, Ryd W, Behrendt M, Bullerdiek J, Mark J, Stenman G. Translocation, deletion/amplification, and expression of HMGIC and MDM2 in a carcinoma ex pleiomorphic adenoma. *Am J Pathol* 2002; 160(2):433–440.
274. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; 55(2):74–108.
275. Cher ML, MacGrogan D, Bookstein R, Brown JA, Jenkins RB, Jensen RH. Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer. *Genes Chromosomes Cancer* 1994; 11(3):153–162.
276. Verderfer I, Hobisch A, Culig Z, Hittmair A, Bartsch G, Erdel M, Duba HC, Utermann G. Combined study of prostatic carcinoma by classical cytogenetic analysis and comparative genomic hybridization. *Int J Oncol* 2001; 19(6):1263–1270.
277. Matsuyama H, Pan Y, Yoshihiro S, Kudren D, Naito K, Bergerheim US, Ekman P. Clinical significance of chromosome 8p, 10q, and 16q deletions in prostate cancer. *Prostate* 2003; 54(2):103–111.
278. Oba K, Matsuyama H, Yoshihiro S, Kishi F, Takahashi M, Tsukamoto M, Kinjo M, Sagiyama K, Naito K. Two putative tumor suppressor genes on chromosome arm 8p may play different roles in prostate cancer. *Cancer Genet Cytogenet* 2001; 124(1):20–26.
279. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaian AM. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 2005; 310(5748):644–648.
280. Tomlins SA, Mehra R, Rhodes DR, Smith LR, Roulston D, Helgeson BE, Cao X, Wei JT, Rubin MA, Shah RB, Chinnaian AM. TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. *Cancer Res* 2006; 66(7):3396–3400.
281. Soller MJ, Isaksson M, Elfving P, Soller W, Lundgren R, Panagopoulos I. Confirmation of the high frequency of the TMPRSS2/ERG fusion gene in prostate cancer. *Genes Chromosomes Cancer* 2006; 45(7):717–719.
282. Eble JN, Sauter G, Epstein JI, Sesterhenn IA. Tumours of the kidney. In: *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs*. Lyon: IARC Press; 2004:9–88.
283. Kovacs G, Frisch S. Clonal chromosome abnormalities in tumor cells from patients with sporadic renal cell carcinomas. *Cancer Res* 1989; 49(3):651–659.
284. Yamakawa K, Morita R, Takahashi E, Hori T, Ishikawa J, Nakamura Y. A detailed deletion mapping of the short arm of chromosome 3 in sporadic renal cell carcinoma. *Cancer Res* 1991; 51(17):4707–4711.
285. Shuin T, Kondo K, Torigoe S, Kishida T, Kubota Y, Hosaka M, Nagashima Y, Kitamura H, Latif F, Zbar B, Lerman MI, Yao M. Frequent somatic mutations and loss of heterozygosity of the von Hippel-Lindau tumor suppressor gene in primary human renal cell carcinomas. *Cancer Res* 1994; 54(11):2852–2855.

286. Gunawan B, Huber W, Holtrup M, von Heydebreck A, Efferth T, Poustka A, Ringert RH, Jakse G, Füzesi L. Prognostic impacts of cytogenetic findings in clear cell renal cell carcinoma: gain of 5q31-pter predicts a distinct clinical phenotype with favorable prognosis. *Cancer Res* 2001; 61(21):7731–7738.
287. Presti JC, Jr, Reuter VE, Cordon-Cardo C, Mazumdar M, Fair WR, Jhanwar SC. Allelic deletions in renal tumors: histopathological correlations. *Cancer Res* 1993; 53(23):5780–5783.
288. Limon J, Mrózek K, Heim S, Elfving P, Nedoszytko B, Babińska M, Mandahl N, Lundgren R, Mitelman F. On the significance of trisomy 7 and sex chromosome loss in renal cell carcinoma. *Cancer Genet Cytogenet* 1990; 49(2):259–263.
289. Emanuel A, Szucs S, Weier HU, Kovacs G. Clonal aberrations of chromosomes X, Y, 7 and 10 in normal kidney tissue of patients with renal cell tumors. *Genes Chromosomes Cancer* 1992; 4(1):75–77.
290. Corless CL, Aburatani H, Fletcher JA, Housman DE, Amin MB, Weinberg DS. Papillary renal cell carcinoma: quantitation of chromosomes 7 and 17 by FISH, analysis of chromosome 3p for LOH, and DNA ploidy. *Diagn Mol Pathol* 1996; 5(1):53–64.
291. Meloni AM, Bridge J, Sandberg AA. Reviews on chromosome studies in urological tumors. I. Renal tumors. *J Urol* 1992; 148(2 Pt 1):253–265.
292. Palmedo G, Fischer J, Kovacs G. Fluorescent microsatellite analysis reveals duplication of specific chromosomal regions in papillary renal cell tumors. *Lab Invest* 1997; 77(6):633–638.
293. Palmedo G, Fischer J, Kovacs G. Duplications of DNA sequences between loci D20S478 and D20S206 at 20q11.2 and between loci D20S902 and D20S480 at 20q13.2 mark new tumor genes in papillary renal cell carcinoma. *Lab Invest* 1999; 79(3):311–316.
294. Zhuang Z, Park WS, Pack S, Schmidt L, Vortmeyer AO, Pak E, Pham T, Weil RJ, Candidus S, Lubensky IA, Linehan WM, Zbar B, Weirich G. Trisomy 7-harbouring non-random duplication of the mutant MET allele in hereditary papillary renal carcinomas. *Nat Genet* 1998; 20(1):66–69.
295. Meloni AM, Dobbs RM, Pontes JE, Sandberg AA. Translocation (X;1) in papillary renal cell carcinoma. A new cytogenetic subtype. *Cancer Genet Cytogenet* 1993; 65(1):1–6.
296. Sidhar SK, Clark J, Gill S, Hamoudi R, Crew AJ, Gwilliam R, Ross M, Linehan WM, Birdsall S, Shipley J, Cooper CS. The t(X;1)(p11.2;q21.2) translocation in papillary renal cell carcinoma fuses a novel gene PRCC to the TFE3 transcription factor gene. *Hum Mol Genet* 1996; 5(9):1333–1338.
297. Heimann P, El Housni H, Ogur G, Weterman MA, Petty EM, Vassart G. Fusion of a novel gene, RCC17, to the TFE3 gene in t(X;17)(p11.2;q25.3)-bearing papillary renal cell carcinomas. *Cancer Res* 2001; 61(10):4130–4135.
298. Clark J, Lu YJ, Sidhar SK, Parker C, Gill S, Smedley D, Hamoudi R, Linehan WM, Shipley J, Colin S. Fusion of splicing factor genes PSF and NonO (p54nrb) to the TFE3 gene in papillary renal cell carcinoma. *Oncogene* 1997; 15(18):2233–2239.
299. Pettinato G, Manivel JC, Wick MR, Dehner LP. Classical and cellular (atypical) congenital mesoblastic nephroma: a clinicopathologic, ultrastructural, immunohistochemical, and flow cytometric study. *Hum Pathol* 1989; 20(7):682–690.
300. Schofield DE, Yunis EJ, Fletcher JA. Chromosome aberrations in mesoblastic nephroma. *Am J Pathol* 1993; 143(3):714–724.
301. Rubin BP, Chen CJ, Morgan TW, Xiao S, Grier HE, Kozakewich HP, Perez-Atayde AR, Fletcher JA. Congenital mesoblastic nephroma t(12;15) is associated with ETV6-NTRK3 gene fusion: cytogenetic and molecular relationship to congenital (infantile) fibrosarcoma. *Am J Pathol* 1998; 153(5):1451–1458.
302. Knezevich SR, Garnett MJ, Pysher TJ, Beckwith JB, Grundy PE, Sorensen PH. ETV6-NTRK3 gene fusions and trisomy 11 establish a histogenetic link between mesoblastic nephroma and congenital fibrosarcoma. *Cancer Res* 1998; 58(22):5046–5048.
303. Knezevich SR, McFadden DE, Tao W, Lim JF, Sorensen PH. A novel ETV6-NTRK3 gene fusion in congenital fibrosarcoma. *Nat Genet* 1998; 18(2):184–187.
304. Watanabe N, Kobayashi H, Hirama T, Kikuta A, Koizumi S, Tsuru T, Kaneko Y. Cryptic t(12;15)(p13;q26) producing the ETV6-NTRK3 fusion gene and no loss of IGF2 imprinting in congenital mesoblastic nephroma with trisomy 11: fluorescence in situ hybridization and IGF2 allelic expression analysis. *Cancer Genet Cytogenet* 2002; 136(1):10–16.

305. Breslow NE, Olson J, Mokness J, Beckwith JB, Grundy P. Familial Wilms' tumour, a descriptive study. *Med Pediatr Oncol* 1996; 27:398–403.
306. Yunis JJ, Ramsay NK. Familial occurrence of the aniridia-Wilms tumor syndrome with deletion 11p13–14.1. *J Pediatr* 1980; 96:1027–1030.
307. Rahman N, Arbour L, Tonin P, Renshaw J, Pelletier J, Baruchel S, Pritchard-Jones K, Stratton MR, Narod SA. Evidence for a familial Wilms' tumour gene (FWT1) on chromosome 17q12-q21. *Nat Genet* 1996; 13:461–463.
308. McDonald JM, Douglass EC, Fisher R, Geiser CF, Krill CE, Strong LC, Virshup D, Huff V. Linkage of familial Wilms' tumor predisposition to chromosome 19 and a two locus model for the etiology of familial tumors. *Cancer Res* 1998; 58:1387–1390.
309. Coppes MJ, Haber DA, Grundy PE. Genetic events in the development of Wilms' tumor. *New Engl J Med* 1994; 331:586–590.
310. Slater RM, Mannens MM. Cytogenetics and molecular genetics of Wilms' tumor of childhood. *Cancer Genet Cytogenet* 1992; 61:111–121.
311. Wang-Wuu S, Soukup S, Bove K, Gotwals B, Lampkin B. Chromosome analysis of 31 Wilms' tumors. *Cancer Res* 1990; 50:2786–2793.
312. Grundy PE, Breslow NE, Li S, Perlman E, Beckwith JB, Ritchey ML, Shamberger RC, Haase GM, D'Angio GJ, Donaldson M, Coppes MJ, Malogolowkin M, Shearer P, Thomas PR, Macklis R, Tomlinson G, Huff V, Green DM; National Wilms Tumor Study Group. Loss of heterozygosity for chromosomes 1p and 16q is an adverse prognostic factor in favorable-histology Wilms tumor: a report from the National Wilms Tumor Study Group. *J Clin Oncol* 2005; 23:7312–7321.
313. Mostofi FK, Sesterhenn IA. Tumours of the testis and paratesticular tissue. In: Eble JN, Sauter G, Epstein JI, Sesterhenn IA, eds. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs*. Lyon: IARC Press; 2004:220.
314. Bosl GJ, Ilson DH, Rodriguez E, Motzer RJ, Reuter VE, Chaganti RS. Clinical relevance of the i(12p) marker chromosome in germ cell tumors. *J Natl Cancer Inst* 1994; 86(5):349–355.
315. Sandberg AA, Meloni AM, Suijkerbuijk RF. Reviews of chromosome studies in urological tumors. III. Cytogenetics and genes in testicular tumors. *J Urol* 1996; 155(5):1531–1556.
316. Atkin NB, Baker MC. Specific chromosome change, i(12p), in testicular tumours? *Lancet* 1982; 2:1349.
317. Suijkerbuijk RF, Sinke RJ, Meloni AM, Parrington JM, van Echten J, de Jong B, Oosterhuis JW, Sandberg AA, Geurts van Kessel A. Overrepresentation of chromosome 12p sequences and karyotypic evolution in i(12p)-negative testicular germ-cell tumors revealed by fluorescence in situ hybridization. *Cancer Genet Cytogenet* 1993; 70(2):85–93.
318. Ottesen AM, Skakkebaek NE, Lundsteen C, Leffers H, Larsen J, Rajpert-De Meyts E. High-resolution comparative genomic hybridization detects extra chromosome arm 12p material in most cases of carcinoma in situ adjacent to overt germ cell tumors, but not before the invasive tumor development. *Genes Chromosomes Cancer* 2003; 38(2):117–125.
319. Rosenberg C, Van Gurp RJ, Geelen E, Oosterhuis JW, Looijenga LH. Overrepresentation of the short arm of chromosome 12 is related to invasive growth of human testicular seminomas and nonseminomas. *Oncogene* 2000; 19(51):5858–5862.
320. Cossu-Rocca P, Zhang S, Roth LM, Eble JN, Zheng W, Karim FW, Michael H, Emerson RE, Jones TD, Hattab EM, Cheng L. Chromosome 12p abnormalities in dysgerminoma of the ovary: a FISH analysis. *Mod Pathol* 2006; 19(4):611–615.
321. Kraggerud SM, Szymanska J, Abeler VM, Kaern J, Eknaes M, Heim S, Teixeira MR, Tropé CG, Peltomäki P, Lothe RA. DNA copy number changes in malignant ovarian germ cell tumors. *Cancer Res* 2000; 60(11):3025–3030.
322. Zahn S, Sievers S, Alemazkour K, Orb S, Harms D, Schulz WA, Calaminus G, Göbel U, Schneider DT. Imbalances of chromosome arm 1p in pediatric and adult germ cell tumors are caused by true allelic loss: a combined comparative genomic hybridization and microsatellite analysis. *Genes Chromosomes Cancer* 2006; 45(11):995–1006.
323. Schneider DT, Schuster AE, Fritsch MK, Calaminus G, Harms D, Göbel U, Perlman EJ. Genetic analysis of childhood germ cell tumors with comparative genomic hybridization. *Klin Padiatr* 2001; 213(4):204–211.

324. Spirtas R, Heineman EF, Bernstein L, Beebe GW, Keehn RJ, Stark A, Harlow BL, Benichou J. Malignant mesothelioma: attributable risk of asbestos exposure. *Occup Environ Med* 1994; 51(12):804–811.
325. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors. mesothelioma. *Cancer Genet Cytogenet* 2001; 127(2):93–110.
326. Hagemeijer A, Versnel MA, Van Drunen E, Moret M, Bouts MJ, van der Kwast TH, Hoogsteden HC. Cytogenetic analysis of malignant mesothelioma. *Cancer Genet Cytogenet* 1990; 47(1):1–28.
327. Murthy SS, Testa JR. Asbestos, chromosomal deletions, and tumor suppressor gene alterations in human malignant mesothelioma. *J Cell Physiol* 1999; 180(2):150–157.
328. Taguchi T, Jhanwar SC, Siegfried JM, Keller SM, Testa JR. Recurrent deletions of specific chromosomal sites in 1p, 3p, 6q, and 9p in human malignant mesothelioma. *Cancer Res* 1993; 53(18):4349–4355.
329. Lee WC, Testa JR. Somatic genetic alterations in human malignant mesothelioma (review). *Int J Oncol* 1999; 14(1):181–188.
330. Halling KC, King W, Sokolova IA, Meyer RG, Burkhardt HM, Halling AC, Cheville JC, Sebo TJ, Ramakumar S, Stewart CS, Pankratz S, O’Kane DJ, Seelig SA, Lieber MM, Jenkins RB. A comparison of cytology and fluorescence in situ hybridization for the detection of urothelial carcinoma. *J Urol* 2000; 164(5):1768–1775.
331. Taguchi T, Zhou JY, Feder M, Litwin S, Klein-Szanto AJ, Testa JR. Detection of aneuploidy in interphase nuclei from non-small cell lung carcinomas by fluorescence in situ hybridization using chromosome-specific repetitive DNA probes. *Cancer Genet Cytogenet* 1996; 89(2):120–125.
332. Schwendel A, Langreck H, Reichel M, Schröck E, Ried T, Dietel M, Petersen I. Primary small-cell lung carcinomas and their metastases are characterized by a recurrent pattern of genetic alterations. *Int J Cancer* 1997; 74(1):86–93.
333. Jenkins RB, Qian J, Lieber MM, Bostwick DG. Detection of c-myc oncogene amplification and chromosomal anomalies in metastatic prostatic carcinoma by fluorescence in situ hybridization. *Cancer Res* 1997; 57(3):524–531.
334. Kakar S, Puangsuvan N, Stevens JM, Serenas R, Mangan G, Sahai S, Mihalov ML. HER-2/neu assessment in breast cancer by immunohistochemistry and fluorescence in situ hybridization: comparison of results and correlation with survival. *Mol Diagn* 2000; 5(3):199–207.
335. Bartlett JM, Going JJ, Mallon EA, Watters AD, Reeves JR, Stanton P, Richmond J, Donald B, Ferrier R, Cooke TG. Evaluating HER2 amplification and overexpression in breast cancer. *J Pathol* 2001; 195(4):422–428.
336. Schwab M. Amplification of N-myc as a prognostic marker for patients with neuroblastoma. *Semin Cancer Biol* 1993 Feb; 4(1):13–18.
337. Shapiro DN, Valentine MB, Rowe ST, Sinclair AE, Sublett JE, Roberts WM, Look AT. Detection of N-myc gene amplification by fluorescence in situ hybridization. Diagnostic utility for neuroblastoma. *Am J Pathol* 1993; 142(5):1339–1346.
338. Mahadevan D, Bearss DJ, Vankayalapati H. Structure-based design of novel anti-cancer agents targeting aurora kinases. *Curr Med Chem Anticancer Agents* 2003; 3(1):25–34.
339. Mortlock A, Keen NJ, Jung FH, Heron NM, Foote KM, Wilkinson RW, Green S. Progress in the development of selective inhibitors of aurora kinases. *Curr Top Med Chem* 2005; 5(2):199–213.
340. Soder AI, Hoare SF, Muir S, Going JJ, Parkinson EK, Keith WN. Amplification, increased dosage and in situ expression of the telomerase RNA gene in human cancer. *Oncogene* 1997; 14(9):1013–1021.
341. Bryce LA, Morrison N, Hoare SF, Muir S, Keith WN. Mapping of the gene for the human telomerase reverse transcriptase, hTERT, to chromosome 5p15.33 by fluorescence in situ hybridization. *Neoplasia* 2000; 2(3):197–201.
342. Cairncross JG, Ueki K, Zlatescu MC, Lisle DK, Finkelstein DM, Hammond RR, Silver JS, Stark PC, Macdonald DR, Ino Y, Ramsay DA, Louis DV. Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. *J Natl Cancer Inst* 1998; 90(19):1473–1479.
343. Houillier C, Lejeune J, Benouaich-Amiel A, Laigle-Donadey F, Criniere E, Mokhtari K, Thillet J, Delattre JY, Hoang-Xuan K, Sanson M. Prognostic impact of molecular markers in a series of 220 primary glioblastomas. *Cancer* 2006; 106(10):2218–2223.
344. Smith JS, Tachibana I, Passe SM, Huntley BK, Borell TJ, Iturria N, O’Fallon JR, Schaefer PL, Scheithauer BW, James CD, Buckner JC, Jenkins RB. PTEN mutation, EGFR amplification, and outcome in patients with anaplastic astrocytoma and glioblastoma multiforme. *J Natl Cancer Inst* 2001; 93(16):1246–1256.

345. Korshunov A, Sycheva R, Gorelyshev S, Golovan A. Clinical utility of fluorescence in situ hybridization (FISH) in nonbrainstem glioblastomas of childhood. *Mod Pathol* 2005; 18(9):1258–1263.
346. Fallon KB, Palmer CA, Roth KA, Nabors LB, Wang W, Carpenter M, Banerjee R, Forsyth P, Rich K, Perry A. Prognostic value of 1p, 19q, 9p, 10q, and EGFR-FISH analyses in recurrent oligodendroglomas. *J Neuropathol Exp Neurol* 2004; 63(4):314–322.
347. Dandachi N, Dietze O, Hauser-Kronberger C. Chromogenic in situ hybridization: a novel approach to a practical and sensitive method for the detection of HER2 oncogene in archival human breast carcinoma. *Lab Invest* 2002; 82(8):1007–1014.
348. Hopman AH, Claessen S, Speel EJ. Multi-colour brightfield in situ hybridisation on tissue sections. *Histochem Cell Biol* 1997; 108(4–5):291–298.
349. Gong Y, Gilcrease M, Sneige N. Reliability of chromogenic in situ hybridization for detecting HER-2 gene status in breast cancer: comparison with fluorescence in situ hybridization and assessment of interobserver reproducibility. *Mod Pathol* 2005; 18(8):1015–1021.
350. Isola J, Tanner M, Forsyth A, Cooke TG, Watters AD, Bartlett JM. Interlaboratory comparison of HER-2 oncogene amplification as detected by chromogenic and fluorescence in situ hybridization. *Clin Cancer Res* 2004; 10(14):4793–4798.
351. Bhargava R, Lal P, Chen B. Chromogenic in situ hybridization for the detection of HER-2/neu gene amplification in breast cancer with an emphasis on tumors with borderline and low-level amplification: does it measure up to fluorescence in situ hybridization? *Am J Clin Pathol* 2005; 123(2):237–243.
352. de Alava E, Kawai A, Healey JH, Fligman I, Meyers PA, Huvos AG, Gerald WL, Jhanwar SC, Argani P, Antonescu CR, Pardo-Mindan FJ, Ginsberg J, Womer R, Lawlor ER, Wunder J, Andrulis I, Sorensen PH, Barr FG, Ladanyi M. EWS-FLI1 fusion transcript structure is an independent determinant of prognosis in Ewing's sarcoma. *J Clin Oncol* 1998; 16(4):1248–1255.
353. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 1992; 258(5083):818–821.
354. Kallioniemi OP, Kallioniemi A, Piper J, Isola J, Waldman FM, Gray JW, Pinkel D. Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. *Genes Chromosomes Cancer* 1994; 10(4):231–243.
355. Shayesteh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, Collins C, Pinkel D, Powell B, Mills GB, Gray JW. PIK3CA is implicated as an oncogene in ovarian cancer. *Nat Genet* 1999; 21(1):99–102.
356. Sakabe T, Shinomiya T, Mori T, Ariyama Y, Fukuda Y, Fujiwara T, Nakamura Y, Inazawa J. Identification of a novel gene, MASL1, within an amplicon at 8p23.1 detected in malignant fibrous histiocytomas by comparative genomic hybridization. *Cancer Res* 1999; 59(3):511–515.
357. Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y, Dairkee SH, Ljung BM, Gray JW, Albertson DG. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. *Nat Genet* 1998; 20(2):207–211.
358. Solinas-Toldo S, Lampel S, Stilgenbauer S, Nickolenko J, Benner A, Döhner H, Cremer T, Lichter P. Matrix-based comparative genomic hybridization: Biochips to screen for genomic imbalances. *Genes Chromosomes Cancer* 1997; 20(4):399–407.
359. Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T. Multicolor spectral karyotyping of human chromosomes. *Science* 1996; 273(5274):494–497.
360. Speicher MR, Gwyn Ballard S, Ward DC. Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nat Genet* 1996; 12(4):368–375.

Contributed protocol section

IMPORTANT: No protocol included in this manual should be used for clinical testing unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements for all chemicals used in the laboratory.

Protocol 12.1 Solid tumor cell culture and harvest

SEQ Adapted from a protocol submitted by the Center for Human Genetics, University of Nebraska Medical Center

I. Principle

Certain solid tumors have been found to be associated with specific chromosome changes and a growing number of consistent cytogenetic abnormalities in both malignant and benign tumors are now being revealed. It is hoped that through continued culture and analysis, the number of such associations will increase. In some cases the histological diagnosis of a tumor may be difficult to make or is unclear, and the chromosomal abnormalities found during cytogenetic analysis of the tumor may be extremely helpful to the pathologist and the clinician.

II. Materials

Reagents

1. Chang Medium MF (Irvine Scientific # T111)
2. Ham's F12 (Irvine Scientific #9058, SOP #88)
3. RPMI 1640 (Irvine Scientific #9160) with 1% HEPES Buffer Solution 1 M (Irvine Scientific #9319) for rinse medium
4. Transport medium (see 12.2.4, Specimen Transport)
5. Collagenase stock solution
6. DMSO (Dimethylsulfoxide) (Sigma)
7. Fetal Bovine Serum (Irvine Scientific #3000)
8. Colcemid® (Irvine Scientific, 10 µg/mL)
9. Sodium Citrate hypotonic, 0.7%
10. Trypsin-EDTA (1×) (GIBCO #610-5300)
11. Methanol
12. Acetic Acid (Glacial)
13. DURO Super Glue-5
14. Liquid Nitrogen (LN₂)

Supplies

15. Gloves
16. Petri dishes (60 × 15 mm; 150 × 25 mm), sterile
17. Culture dishes (35 × 10 mm), sterile
18. Plastek coverslip kit, Type CSGK/F (MatTek Corp)
19. Sterile forceps
20. Disposable scalpels, sterile
21. Tissue culture flasks (Falcon, T-25, T-12.5), sterile
22. Pasteur pipettes, (5¾ inch and sterile 9 inch)
23. Serological pipettes (5 mL, 10 mL, 25 mL), sterile
24. Screw-cap centrifuge tubes (Corning, 50 mL and 15 mL), sterile
25. Microscope slides (3 inch × 1 inch thickness 0.97–1.07 mm)
26. 2 µL bulbs for Pasteur pipettes
27. Specimen container, sterile with lid
28. Cryovials (1 mL) (Nunc)

29. Cane(s) (for cryopreservation) or boxes
30. Shield(s) for cryopreservation cane(s)
31. Cryoboxes
32. Permapoint marker
33. Kimwipes
34. Accession labels

Equipment

35. Incubator at 37 °C and 5% CO₂
36. Oven, Blue-M (60 °C, 90–96 °C)
37. Incubator, dry (37 °C)
38. Freezer, -70 °C
39. Refrigerator/Freezer, 4 °C and -20 °C
40. Pipetman, P-20 (Rainin)
41. Pipetman pipette tips (1-100 µL)
42. Automatic pipettor
43. Centrifuge
44. Hygrometer
45. Veristaltic pump
46. Cyto MiniPrep 60 Harvester (Tecan, USA, Inc.)
47. Thermotron Environmental Chamber
48. Biological safety cabinet, Class A Type II
49. Inverted microscope with removable phase contrast ring and plan 2.5× and plan 6.3× objectives
50. Nalgene Cryo 1 °C Freezing Container (Mr Freeze #5100-0001)
51. Liquid nitrogen storage tank (Dewar)
52. Nalgene filter units: 0.45 µm, 0.22 µm

Specimen

1. Tumor tissue, 0.5–1.0 g or 5–10 mm³ of viable tissue is sufficient, in most cases.
2. Much smaller biopsy specimens such as fine-needle aspirates (FNA) or needle biopsies may also be successfully analyzed. However, it will require fewer cultures and they may require prolonged culture periods in order to obtain enough mitotic cells for analysis (2–3 mL of FNA is desirable).
3. If the tumor is large (greater than 3 cm³) and cannot be covered adequately by the transport medium in one container, it may be cut into smaller 1–2 cm³ pieces and divided into multiple transport containers.

Transportation of tumor samples

1. Tumor samples should be part of the same specimen that is submitted for pathological studies.
2. If several specimens from the same tumor are available, each should be processed separately.
3. Tumors should be handled as aseptically as is practical and trimmed of any extraneous tissue.
4. Samples which will be received in the Cytogenetics laboratory within one hour of being removed from the patient may be placed in a sterile Petri dish which contains a piece of sterile gauze soaked in sterile saline. The gauze is used to help maintain a humid atmosphere in the Petri dish.
5. Samples which will be received after one hour, but within the same day as the surgery, should be transported in sterile transport medium.
6. Deliver specimens to the Cytogenetics laboratory as soon as possible. During the summer months it is helpful to place the specimen container in a Styrofoam box with an ice pack to protect it from the heat if the specimen is in transit overnight. Do not allow the specimen to come in contact with the ice pack.
7. Although tumor samples that are processed immediately yield better results in tissue culture, successful growth of cells may also be obtained from samples that are processed up to 24–48 hours after collection. Tumor specimens can be sent to the laboratory by overnight package express services and processed on the following day. Place these samples in sterile containers filled with sterile transport media.

8. **IMPORTANT:** If neural tissue (i.e., brain tumor) is received late in the day, all attempts should be made to process it immediately. Following thorough mincing, collagenase time may be reduced to 30 minutes. If the tissue cannot be processed the same day, leave the sample at room temperature in complete medium. Do not refrigerate.

III. Method

Note: Label all receptacles into which the specimen is transferred with two patient identifiers, e.g., patient name and accession number.

Tissue mincing

1. Upon a tumor specimen's arrival in the laboratory, transfer the tumor tissue using sterile procedure to a Petri dish (60×15 mm or 13×10 mm) containing ~1 mL of unsupplemented RPMI medium. Unless the tissue appears fragile*, rinse gently. Note the size and appearance of the tissue on culture sheet. (If tissue is bloody, rinse off as much blood as possible.)
*If the tissue is fragile, omit the rinse step and centrifuge the transport medium to collect any suspended cells. This cell pellet should be included with the minced tissue for culture.
2. Check for the following exceptions before proceeding:
 - a. If diagnosis is Ewing sarcoma/PNET, rhabdomyosarcoma, or synovial sarcoma, check with your laboratory's policy in determining whether the specimen should be considered for RT-PCR studies (see Note 1, Insufficient quantity for RT-PCR).
 - b. **If tissue is extremely small (FNA), check with laboratory policy before using the entire tissue for culture.**
 - c. If interphase FISH is indicated, touch preparations can be made from the tissue before it is frozen (see Note 2, Preparing touch preps for interphase FISH).
3. Using two crossed scalpels, mince the tissue in a second Petri dish containing a few drops (up to 0.5 mL) of Chang Medium MF. Mince only the volume of tissue necessary to establish the desired number of cultures. For example,
 - a. Establishing only one culture ("A") should be considered if the tissue measures 0.5 cm^3 or smaller.
 - b. Tissues measuring $1\text{--}1.5 \text{ cm}^3$ are typically adequate to establish both "A" and "B" cultures.
 - c. If the tumor size exceeds 1.5 cm^3 , place the remaining portion of the tumor in one to four cryovials and "snap freeze" in liquid nitrogen for future studies (see Note 3, Snap freeze). Record the freeze in the cryopreservation logbook and in the Cytogenetics database (see Note 4, Numbering code for cryopreservation). If there is remaining tissue after the snap freeze, see Note 5, Tumor bank, regarding distribution of tissue to the Tumor Bank.
4. Collect minced tissue with a scalpel blade and place into the appropriate "A"/"B" T-25 flask containing 2 mL Chang MF medium and 2 mL 0.8% collagenase solution. Exceptions include:
 - a. If the specimen is a cartilage tumor, use Ham's F-12 complete medium in place of Chang MF medium throughout the procedure.
 - b. If the specimen is very small, like a FNA, use a T-12.5 flask ("A") with 1 mL of complete medium and 1 mL of collagenase solution.

Direct/overnight/supernatant harvest

1. If the specimen is of an inadequate size, a direct or supernatant harvest can be considered for specific tumor types (see Note 6, When to set up a direct or supernatant harvest).
 - a. For a "Direct" culture, place a portion of the minced tissue into a 15 mL centrifuge tube containing 10 mL Chang MF medium. Add 0.2 mL Colcemid® for 45 minutes. Proceed to step "d."
 - b. For a "Direct Overnight" culture, place minced tissue in a 15 mL centrifuge tube containing 10 mL Chang MF medium and add 10 µL Colcemid®. The next morning, add 0.2 mL Colcemid® for 45 minutes. Proceed to step "d."
 - c. If a "Supernatant" harvest is required, wait approximately 24–48 hours after setup. Once growth in the in-situ/flask cultures is established, remove the media from the flasks and coverslips, which were set up from the collagenased tissue, and place in a labeled 15-mL centrifuge tube. Add new complete media back onto the cultures and place back in the incubator for long-term culture. To the centrifuge tube, add 3 µL of Colcemid® for every 2 mL of medium and incubate overnight. Proceed to step "d."
 - d. For each harvest, the remainder of the harvest procedure is the same.
 - i. Centrifuge the centrifuge tube for 6 minutes at 1000–1200 RPM.
 - ii. Remove the supernatant. Resuspend pellet.

- iii. Add 6 mL of sodium citrate (0.7%) hypotonic saline solution. Incubate at 37 °C for 24 minutes.
- iv. Prefix with 2 mL freshly made 3 : 1 fixative (methanol–acetic acid) (see Note 7, Fixative). Spin for 6 minutes at 1000–1200 RPM.
- v. Remove the supernatant and resuspend the pellet in 4 mL of 3:1 fixative.
- vi. Spin again for 6 minutes at 1000–1200 RPM and after aspirating the supernatant, resuspend the pellet in 4 mL of 3 : 1 fixative. Omit the 2nd fixative change if the pellet is extremely small.
- vii. Refrigerate the pellet at 4 °C until slides can be made for analysis.

Tissue disaggregation with collagenase

1. Label the T-25 or T-12.5 flask with patient's name, date, accession number.
2. Record set-up information on culture sheet and enter appropriate data into your laboratory's LIS database.
3. Incubate 4–48 hours in 5% CO₂ at 37 °C (all incubations unless otherwise stated are at 37 °C in 5% CO₂). The timing of the enzymatic treatment depends upon the tumor type.
 - a. Samples which are soft, loose and mince easily generally require only 4 hours of collagenase treatment.
 - b. **Collagenase time on brain tissue may be reduced to 30 minutes if adequate disaggregation has occurred. Brain tissue should not be exposed to collagenase for more than 2–3 hours.**
 - c. Samples which are very firm generally require an overnight incubation.
 - d. In *extreme* cases, samples which contain large amounts of bone may require up to one week in collagenase.
 - e. If the sample is not adequately disaggregated after three days, add a second aliquot of sterile collagenase (2000 µ/mL) to the flask.
4. The "A" and "B" flasks are typically taken out of collagenase on two consecutive days; however, in the case of soft or loose tissue samples, both flasks may be taken out of collagenase at the same time. At the end of the collagenase treatment, pipet the sample **vigorously** up and down in the same flask. If the tissue does not break up appropriately, return the flask to the incubator and continue the collagenase treatment.
5. After disaggregation, remove the tissue and culture medium from the flask with a pipette and place into a sterile 15 mL, capped centrifuge tube. Centrifuge the cell suspension at 1000–1200 RPM for 6 minutes. Add fresh complete medium to the original flask and reincubate the flask in 5% CO₂ at 37 °C.
6. After centrifugation, aspirate the supernatant and resuspend the washed cells in complete medium. If a fat layer is present at the top of the medium after centrifugation, set up a fat culture according to Note 8, Fat layer.
7. The volume of medium used for the resuspension will depend on the amount of tissue present and also on how completely the tissue was disaggregated. The number and size of the cultures will depend upon the size and expected viability of the cell pellet. Typically we establish four coverslips (labeled A1/B1, A2/B2, A3/B3, A4/B4) and two T-25 flasks (labeled A7/B7 and A8/B8) from each flask of collagenased tissue. If you need to vary from this protocol because of the limited size of the pellet, check with your laboratory's protocol to determine the number and type of cultures most appropriate. An example would be to set-up only 2–3 coverslips on a FNA. Viability will vary with the tumor type. In general, sarcomas will have a high plating efficiency or viability, and carcinomas will have a low plating efficiency or viability. Use 1 mL of cell suspension and 3 mL of complete medium for each T-25 flask culture and 0.5 mL of cell suspension for each coverslip culture.
8. In instances where there is more than enough sample to establish the typical 4 coverslips and 2 flask cultures, use a portion of the cell suspension for freezing. To do this step, recentrifuge remaining cell suspension, aspirate off the medium, resuspend in 1 mL FBS and 0.1 mL DMSO, mix well and place in 1.0 mL cryovial. Cryopreserve in liquid nitrogen (see Chapter 4, Protocols 4.27–4.30).
9. Label each culture with patient name or initials, accession number, date and a culture identification letter or number. Record cultures on patient culture sheet and enter data into the laboratory's workflow documentation (e.g., LIS) system. Incubate the cultures at 5% CO₂ and 37 °C.

Observation of cultures for cell growth

1. The following day, check cultures for cell attachment using a phase microscope.
 - If cell attachment is observed, flood the coverslip cultures with 1.5 mL of complete medium.
 - If no growth is observed you may wait another 24 hours before flooding the cultures.
 - If the core technologist or senior culture tech has determined that the cultures were set too thin, aspirate the suspended cells and medium from all cultures, centrifuge, discard supernatant, resuspend cell in appropriate volume of medium and reinoculate onto fewer coverslips/flasks.

- If growth is too heavy on the day the cultures are flooded, rinse and re-feed the cultures with pre-warmed medium in order to remove some of the floating cells and reduce the potential for additional cells to attach. On subsequent days in culture, it may be necessary to rinse the coverslips one or more times to remove any floating cells and debris prior to re-feeding. Flask cultures may also need to be rinsed at this time (see Note 9, Culture drawback).
2. Note any mitotic activity in each culture. Record on the specimen's culture sheet.
 3. The date of harvest of the cultures is individualized for each flask or dish and is carried out when "peak" mitotic activity is observed.
 4. In order to avoid overgrowth with normal cells, most cultures should be harvested within 10 days of culture initiation.
 5. Occasionally, tumor cells will attach, divide a few times, die, and lift off the bottom of the flask, while the fibroblasts steadily multiply. This is especially true in the culture of bladder tumors. Therefore, do not wait more than 48 hours after seeding bladder tumor cells before beginning harvest of at least one culture (see Note 10, Normal findings in bladder specimens).
 6. Once adequate cultures have been harvested for completion of analysis, cryopreserve the remaining cultures (see Note 3, Snap freeze).

Harvest procedures for flasks

1. Two harvesting methods for flasks may be employed depending on the proliferation characteristics of the individual tumor samples. It is preferable to harvest flasks when they have reached 60-85% confluence.
 - a. Short-term Colcemid® treatment to be used on rapidly growing cultures: Colcemid® is added to each culture at a final concentration of 0.1 µg/mL (40 µL/4 mL of medium). Re-incubate. Record initiation of harvest on culture sheet and in the LIS database. Harvest after 1–2 hours incubation (37 °C).
 - b. Prolonged Colcemid® treatment to be used on slow-growing cultures: Colcemid® is added to the culture at a final concentration of 0.015 µg/mL (6 µL/4 mL of medium) at 4:00 p.m. Re-incubate overnight. Record initiation of harvest on culture sheet and in the LIS database. Harvest the following morning.
2. Using either method, the remainder of the harvesting procedure is the same.
 - a. Remove the supernatant from the flask and place in a labeled 15-mL centrifuge tube.
 - b. Rinse the flask with 2 mL rinse medium and add this rinse to the same centrifuge tube.
 - c. Add 2 mL of trypsin-EDTA to the flask and incubate at 37 °C for 5–6 minutes until all of the cells have detached from the flask. It may require physical force to help detach the cells, such as knocking the side of flask or vigorously pipetting the trypsin up and down in the flask.
 - d. Once the cells are detached, remove the trypsin/cell suspension and add it to the medium in the centrifuge tube.
 - e. Rinse the flask once more with medium to remove the remaining cells and add to centrifuge tube.
 - f. The remainder of the harvest is completed as previously described in Direct/Overnight/Supernatant Harvest above.
3. Once the harvest is complete, slides can be made in the Thermotron drying chamber or manually (see Note 11, Humidity).
4. Store the remaining cell pellet in 3 : 1 methanol–glacial acetic acid fixative at 4 °C in a tightly closed 15 mL centrifuge tube sealed with parafilm. This insures that cells are available if needed for further banding studies or in situ hybridization. If directed by the geneticist, the cell pellet can be "snap-frozen" in 1 mL of 3 : 1 methanol–glacial acetic acid fixative in liquid nitrogen (see Note 1, Insufficient quantity for RT-PCR and Note 3, Snap freeze).

Harvest procedure for coverslips

1. Coverslips must be harvested before they are confluent (see Note 12, Confluent coverslip cultures). However, you will most likely not obtain adequate metaphases from a coverslip that is harvested before day 3 after setup.
2. Two harvesting methods for coverslips may also be employed depending on the proliferation characteristics of the individual tumor samples:
 - a. Short-term Colcemid® treatment to be used on rapidly growing cultures: Colcemid® is added to each culture at a final concentration of 0.1 µg/mL (20 µL/2 mL medium). Re-incubate. Record initiation of harvest following laboratory guidelines. Harvest after 1–2 hours.
 - b. Prolonged Colcemid® treatment to be used on slow-growing cultures: Colcemid® is added to the culture at a final concentration of 0.015 µg/mL (6 µL/4 mL medium) at 4:00 p.m. Re-incubate overnight. Record initiation of harvest following laboratory guidelines. Harvest the following morning.
3. Harvest using the solid tumor coverslip program on the Tecan Cyto MiniPrep 60 or manually (see Note 13, Adding solutions).

4. After completion of the harvest, remove the coverslip from the dish, dry the back of the coverslip with a Kimwipe and label the top corners on back of the slip with accession number and culture number.
5. With a permanent marker, write the culture and harvest date on the patient's accession label. **Check and verify that the patient's name and accession number match the culture dish labels before placing labels on the slides.**
6. After labeling slides, remove the remaining labels for that patient from the slide-making area.
7. Glue the coverslip cell-side up on the labeled glass microscope slide. Place in a 60 °C oven overnight. For staining techniques, see **Chapter 6**, Chromosome stains).
8. Analyze banded cells (see Note 10, Normal findings in bladder specimens and Note 14, Chromosomal changes).

IV. Notes

1. **Insufficient Quantity for RT-PCR.** If there is an insufficient amount of tissue available for RT-PCR, culture supernatant or trypsinized cultured cells can also be sent for study.
2. **Preparing Touch Preps for Interphase FISH.** Touch preps are made by touching the tissue multiple times to a clean slide. When possible, freshly cut edges of the tissue should be used. The slides are then air-dried and fixed in 3 : 1 methanol–acetic acid for 10 minutes. The slides are stored at -20 °C.
3. **Snap Freeze.** Place tissue or fixed cells directly into labeled cryovial and seal. Place in the proper box and tower in the liquid nitrogen tank. Record in the cryopreservation logbook, the cryopreservation database and in the cytogenetics lab database.
4. **Numbering Code for Cryopreservation.** The codes for cryopreservation of solid tumor tissue and cells are as follows: The first number in the code corresponds to the number of the nitrogen tank (Dewar), the second number corresponds to the canister number, the third letter is the box label (this is sequential A, B, C, etc., with the next letter that is to be used recorded in the cryopreservation log). The fourth (last) number will indicate the location of the ampule on the cane or in the box with No. 1 at the bottom of the cane and No. 6 the top of the cane. One patient's specimens may be located in more than one box. Code 62H33 would thus represent a sample cryopreserved in liquid nitrogen tank #6, rack #2, box letter H and position #33.
5. **Tumor Bank.** Individual laboratories must establish an approved protocol if excess material is to be sent to a tumor bank. Protocol should include contact details of essential bank personnel and instructions for tumor eligibility. If a Patient Consent and Authorization form is received for a Tissue Bank donation, laboratory policy should stipulate whether the form is to be copied before forwarding with the specimen. Laboratory policy should also stipulate (a) whose responsibility it is to determine if the patient has consented to storage of the specimen in the Tumor Bank; (b) who will properly dispose of material and how, if consent is not given by the patient; (c) and what details regarding the distribution of tissue to a Tumor Bank must be documented on your specific laboratory's records.
6. **When to Set Up a Direct or Supernatant Harvest.** The following list defines the tumors which should be considered for direct harvest, direct overnight culture, or supernatant harvests:
 - a. Ewing sarcoma/Peripheral primitive neuroectodermal tumor (PPNET or PNET)/Askin tumor
 - b. Tumors involving hematopoietic cells or lymphoid tissue, such as plasmacytoma or lymphoma
 - c. Tumors of the nervous system, such as neuroblastoma and medulloblastoma
 - d. Retinoblastoma
 - e. Rhabdomyosarcoma
 - f. Germ cell tumors
 - g. Epitheloid sarcoma
 - h. Carcinomas
 - i. Unknown diagnosis
7. **Fixative.** The fixative must be prepared fresh daily prior to each harvest. Fixative must be kept capped between uses. Keep the fixative out of the harvest area or the hood at the time hypotonic should be added. Do not make the fixative until it is to be used. This is to prevent the accidental addition of fixative to cultures during the addition of hypotonic.
8. **Fat Layer.** If a fat layer is present at the top of the medium after centrifugation, carefully pull this layer off with a 2 mL pipet and place into a T-12.5 flask. Fill this flask completely with Chang MF medium and incubate at 37 °C for long-term culture. Label this culture as A9 or B9 with the patient name, date and accession number and record on culture sheet and in the LIS database. Once growth has been established in this culture, the medium may be discarded and replaced with 2 mL of Chang MF. Monitor growth and harvest according to the standard flasks procedures previously stated in this SOP.
9. **Culture Drawbacks.** The culture requirements are not known for all types of tumors. Culture conditions used in this procedure have been found to be satisfactory for the majority, but not all, urological tumors (bladder, kidney, testicular),

- soft tissue tumors and some benign tumors. Therefore, some tumor cultures may be expected to yield only normal metaphases. In these cases, it may not be possible to distinguish between the following situations:
- a. Only normal cells grew in culture. The tumor cells did not proliferate in vitro.
 - b. The tumor cells are chromosomally normal.
 - c. Chromosomal abnormalities are present in the tumor cells, but cannot be detected with these current procedures.
10. **Normal Findings in Bladder Specimens.** Normal cells are present in almost every tumor. In bladder tumor cultures these cells tend to predominant after 72 hours incubation. Therefore, no matter how slowly a bladder tumor culture seems to be growing, do not wait more than 48 hours before beginning harvest of at least one culture.
 11. **Humidity.** The relative humidity of the harvest room is an important factor in acquiring good spreading of the chromosomes. The optimal humidity is 55%. To the centrifuge tube, as measured by a hygrometer. The humidity may range from 50% - 60%.
 12. **Confluent Coverslip Cultures.** Since the cells are not removed from the growing surface, there must be sufficient space between the cells on the coverslip in order to permit swelling during the hypotonic treatment and subsequent spreading of the chromosomes during drying. Coverslips must therefore be harvested before they are confluent.
 13. **Adding Solutions.** It is *very important* in the harvest of in situ coverslip cultures not to add solutions too fast, or to joggle the culture dishes. The mitotic cells are attached tenuously and may become detached from the colonies. Be very careful during all aspects of checking, adding Colcemid® and harvest.
 14. **Chromosomal Changes.** In malignant cells any modal number or chromosome changes are possible.

Protocol 12.2 Solid tumor cell culture and harvest

Adapted from a procedure contributed by Ohio State University, Department of Pathology, Columbus, OH

I. Principle

Clonal chromosomal aberrations have been identified in virtually all malignant tumors. Through cytogenetic evaluation, the frequency and diagnostic relevance of specific aberrations can be correlated with specific tumors to confirm or establish a diagnosis or prognosis. Special mechanical dissociation, enzymatic disaggregation, short- and long-term culture, and harvest methods are used to maximize the number and quality of metaphases for study. Variations of these methods may be required for the success of specific tumor types.

Specimen

Using aseptic technique, excise a viable non-necrotic, 0.5–1.0 cm³ or 1–5 g piece of tumor. Place fresh tumor tissue in a 10–25 mL tube filled with sterile isotonic medium (RPMI, HBSS, MEM) preferably supplemented with antibiotics (penicillin, streptomycin, gentamicin, fungizone). Transport to cytogenetics lab as soon as possible at *room temperature*. If more than one tumor site is sampled, place each sample in a separate container to minimize the likelihood of contamination. Although specimens should not be frozen or refrigerated, tissue can be stored in growth medium for up to 24 hours at room temperature. Deliver tumor at room temperature as soon as possible after collection.

II. Materials

Reagents and solutions

1. RPMI 1640
Store at 2–8 °C. Date and initial bottle when opened. Discard in sink with running water at manufacturer's expiration date.
2. MEM
Store at 2–8 °C. Date and initial bottle when opened. Discard in sink with running water at manufacturer's expiration date.
3. Fetal Bovine Serum
Store frozen between –5 °C and –30 °C. Discard in biohazard container at manufacturer's expiration date. Keep in protective yellow bag as much as possible during medium preparation to block harmful UV light. Thaw completely in 37 °C waterbath before use. Avoid repeated freezing and thawing.

4. L-Glutamine

Store stock and aliquots frozen between –5 °C and –30 °C. Aseptically dispense in 5 mL aliquots. Label aliquots “L-glut” with lot #, preparation and expiration dates, and preparer’s initials. Before use, thaw completely and mix well. Discard in sink with running water at manufacturer’s expiration date.

5. Penicillin–Streptomycin

Aseptically dispense in 5-mL aliquots. Place aliquots in container labeled “Pen-Strep” with lot #, preparation and expiration dates, and preparer’s initials. Perform QC testing according to your laboratory protocol (see Note 1, Reagent QC testing). Store frozen between –5 °C and –30 °C. Thaw completely and mix well before use. Discard in red plastic pharmaceutical container.

6. Fungizone

Aseptically dispense in 2-mL aliquots. Place aliquots in container labeled “Fungizone” with lot #, preparation and expiration dates, and preparer’s initials. Perform QC testing according to your laboratory protocol (see Note 1, Reagent QC testing). Store frozen between –5 °C and –30 °C. Thaw completely and mix well before use. Discard in red plastic pharmaceutical container.

7. Tumor RPMI

Medium is prepared aseptically in laminar flow hood. Aseptically add to a sterile, capped, 250 mL Corning #430198 tissue culture flask:

- 100 mL RPMI 1640
- 12.5 mL Fetal Bovine Serum (FBS)
- 2.5 mL L-glutamine
- 2.5 mL penicillin–streptomycin
- 1.25 mL ITS supplement
- 12.5 mL BMC

Expiration date is 3 weeks.

Label flask as “Tumor RPMI medium” with batch number, preparation date, expiration date, storage requirements, and preparer’s initials. Record above label information, and lot numbers of reagents that had been used in medium preparation, on quality control log sheet. Test each batch for sterility following your laboratory’s protocol for Quality Control on medium (see Note 1, Reagent QC testing for suggestions). Store at 2–8 °C. Discard any unused tumor medium in sink with running water after three weeks or at RPMI expiration date.

8. Tumor MEM

- 100 mL MEM Medium
- 12.5 mL FBS
- 2.5 mL penicillin–streptomycin
- 2.5 mL L-glutamine
- 12.5 mL BMC

Label tubes as “Tumor MEM,” with batch number, preparation date, expiration date, storage requirements, and preparer’s initials. Record above label information, and lot numbers of reagents that had been used in medium preparation, on quality control log sheet. Test each batch for sterility following your laboratory’s protocol for Quality Control on medium (see Note 1, Reagent QC testing for suggestions).

Store frozen at 2–8 °C. Discard any, unused Tumor MEM medium in sink with running water after three weeks or at Tumor MEM expiration date.

9. Tumor Transport Medium

NOTE: Medium is prepared aseptically in laminar flow hood.

Aseptically add to a sterile, capped, 750 mL Falcon® #3028 tissue culture flask:

- 200 mL RPMI 1640
- 15 mL penicillin–streptomycin
- 2 mL fungizone
- 2 mL gentamicin

Aseptically aliquot 5 mL of transport medium into 15-mL sterile Sarstedt centrifuge tubes. Label tubes as “Tumor transport media” with batch number, preparation date, expiration date, storage requirements, and preparer’s initials. Record above label information, and lot numbers of reagents that had been used in medium preparation, on quality control log sheet. Test each batch for sterility following your laboratory’s protocol for Quality Control on medium (see Note 1, Reagent QC Testing for suggestions). Discard unused medium in sink with running water at manufacturer’s expiration date.

10. Collagenase II-S

Activity varies by lot number and is on the bottle. Reconstitute according to specific activity. Volume RPMI to add to vial = $50 \text{ mg} \times \text{activity (units/ng)} \div 500 \text{ units/mL}$. Aliquot 1 mL/tube and store frozen. Expiration date is 6 months.

11. 1× Trypsin/EDTA

Aseptically dispense in 2-mL aliquots. Place aliquots labeled as "Trypsin" in a container labeled with lot #, preparation and expiration dates and preparer's initials. Store stock and aliquots frozen between -5°C and -30°C . Thaw completely and mix well before use. Discard in sink with running water at manufacturer's expiration date.

12. Colcemid® 10 µg/mL in HBSS

Label with date opened and expiration date. Store at $2\text{--}8^\circ\text{C}$. Discard open bottle in red plastic pharmaceutical container after one (1) week or at manufacturer's expiration date.

13. Potassium Chloride (KCl)

Prepare 0.075 M KCl hypotonic solution by dissolving 5.59 g of KCl in 1000 mL double-distilled demineralized water. Use a 1000-mL volumetric flask. Store in white plastic bottle labeled "0.075 M KCl," with lot #, preparation date and preparer's initials. Store at $2\text{--}8^\circ\text{C}$. Discard any unused stock in sink with running water after one (1) week.

14. Methanol, Analytical Reagent Grade

Store in OSHA-approved flammables safety cabinet. Date and initial bottle when opened. Cap tightly. Discard in hazardous waste container at manufacturer's expiration date. Mark date, type and concentration of compound, quantity discarded, and initials on tag attached to safety can.

15. Glacial Acetic Acid, Analytical Reagent Grade

Store in OSHA approved flammables safety cabinet. Date and initial bottle when opened and keep in laminar flow hood. Cap tightly. Discard in hazardous waste container at manufacturer's expiration date. Mark date, type and concentration of compound, quantity discarded, and initials on tag attached to safety can.

16. Fixative (3:1 Methanol–Acetic Acid)

CAUTION: Fixative is corrosive and flammable. Wear gloves, protective clothing and prepare in fume hood to avoid contact with skin and mucous membranes.

Measure 3 parts methanol (e.g., 75 mL) to 1 part glacial acetic acid (e.g., 25 mL) using a graduated cylinder. Store in a 100-mL bottle labeled as "3 : 1 Fix" with CORROSIVE/FLAMMABLE warning labels, preparation and expiration dates and preparer's initials. Keep tightly capped. Prepare fixative immediately before use. Use in laminar flow hood.

Discard daily in hazardous waste can. Mark date, type and concentration of compound, quantity discarded, and initials on tag attached to safety can.

Supplies

1. Coverslip Dishes
2. Sterile Fisherbrand® 100 × 15 mm Petri dish
3. Sterile scalpels
4. Graduated 15 mL conical Sarstedt centrifuge tubes
5. Sterile disposable 10 mL pipettes
6. 2.0 mL Nalgene® cryovials
7. Sterile Corning® 75 cm² tissue culture flasks
8. Sterile Corning® 50 mL conical centrifuge tubes
9. Sterile disposable 5-mL pipettes
10. Sterile disposable 1-mL pipettes
11. Solid Tumor Processing Worksheet
12. Sterile Falcon® 3023 Tissue Culture flasks
13. 35 mm × 10 mm sterile Petri plates

Equipment

14. AND balance
15. Precision waterbath
16. Whirlpool refrigerator
17. Forma incubator
18. Pipetman

19. Safety Cabinet
20. Hettick Rotina 35 centrifuge
21. Eppendorf pipettor

Quality Control

Follow your laboratory's Quality Control for Specimen Identification, Media Preparation, and Analysis of Culture Failures (see Note 1, Reagent QC testing).

III. Procedure

Cell culture and harvest

Caution: All specimens must be considered potentially infectious and therefore should be processed only in a laminar flow hood. Wear gloves and protective clothing during entire culturing and harvesting procedure.

Mechanical dissociation

1. Upon receipt, centrifuge tumor at 1000 RPM for 8 minutes. Remove all but 1 mL of media. Resuspend the pellet.
2. Place tumor tissue in sterile Petri dish. Gently mince tissue into 2–3 mm pieces using sterile scalpels. Remove fat and necrotic areas (see Note 2, Necrotic cells affect cultures) from tissue to be cultured. If tumor contains areas of differing morphology, these areas should be dissected apart and cultured separately.
3. Remove media from solid pieces of tissue and culture in BMC to be harvested at 24 hours.
4. Record required information on tumor processing sheet.

Enzymatic disaggregation

1. Thaw 2 mL of Collagenase II-S aliquot. Mix well.
2. Pour thawed collagenase into a 35 mm × 10 mm Petri plate. Label plate with patient's name and log number. Add a maximum of 2.5 cm³ of minced tumor to collagenase (see Note 3, Use of collagenase) using sterile 10-mL pipette (pieces should be small enough to fit through bore). Flush Petri dish with collagenase to insure all dissociated cells are removed.
3. Incubate at 37 °C in 5% CO₂ incubator until tissue is completely dissociated (approx. 2 hours: solution should turn an orange to yellow color with no or tiny pieces of tissue present). Periodically check progress.
4. Record required information on tumor processing sheet

Cell culture

1. Place Collagenase II-S/tumor suspension in a 15-mL sterile conical centrifuge tube.
2. Wrap tightly capped tube containing specimen and collagenase II-S in Parafilm M® and centrifuge at 1000 RPM for 8 minutes. Aseptically remove supernatant with a sterile pipet until ⅛ to ¼ inch supernatant remains above cell pellet. Try not to disturb cell pellet. Replace cap on centrifuge tube and evenly resuspend cells by gently flicking bottom of tube with finger. Make sure entire pellet is resuspended.
3. Inoculate one to four coverslip dishes with 1–2 drops of tumor suspension and one or two 25-mL flasks with approximately 10 drops of suspension. One-half of flasks are set up in Tumor RPMI and one-half in Tumor MEM. One-half of coverslips are set up in 2 mL of Tumor RPMI and one-half in 2 mL of Tumor MEM. If specimen appears to have low cellularity, gently apply tumor with 1 mL of media on the coverslip creating a dome, 2–4 hours later add 1 mL of media to coverslip dish.
4. Label coverslips/flasks with patient name, log number, and culture number (A, B, C, D, etc. for the coverslips).
5. Incubate cultures with caps loosened at 37 °C in 5% CO₂ incubator.
6. After 24 hours, add additional 1 mL of warmed tumor medium to flasks.
7. Monitor daily under inverted phase contrast microscope for cell growth and contamination (see Note 4, Contamination considerations). Record observations and required information on Tumor Processing Worksheet.

8. Aseptically replace tumor medium (i.e., feed flasks) when medium becomes orange/yellow and/or cell debris obstructs view of progressing cell attachment and colony growth. Gently pour off old medium, being careful not to disturb attached cells and colonies (see Note 5, Reseeding floating cells). Add 2 mL of fresh 37 °C appropriate tumor medium. Replace flask and coverslips in 37 °C, 5% CO₂ incubator until harvest.
9. If cells and/or colonies approach confluence, cultures can be split to re-initiate growth cycle (see Note 6, Harvest tumor cultures early).

Note: Cell culture for pediatric tumors (i.e., brain, small round cell, and neuroblastoma tumors) are to be harvested at 24 and 48 hours.

Harvest

Flasks

1. Flask(s) are harvested individually at staggered intervals, dependent upon time of maximum tumor cell growth and mitotic activity (see Note 6, Harvest tumor cultures early). Harvest flask when a high proportion of cells appear rounded, dark, three-dimensional, and actively dividing (see Note 7, Lengthening chromosomes). Record required information on Tumor Processing Worksheet.
2. Warm KCl to 37 °C.
3. Using Eppendorf® pipettor, add 40 µL of Colcemid® (0.08 mg/mL) to flask (see Note 8, Colcemid® time or concentration). Swirl gently to mix. Incubate with cap loosened for 3 hours in 37 °C, 5% CO₂ incubator.
4. Remove flask from incubator, pour off medium with Colcemid® into labeled 15 mL, conical centrifuge tube.
5. Add 0.5 mL trypsin-EDTA and place at 37° C for 1 minute. Monitor for detachment of cells using inverted scope.
6. Introduce a small amount of the poured off medium back into the flask to stop the action of the trypsin-EDTA.
7. Pour all the media from the flask back into the labeled 15 mL centrifuge tube. Centrifuge at 800 RPM for 8 minutes. Discard supernatant.
8. Add 5 mL warmed (37 °C) KCl to the centrifuge tube (see Note 9, Hypotonic effect). Slant tube at 45° angle in 37 °C incubator for 20 minutes.
9. Add 0.5 mL of 3:1 methanol: acetic acid fixative.
10. Wrap tightly capped tube with Parafilm "M" and centrifuge at 800 RPM for 8 minutes.
11. Aspirate supernatant using Pasteur pipet attached to vacuum aspirator assembly until 1/8-1/4 inch supernatant remains above cell pellet. Do not disturb pellet. *Caution:* Cell pellet may be invisible.
12. Gently and thoroughly resuspend pellet (see Note 10, Cell clumping after fixation). Slowly add 5 mL 3 : 1 fixative, evenly resuspending concentrated cells with constant gentle agitation. Place tube in refrigerator for 30 minutes.
13. Centrifuge at 800 RPM for 8 minutes.
14. Repeat step 11. Resuspend pellet. Slowly add 2 mL 3 : 1 fixative, evenly resuspending concentrated cell with constant gentle agitation.
15. Centrifuge. Refrigerate until ready for dropping.

Coverslips

1. Coverslips are harvested individually at staggered intervals, dependent upon time of maximum tumor cell growth and mitotic activity (see Note 6, Harvest tumor cultures early). Harvest when a high proportion of cells appear rounded, fluorescent, three-dimensional, and actively dividing. Record required information on Tumor Processing Worksheet.
2. Add 40 µL of Colcemid® (0.08 mg/mL) to each coverslip (see Note 8, Colcemid® time or concentration). Swirl gently to mix. Incubate at 37 °C for 3 hours.
3. Warm KCl to 37 °C.
4. Pipet off media and add 2 mL of prewarmed KCl for 23 minutes (see Note 9, Hypotonic effect). Allow to stand at room temperature.
5. Prefix, by adding 1 mL of 3 : 1 fixative dropwise. Wait 1 to 2 minutes before pipetting off KCl/fix solution.
6. Pipet off fix solution. Add 2 mL of fixative for 15 minutes.
7. Repeat step 6 above, 2–3 times.
8. Pull coverslip out of dish with forceps. Hold corners between your fingers, keeping the side that was facing up in the dish towards you. Alternate huffing and blowing until coverslip is pretty much dry (1–2 minutes). You should be able to see a rainbow sheen as it is drying.

9. Flip coverslip over onto a dry blank slide so that the coverslip is face down. Write the first 3 letters of the last name and the letter of the coverslip on the back side of the coverslip. Scan the coverslip looking for metaphases and document the appropriate information on the chart.
10. Place coverslip back in dish face up. Place in box and put in 90 °C oven for 1 hour to dry.

IV. Notes

1. **Reagent QC Testing.** Each prepared batch of culture media must be tested before being used on patient specimens.
 - a. Label flasks with the following information: name of reagent (e.g., “Tumor RPMI medium” or “L-glut”), batch number, preparation date, expiration date, storage requirements, and preparer’s initials.
 - b. Record the label information on the laboratory’s quality control log sheet, along with the lot numbers of all reagents that were used in the medium’s preparation. Expiration dates for each reagent should also be recorded if not accessible elsewhere. Note: Even though the anticipated expiration date for prepared medium is defined within laboratory protocol, this date must default to any individual component’s expiration date that expires prior to that date.
 - c. Each batch must be tested for sterility following laboratory protocol for quality control on medium (for suggestions, see Chapter 4, Protocol 4.2A, Note 1, Media Testing for sterility and growth support). New lots, or the same lot but a different shipping date, may also require additional testing to ensure that the new lot or batch supports cell growth, especially if shipping conditions could adversely affect the performance of that reagent.
2. **Necrotic cells affect cultures.** Unremoved necrotic areas of the specimen release deleterious toxins which can damage or render the remaining tissue nonviable.
3. **Use of collagenase.** The required duration for collagenase disaggregation is dependent on tumor type. Collagenase destroys the connective tissue without damaging the cells; therefore, prolonged incubation (≥ 20 hours) is safe for unusually fibrous specimens. Most tumors can be cultured after enzymatic disaggregation of about 2 hours in collagenase. Complete disaggregation is not always necessary.
4. **Contamination considerations.** Specimens not collected or cultured aseptically may be destroyed by bacterial or fungal overgrowth. If contamination occurs in one or all culture flasks, check your laboratory’s Quality Control procedure for analyzing culture contamination.
5. **Reseeding floating cells.** Floating cells in tumor cultures can be removed and used to initiate new cultures.
6. **Harvest tumor cultures early.** Tumor specimens are frequently a combination of the desired tumor cells and often indistinguishable non-neoplastic elements (e.g., connective tissue, organ tissue, fat, blood). Overgrowth by normal cell populations will obscure or mask the cytogenetic aberrations of the tumor. Cultures should be harvested as soon as sufficient tumor growth and mitotic activity is observed under the inverted microscope.
7. **Lengthening chromosomes.** Ethidium bromide, an intercalating agent, can be used to lengthen chromosomes.
8. **Colcemid® time or concentration.** Colcemid® time and/or concentration can be increased and/or decreased to arrest a maximum number of cells in metaphase. Longer exposures and higher concentrations will cause chromosomes to contract making analysis difficult or impossible.
9. **Hypotonic effect.** Hypotonic time can be lengthened to give metaphases better chromosome spreading and less cytoplasm. Shorter times can be used if breakage problems arise during slide making. Typical range is 10–30 minutes. Optimal exposure times will vary for different tumor types.
10. **Cell clumping after fixation.** Failure to resuspend cells thoroughly before hypotonic exposure or fixation may lead to irreversible cell clumping which can adversely alter chromosome morphology. Recap and slightly invert tube to mix, if necessary.

V. Additional readings

1. Fletcher JA, Kozakewich HP, Hoffer FA. Diagnostic relevance of clonal cytogenetic aberrations in malignant soft tissue tumors. *N Engl J Med* 1991;324:436–442.
2. Limon J, Dal Cin P, Sandberg AA. Application of long term collagenase disaggregation for the cytogenetic analysis of human solid tumors. *Cancer Genet Cyogenet* 1986;23:305–.
3. Trent J, Crickard K, Gibas Z, Goodacre A, Pathak S, Sandberg AA, Thompson F, Whang-Peng J, Wolman S. Methodologic advances in the cytogenetic analysis of human solid tumors. *Cancer Genet Cyogenet* 1986;19:57–66.
4. Thompson FH. Cytogenetic methodological approaches and findings in human solid tumors. In: Barch M (ed.). *The Association of Cytogenetic Technologists: Cytogenetics Laboratory Manual*, 2nd ed. 1991, pp. 454–463.
5. Lawce H. Cytogenetics of solid tumors: results of a survey. *Applied Cytogenetics* 1994;20(1):1–14.

Protocol 12.3 Solid tumor culture

Contributed by Children's Mercy Hospital, Clinical Laboratory Genetics, Kansas City, MO

I. Principle

Conventional cytogenetic analysis can only be performed on chromosomes captured in the metaphase or prometaphase stages of mitosis. Culturing of the living cells enables capture of the cells in these stages of mitosis and provides better morphology chromosomes for analysis than chromosomes obtained by direct harvest.

Specimen

Solid tumor material will be variable in source, amount, sterility, and type. It should be transported in tissue culture medium as soon as possible after removal. If delayed at an outside institution overnight, the sample should be refrigerated.

II. Materials

1. Complete alpha-MEM (prepared with 10% FBS, 10% Boehringer- Mannheim Conditioned Medium (BM Condimed), 1% L-glutamine)
2. Complete RPMI 1640 medium (as prepared for bone marrow culture)
3. P35 Petri dishes containing coverslips
4. T-12 or T-25 tissue culture flasks
5. Pipettes
6. 15-mL centrifuge tubes
7. Scalpel blades
8. Petri dishes
9. Collagenase (Type IV)
10. Alpha-MEM or RPMI without FBS or supplements
11. Complete alpha-MEM (used for all monolayer tumor except liver tumors)
 - 80 mL MEM
 - 10% FBS
 - 10% Boehringer-Mannheim Conditioned Medium (BM Condimed)
 - 1% L-glutamine
12. Complete RPMI 1640 with (filter sterilized) used for suspension tumors
 - 80 mL RPMI 1640
 - 10% FBS
 - 10% BM Condimed
 - 1% L-glutamine

** Complete RPMI prepared for bone marrow cultures may be used for tumors.
13. Complete alpha-MEM for Liver Tumors
 - 90% mL MEM
 - 10% FBS
 - 1% L-glutamine

III. Method

Procedure note

Tumors can be divided into small round cell tumors (SRCTs) that grow in suspension and others that grow as a monolayer attached to plastic or glass. For suspension culture, use complete RPMI; for monolayer culture, use complete MEM. If you are unsure which culture method is best for the tumor you are working with, consult the director.

1. Wear gloves at all times when handling fresh, unfixed tissue samples.
2. Initiate cultures as soon as possible after receipt of the sample.
3. Check the specimen label with the paperwork to assure the correct specimen has been received. Check and compare name and lab number from container and paperwork.

4. Read the intake sheet for information about the patient and disease process to help you select the best culture conditions. Note the following:
 - a. age of patient, previous therapy, history, or clinical information
 - b. tumor type and source
 - c. mitotic activity as seen on histology or pathologist's comments
5. If the sample is from a contaminated region of the body (e.g., bowel, nasopharynx), wash the specimen in antibiotic medium prior to mincing. Make up a solution of medium (RPMI or MEM without FBS) with 1% Fungizone (Amphotericin B), 1% Penicillin/Streptomycin, and 1% Gentamicin. In the laminar flow hood, transfer contaminated specimen to antibiotic medium and allow the sample to sit for 2-4 hours before mincing. Aspirate supernatant and proceed with processing of sample.
6. Place tissue in a P35 Petri dish with a coverslip using sterile technique. Mince and/or scrape the tissue piece(s) with 2 sterile scalpel blades until pieces are 1 mm or less. Using the inverted scope, look for tissue dissociation. Place tissue pieces in collagenase solution (0.5 mL Collagenase type IV in 1.5 mL of alpha-MEM) and place in 37 °C incubator until there is loosening of the peripheral cells on the tissue pieces. Leave in the collagenase until desired effect has been obtained. To facilitate disaggregation, aspirate cells and medium a few times with a pipette. For tumors that arrive late in the day and require collagenase treatment, mince as above, place in complete medium in the incubator and finish processing with collagenase in the morning of the next day.
7. Once tissue is disaggregated, pipet medium, tissue pieces and cells into a centrifuge tube; spin for 5 minutes. Aspirate and discard medium with collagenase in it. Add 2 mL medium to the dish and coverslip used for disaggregation, label as OT (original tumor dish), and place in incubator.
8. For tumors that grow as a monolayer, resuspend cells in complete MEM medium. The size of the cell pellet and the cellularity will determine the number of cultures initiated. A minimum of two cultures will be initiated on each specimen. Estimate the dilution by gauging the pellet size. Place 0.5 mL of diluted pellet on a coverslip and look at it with the inverted microscope to determine whether the dilution is appropriate. If it is too concentrated, aspirate the cells from the coverslip and return to the centrifuge tube for further dilution. Repeat until cell solution is appropriate for cell type. If you are unsure of optimal concentration, consult the director. Place some cells or remaining tissue pieces into a flask. Use minimal medium and distribute the cells over the bottom of the flask or dish. Label flasks and the top of P35 dishes with patient name, lab #, date, and culture #. Also label the bottom of the coverslip dish with the lab #. Place culture dishes in the incubator.
9. Flood cultures using 1.5 mL medium only after attachment is seen. After one or more days in culture, if there is excess tissue or debris, gently draw off the medium and replace with fresh medium. If additional cultures are needed, put the medium removed from the cultures into a flask for back-up. Observe the cultures each day for growth and mitotic activity. Change medium or add medium as needed to keep cells hydrated and healthy.
10. For suspension type cultures, divide the tumor cells (~0.2–0.4 mL of cell pellet per flask and add complete RPMI medium to a total of 10 mL per flask). Or you may count the cells and set up with approximately 2 million cells per mL medium.
11. Save the original tube with the specimen label. Place unused sample back in the original container with medium and store in the refrigerator. This will be discarded after the study is completed. Discard used blades, tissue culture dishes and pipettes appropriately. Clean work surface with a 10% Chlorox® solution followed by a 70% alcohol solution.
12. Check monolayer cultures daily for growth and contamination. Select cultures for harvest when mitotic activity is noted. Maintain cultures until adequate metaphase cells are obtained, and the case is finalized by the Director.

Protocol 12.4 Solid tumor harvest: monolayer and flask methods

Contributed by Children's Mercy Hospital, Clinical Laboratory Genetics, Kansas City, MO

I. Principle

To process living cell cultures to result in fixed, swollen metaphase cells that yield analyzable chromosome spreads when the cells are applied to glass slides.

II. Materials

1. Methanol-acetic acid (glacial) 3 : 1 mixture
2. 0.075 M KCl

3. Colcemid® (10 µg/mL)
4. Pipettes, sterile
5. 15-mL centrifuge tubes
6. Beaker for waste
7. Chlorox (10%)
8. Trypsin/EDTA (0.025%)
9. 0.8% sodium citrate
10. BrdU (3 mg/mL)
11. Stock Velban Solution

Calculations

1. 0.075 M KCl: mix 2.796 grams of KCl crystals in 500 mL deionized (type 1) water.
2. 0.8% sodium citrate: mix 2.0 grams of sodium citrate in 250 mL deionized (type 1) water.

QC

1. Observe sterile technique as harvested flasks are recultured in case a second harvest is necessary.
2. All procedures are carried out under the hood until cells are in the first fixative.

III. Method

Flask harvest – suspension procedure

Overnight harvesting method for a 5-mL flask culture

1. Add appropriate concentration of Colcemid® to each culture flask to be harvested, e.g., for full-strength Colcemid® use 50 µL of full strength (10 µg/mL) Colcemid®; for ½-strength Colcemid® use 25 µL; for ¼ strength use 12 µL.
2. Incubate overnight (11–14 hours) in 37 °C incubator with a humidified atmosphere of 5% CO₂.

Same-day harvesting method for a 5-mL flask culture

1. Add 50 µL of 10 µg/mL Colcemid® (full strength) to each culture flask to be harvested.
2. Incubate for 25 minutes in 37 °C incubator with a humidified atmosphere at 5% CO₂.
3. Discard medium. Rinse cell layer with 0.25% trypsin-EDTA solution (2–4 mL) and discard.
4. Add enough 0.25% trypsin-EDTA (0.5 to 1 mL) to cover cell layer in flask and treat until cells detach (1–2 minutes).
5. Add 3–4 mL complete culture medium, swirl to remove cells and transfer cell suspension to tube. Leave 0.5 to 1 mL of cell suspension in flask. Add up to 4 mL total complete culture medium to flask and return to incubator for regrowth.
6. Centrifuge harvested cells at 200g for 10 minutes.
7. Remove and discard supernatant, resuspend cell pellet and add 2–5 mL 37 °C hypotonic solution (1 : 1 KCl/sodium citrate). Place tubes in 37 °C incubator for 20–30 minutes.
8. Add 1 mL of fixative to cell suspension, gently mix, then centrifuge at 200g for 10 minutes.
9. Remove and discard supernatant. Resuspend cell pellet by tapping on the tube, then add 3 mL of fixative.
10. Centrifuge and discard supernatant. Resuspend pellet and add fresh fixative as before. Cells are (usually) adequately fixed after 2–3 fix changes as described above.
11. Following fixation, resuspend cells in enough fix to make a turbid suspension (typically about 0.3 mL). Follow slide-making procedure.
12. Bake slides at 65 °C overnight. Band according to banding procedure.

In situ (coverslip) harvest – monolayer procedure

Overnight harvesting method for a 2-mL coverslip culture

1. Add appropriate concentration of Colcemid® to each culture dish to be harvested, e.g., for full-strength Colcemid® use 20 µL of full strength (10 µg/mL) Colcemid®; for half-strength Colcemid® use 10 µL; for quarter-strength use 5 µL. Incubate overnight (11–14 hours) in 37 °C incubator with a humidified atmosphere of 5% CO₂ and 5% O₂.

Same-day harvesting method for a 2-mL coverslip culture

1. Add 20 µL of Colcemid® (full strength) to each culture dish to be harvested.
2. Incubate for 25 minutes in 37 °C incubator with a humidified atmosphere at 5% CO₂ and 5% O₂.
3. Remove medium and add 2 mL of 37 °C hypotonic (1 : 1 KCl/sodium citrate). Let sit at room temperature for 18–30 minutes; the time will need to be adjusted in different relative humidity conditions.
4. Add 1 mL of fresh fixative to the hypotonic in each dish. Let sit for 2 minutes.
5. Remove hypotonic and fix solution and replace with 2 mL of fix. Wipe condensation from inside of lid.
6. Remove fix and add 2 mL of fixative. Let sit for 20 minutes.
7. Remove fix and add 2 mL of fixative. Let sit for 10 minutes. Repeat once.
8. Following the last fixative step, the coverslip may be dried using either the controlled environment “Drying Chamber” (preferred) or on the hot plate (see Note 1, Slide-making tips).

Drying chamber

1. Turn “Drying Chamber” on at the beginning of the harvest to allow the chamber to equilibrate. Generally, the optimal conditions are with a temperature of 28 °C and humidity set at 55%. The fan blower setting should be at 60, and both fans turned on.
2. Initially, work with only one coverslip. After drying pattern has been established, then 2 coverslips may be dried at the same time.
3. Record the patient and coverslip number of the culture being dried in harvest book.
4. Aspirate fixative from inside dish by tipping and aspirating with pipette tip, then using the tip of the pipette, dry around the edge of the dish ~6 times to aspirate remaining fix. Next, aspirate with the tip of the pipette around the edge of the coverslip (all 4 edges) 3 times. Put lid back on and let sit ~30 seconds. Aspirate around coverslip again to remove any remaining fixative. Replace lid.
5. Put coverslip into drying chamber while humidity is rising. Remove lid from dish when the humidity reaches ~62% and close the door.
6. Aspirate the next dish as described in step 4.
7. Remove the first coverslip from the drying chamber when the coverslip is dry (humidity will be ~59%).
8. Examine this coverslip with the phase microscope to assess chromosome spreading. If spreading is good, continue. Otherwise, make adjustments as necessary.
9. Extract coverslip from dish and wipe excess fixative from back of coverslip.
10. Write last 2 digits of lab number on the noncell side in one corner of the coverslip and the culture symbol (T, etc.) in the opposite corner.
11. Place on 54 °C hot plate, cell side up, overnight. Place dish with lid belonging to coverslip over the coverslip.
12. If rapid aging is needed, place coverslips in 90 °C oven for 45 minutes. Never rapid age all coverslip cultures from the same patient.

Hot plate method

1. Remove the coverslip with scalpel and wipe excess fix from back of coverslip.
2. Hold coverslip over 37 °C slide warmer and let dry. Observe using phase contrast to evaluate spreading. Write last 2 digits of lab number in one corner of the coverslip and the culture label (T, etc.) in the opposite corner.
3. Replace coverslip in culture dish top which also has patient name and lab#.

IV. Notes

1. **Slide-making tips.** Room temperature of 26 °C and relative humidity of 50–60% seem optimum for good spreading. Sometimes waving the coverslips over the warming plate or gently blowing on the coverslips will improve the spreading. When the humidity is very low (10–30%), try drying the coverslips over damp paper towels.

Protocol 12.5 Solid tumor culturing and harvesting

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

Cytogenetic analysis of human solid tumors is clinically useful for the diagnosis and prognosis of many tumors. (1, 2, 3) The method involves sterile collection of the tumor in medium or saline, followed by enzymatic dissociation of the cells and culturing until ready to harvest. Appropriate culturing generally provides better chromosome morphology than direct harvest

methods. It is preferable to harvest cells within 10 days, if possible, to avoid clonal in vitro changes of abnormal karyotypes and to avoid harvesting normal supporting cells, which generally take longer to establish in the culture. The harvest method involves enzymatic removal of cells from the growth surface of the flask and swelling and fixing cells which have been arrested in metaphase with Colcemid®. Slides are made and cells are analyzed using trypsin G-banding, plus other appropriate stains, depending upon the clinical and cytogenetic findings.

Safety warnings

All tissue specimens should be handled as biohazardous, using Universal Precautions. Use the laminar flow hood for all steps up to harvest spin. Wear a laboratory coat and protective gloves for all steps through slide-making. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with fresh Sanimaster 4, made fresh weekly. Cover cuts with protective bandages even when gloves are worn. Dispose of Pasteur pipettes, needles and syringes in sharps container. Wash hands thoroughly after removing gloves.

Specimen

Ideally, tumor specimens of at least 1 cm² (up to 3 inches in diameter) are immediately collected with sterile methods into closable containers with sterile culture medium supplemented with serum and antibiotics (enough to cover specimen) and delivered to the laboratory within hours (up to a day or two later, but with decreased success) well protected from temperature extremes. If medium is not available, sterile Ringer's solution (second choice; either lactated or nonlactated) or sterile isotonic saline (3rd choice) is acceptable, although some compromise of viability will be likely, especially with prolonged contact.

Solid tumor samples should be selected from viable areas, with as little normal or necrotic material as possible. It is preferable to collect the specimen before initiation of chemotherapy in the patient. Fluid samples (e.g., ascitic or pleural fluid) do not require culture medium for collection, but should be collected and transported aseptically.

Unacceptable specimens are acellular, necrotic, septic, specimens in fixative or frozen without cryopreservatives, or specimens collected more than 2 weeks previously. The laboratory will log-in unacceptable specimens, notify physician of unacceptability, and send report as "study canceled."

Check specimen label assuring correct specimen. Retrieve clinical/pathology information. Follow laboratory protocol for accessioning specimen, e.g., log specimen into laboratory accession book and onto tumor flow sheet and log sheet.

Wear protective gloves and coat.

II. Materials

Materials for setup and culture

1. 35 mm² sterile plastic Petri dishes (Corning)
2. 1 mL TB syringes
3. Sterile scalpel handles and blades
4. Sterile forceps
5. Sterile plastic screw cap centrifuge tubes (Corning, any type)
6. Corning sterile 25 mm² tissue culture flasks (#25100)
7. Corning sterile 75 mm² tissue culture flasks (#430720)
8. Sterile, cotton-plugged Pasteur pipettes
9. 5 mL and 10 mL sterile graduated pipettes
10. Chang-C medium with Chang supplement, 0.1% gentamicin and 2% L-glutamine. Shelf life 10 days.
11. RPMI 1640, 15% FBS, with 0.1% gentamicin, 2% L-glutamine, and 1 % of insulin-transferin-selenium (ITS) added. Shelf life 14 days.
12. (Optional): Half RPMI 1640 supplemented with 15% FBS, 0.1% gentamicin and 2% L-glutamine, and half Chang-C complete. Shelf life 10–14 days.
13. Insulin–transferin–selenium (ITS), Gibco #41400-011, 100X. Store at 2 to 8 °C. Shelf life indefinite.
14. Trypan blue viability stain; mix the following:
0.4 g Trypan blue
0.81 g NaCl

0.06 g K₂HPO₄
95 mL H₂O

Heat to boiling until it is completely dissolved. Cool. Adjust pH to 7.2 with 1 N NaOH. Bring volume to 100 mL with water. Trypan blue may also be purchased from Gibco®, #15100-043. Indefinite shelf life.

15. Collagenase from either Sigma or Worthington, with Worthington preferred:
 - a. Worthington Type I Collagenase, #LS004196, 125–150 units/mg. Dilute with incomplete medium with 0.1% gentamicin to a final concentration of about 470 units per mL, rounding up to the closest multiple of 10 (e.g., for 142 units/mg, use 150 mL medium). Filter through a vacuum filtration unit with 0.2 µm pore size. Aliquot into 5-mL snap cap sterile tubes and freeze. Shelf life 1 year.
 - b. Stock Collagenase (Sigma, type IV-S, 435 units/mg, reconstituted with 5 mL sterile RPMI 1640 medium with antibiotics if needed but without supplements. Shelf life 1 month, refrigerated. Working solution is 0.25–0.3 mL of stock collagenase added to 5 mL incomplete RPMI medium (no serum added).

Materials for harvest

1. Test tube rack with a test tube for each Pasteur pipette.
 2. Pasteur pipette with amber bulb, 1 per flask.
 3. 1 and 5 mL sterile disposable plastic pipettes.
 4. Polypropylene centrifuge tubes with screw caps.
 5. Colcemid®, Gibco®, 10 µg/mL. Shelf life 1 year.
 6. 1× saline (BSS). Shelf life 6 months.
 7. Fresh trypsin-EDTA (2 mL of 10× Gibco® trypsin-EDTA diluted in 18 mL of 1× saline). Shelf life 1 week.
 8. 0.075 M KCl (0.559% in distilled water), prewarmed to 37 °C. Shelf life if stored at 4 °C is 6 months.
 9. Fixative: 3 : 1 methanol–acetic acid, freshly made before use. Shelf life is 1 day. Methanol is kept in flammables cabinet.
 10. Ethidium bromide, 10 µg/mL (Sigma #E1510, aqueous solution, 10 mg/mL). Dilute stock 1 mL in 9 mL Hanks' BSS. Store in foil-covered vacutainer in refrigerator. Shelf life 1 year. Use 0.05 mL per 5 mL culture.
 11. Amethopterin (Methotrexate, Lederle) stock = 0.8 mL Methotrexate + 4.4 mL sterile water. Working solution = 0.1 mL stock + 9.9 mL sterile water (10⁻⁵ M concentration). Use 0.05 mL/5 mL culture (10⁻⁷ M). Shelf life 1 year.
 12. Thymidine (Sigma #T-5018) 0.02 g in 80 mL Hanks' BSS (10⁻³ M). Filter sterilize with a 0.2 µm filtration unit. Aliquot in 20-mL aliquots into a T-12.5 blue-capped flask. Store at 4 °C. Shelf life 12 months. Use 0.1 mL (100 µL)/5 mL culture with Colcemid® for better mitotic index.
 13. BrdU (Sigma #B-5002), 25 mg in 10 mL RPMI (10⁻² M). Use 0.05 mL/5 mL culture. Shelf life 6 months.
 14. Caffeine (Sigma): 2 mM solution is 0.97 g/50 mL distilled water. Store at room temperature. Shelf life 6 months.
- Wear protective gloves and coat.

III. Method

Setup and culture

1. Place tumor in a Petri dish. Note size and appearance. Write GL# and patient name on Petri dish tops. Dissect any different-appearing portions apart with scalpel and forceps; keep separate. Write GL#, piece description (e.g., red, soft, etc.), and patient name on Petri tops.
2. Assess remaining transport medium for presence of any cells which have separated out; these may be centrifuged and seeded just as the collagenased cells are (see below no. 4), or used for a direct (unattached or suspension culture) harvest. Fluids (ascitic, pleural, bladder washings) are centrifuged, and seeded as in #4 below (see Note 1, Setting up lymph nodes).
3. Remove the tumor pieces from the dish with transport medium into a new Petri dish(es) for collagenase treatment. Use the Worthington collagenase as the primary enzyme, and the Sigma collagenase only in cases where there is plenty of material, and some of the tissue has been dissociated with the Worthington collagenase.

Worthington collagenase method: Defrost 4–5 mL of working collagenase. Pour into a Petri dish and mince tissue into small fragments. Incubate at 37 °C for 1–4 hours.

Sigma collagenase method: For each tissue portion (generally one or two), add 5.0 mL of unsupplemented RPMI and 0.25–0.3 mL stock collagenase to each Petri dish. Mince with sterile scalpel. Incubate 1–3 hours at 37 °C in a 5% CO₂

incubator. The tougher the tissue is, the more collagen it contains and the longer collagenase time will be required. Brain tissue does not have any collagen and will dissociate quickly from digestion by other proteases present in the collagenase.

4. When enzyme treatment is complete, tissues will appear loosely aggregated under the inverted microscope, and will pipet apart with a minute or two of trituration in a centrifuge tube with a 5–10 mL pipette. Use the last drop of cell suspension in the pipette to do a Trypan blue viability stain; add 1 drop cell suspension plus 1 drop (Pasteur pipette) of Trypan blue to a slide; coverslip and observe for viability, cellularity. Viable cells are clear, and dead cells appear blue. Use the estimated viability (e.g., 50%, 90% viable) to adjust the concentration of the culture inoculum. Caution: The presence of large numbers of red cells may be misleading, as they also exclude dye.
5. Centrifuge cells out of collagenase at 1000 RPM for 10 minutes. For each piece, set up 1–3 flasks. If sample is highly cellular, it is a good idea to set up one flask in a T-75 flask with a dilute cell inoculum to ensure that cells do not overgrow all cultures. If sample is very small, a T-12.5 flask may be used. Very tough specimens that are mostly collagen will have few cells compared to other tumors, and will need more cell inoculum.
6. Remove supernatant; resuspend $\frac{1}{4}$ –1 mL of inoculum in 1 or more mL of medium per flask. Usually some will be set up in Chang, some in RPMI, and if desired, some in half Chang, half RPMI. It is helpful to vary the cell concentrations from flask to flask. If T-25 flasks are used, add ~4 more mL of appropriate medium to the $\frac{1}{4}$ –1 mL of cell-medium suspension; gas with 5% CO₂, 5% O₂, 90% N₂ for 8–10 seconds; cap tightly; and incubate at 37 °C in a 5% CO₂ incubator. (Label on flask should include flask number, accession number, name, date, and medium used.)
7. Check daily for harvest (see Note 2, Removing dead or dying cells). Feed twice weekly by pouring off media, re-feeding with 5 mL of appropriate complete medium, gassing with 5% O₂, 5% CO₂, 90% N₂ briefly, and capping tightly. Subculture only if necessary, to get cells at an ideal density for harvest or freezing.

Harvest

Direct or suspension harvests

(See Note 3, When a direct or suspension harvest is appropriate.)

1. Add 15 µL of Colcemid® (10 µg/mL stock solution) to each 5 mL of suspension culture. Incubate for 1 hour to overnight at 37 °C.
2. Add cells to be harvested to centrifuge tube, if needed, and centrifuge 10 minutes at 1000 RPM.
3. Remove supernatant to $\frac{1}{4}$ mL. Pipet to resuspend.
4. Add 2–5 mL of warm KCl. Let tube sit at room temperature for 15–20 minutes.
5. Add 4–5 drops of fresh fixative to the hypotonic. Centrifuge as before.
6. Remove supernatant to $\frac{1}{4}$ mL. Pipet to resuspend. Add 5–6 drops fixative, mix, and add up to 2–5 mL of fixative. Place in freezer for 20 minutes to overnight.
7. Centrifuge cells, remove supernatant, and make slides using fresh fixative.

Cultured cell harvests

After attached cells have grown for several days (the time is tumor and patient-specific and ranges from 3 to 15 days), mitotic cells will become more frequent as the cultures approach log-phase division. When sufficient mitotic figures are seen on the tissue culture microscope, a harvest is attempted. If cells continue to increase in number but no mitotic figures are evident, the cells are probably dividing during the night, and harvest may be attempted with an overnight Colcemid® exposure, even in the absence of mitotic cell types.

Note: Harvest method steps 1–7 are carried out by sterile technique in a flow hood.

1. When at least 25% of the flask growth-surface is covered with cells displaying mitotic activity, add 15 µL of Colcemid® to each 5 mL culture. Incubate at 37 °C for 3 hours to overnight.
2. Check cells on phase scope to determine success in mitotic arrest; look for an increase in rounded up, refractile cells, and large cells held to the flask surface by threads which have a visible metaphase plate on high power.
3. Pour medium into centrifuge tube.
4. Rinse 2 times with 2 mL of Hanks' BSS, adding rinse to centrifuge tube.
5. Add 1 mL trypsin-EDTA to flask and incubate 3–5 minutes at 37 °C. Monitor cell detachment microscopically. Rap flask on benchtop to encourage cell detachment.
6. When cells are detached, pipet a few times with a 1-mL pipette to separate cells, and add suspension to tube. Re-feed and gas flask; re-incubate for further growth.

7. Centrifuge tubes at 1000 RPM for 10 minutes. Remove supernate. Resuspend gently but thoroughly with Pasteur pipette.
8. Add the initial 3 drops of warm KCl; pipet well to mix. Repeat and then add 3 mL of KCl, pipet, let stand (room temperature) for 15 minutes.
9. Add 3–5 drops fresh 3 : 1 fixative, pipet to mix, and centrifuge as before.
10. Remove supernatant. Resuspend gently but thoroughly with pipette. Add 3 drops fixative, pipet. Repeat 2–3 times. Add 3 mL fixative and let stand 10 minutes minimum.
11. Centrifuge cells and place in freezer for 20 minutes to overnight.
12. Centrifuge cells, remove supernatant, and make slides using fresh fixative.
13. Slides are made as usual, baked at 90–95 °C for 20 minutes, and G-banded with trypsin (see Note 4, If the number of metaphases is insufficient). Trypsin time is usually short (about $\frac{1}{2}$ of time for blood slides). Additional stains may be used as needed.

Variations on the standard harvest

1. For longer chromosomes, add 0.05 mL of ethidium bromide per 5 mL culture for 45 minutes, then Colcemid® (25 μ L) for 45 minutes. Harvest as usual. This will result in a lower mitotic index than the standard harvest, but can be useful for clarification of breakpoints, etc.
2. For better banding, or for cultures that either divide very slowly or have no or few abnormal cells, synchronize 5-mL cultures overnight with 0.05 mL of methotrexate (amethopterin) at 4:30–5:00 p.m. At 8:30–9:00 a.m. the following day, add 0.1 mL of thymidine, incubate 6–7 hours, with Colcemid® (15 μ L) the last 3 hours. Harvest as usual.
3. For problem specimens, use Colcemid® overnight (even up to 2–3 days) to help collect enough abnormal cells to perform a study. At 4:00–5:00 p.m., add 15 μ L Colcemid® to each 5 mL culture. Swirl and incubate overnight. Harvest in the morning (or later in the day) as usual. If cultures do not have sufficient metaphase figures on phase microscope after overnight Colcemid®, try waiting until the following morning (48 hours) or later to harvest cultures.
4. For cultures that will not divide, try adding 0.1 mL of 10^{-3} M thymidine solution along with the Colcemid® to help cells enter mitosis.
5. For cultures that will not divide even with thymidine, try adding caffeine (0.1 mL of 2 mM caffeine per 5 mL culture) with the Colcemid®.
6. For cultures that will not divide even with thymidine or caffeine, add both thymidine and caffeine with the Colcemid®.

Chromosome analysis for solid tumors

1. Usually, at least 2 different cultures are set up, harvested and analyzed using G-banding (if a suspension 24-hour culture is available, both attached and suspension cultures should be analyzed). If only one culture is available, all 20 cells may be from a single flask.
2. All 20 metaphases are usually photographed (or digitally captured), and a minimum of three are karyotyped. If multiple clones are present, prepare two karyograms per clone, e.g., Solid tumor chromosome analysis
Number of cells:
Counted 20
Analyzed 5–20
Photographed 20
Karyotyped 3+
- Usually, at least two different cultures are harvested and analyzed. If only one culture is available, all 20 cells may be from a single flask.
3. Additional staining methods, such as Q-bands, C-bands, fluorescent in situ hybridization (FISH) and others, may be used to further identify tumor karyotypes. Perform FISH for n-myc on all neuroblastomas, as well as FISH for the 1p36 deletion in most neuroblastomas.

IV. Notes

1. **Setting up Lymph Nodes.** Lymph node specimens can represent solid tumors that have metastasized to node, or they can be referred to the laboratory as a lymphoma or leukemia. This means that, in absence of a reason for referral, they should be set up both as attached and suspension cultures to cover both possibilities.

2. **Removing Dead or Dying Cells.** The presence of many dead and dying cells, or even too many viable cells, is undesirable. Medium may be removed to a new flask to allow continued attachment and the old flask may be fed at day 1 or 2. Subculturing may be performed if growth in flask is too heavy.
3. **When a Direct or Suspension Harvest is Appropriate.** Some tumors are actively dividing when they arrive in the laboratory, and while the quality of the harvest is generally better after cells have attached and grown for 3–7 days, a quick answer derived from a direct or overnight suspension culture can save labor and aid turnaround time, especially important with pediatric tumors. Furthermore, many small round blue cell tumors and other types will fail to grow attached, and the only way to get a result is the immediate harvest.

The rules of thumb for when to do a suspension culture are:

- All small round blue cell tumors, especially neuroblastomas, unless they are too tough to get cells out without collagenase.
- Any tumor that is friable and sheds viable cells into the transport medium.
- Any tumor on which the pathologist reports a high mitotic index.

Hint: If cells are not shedding into transport medium and a direct harvest needs to be undertaken, add 15 µL of stock Colcemid® to the collagenase and harvest some of the released cells at the end of the incubation time (1–3 hours or overnight). Or, set up attached-type cultures as usual and add Colcemid® to any floating cells within the first 2–3 days of culture.

4. **If the Number of Metaphases is Insufficient.** If insufficient metaphases are obtained for analysis, repeat harvest from another culture. Longer Colcemid® times may help with a low mitotic index (4 hours to overnight).

V. Additional readings

1. Thompson FH. Cytogenetic methods and findings in human solid tumors. In Barch M, Knutsen T, Spurbeck J, eds. *AGT Cytogenetics Laboratory Manual*, 3rd ed. 1997. NY: Raven Press; 375–430.
2. Heim S, Mitelman F. *Cancer Cytogenetics*. 1995. New York: Wiley-Liss.
3. Mitelman F, ed. *Catalog of Chromosome Aberrations in Cancer*, 5th ed. 1994. NY: Wiley-Liss.

CHAPTER 13

Chromosome instability syndromes

Yassmine Akkari

Cytogenetics, Molecular Pathology, FISH and Legacy Health, Portland, OR, USA

13.1 Introduction

Chromosome instability syndromes are a group of mostly recessive disorders characterized by an increased frequency of spontaneous or induced chromosomal changes leading to breakage and neoplasia. Under normal physiological conditions, DNA is continuously being damaged. Examples of such damage include the spontaneous deamination of bases, oxidation of nucleotides, and strand breaks by reactive oxygen species (ROS). These errors may be due to normal metabolism, environmental stress, or replication errors resulting from mismatched nucleotides. Maintenance of genome stability is vital for all living organisms, and its pathways are highly conserved from yeast to mammals (see Figure 13.1). Sustained genomic integrity requires repair of these DNA lesions through a multitude of DNA repair pathways that are temporally and spatially regulated and requires appropriate responses to DNA alterations. DNA lesions that cause chromosome breakage are mainly double-strand breaks, stemming primarily from defects in recombinational repair. Compared to single-strand breaks, double-strand breaks are a problematic form of DNA damage because of the lack of a direct template to repair them. They may be caused by external agents, such as ionizing radiation and chemical agents, or internal agents, such as recombination in meiosis, recombination in the T-cell receptor and immunoglobulin genes in T- and B-cells, or simply maintenance of the replication fork. Telomere shortening can also be considered an example of double-strand breaks. Repair of double-strand breaks is performed by cells at a low level at all times. They are repaired by error-free recombination repair called homologous recombination (HR) or error-prone nonhomologous end-joining repair (NEJR) [1]. Many proteins involved in these repair pathways (such as *BRCA2* and *ATM*) have been shown to be mutated in human breakage syndromes and cancer [2].

Rare pediatric chromosome instability syndromes, such as Fanconi anemia and ataxia–telangiectasia (A-T), have provided insights into the function of the various DNA repair pathways. Children born with these disorders generally have multiple congenital anomalies, cellular sensitivity to DNA damaging agents, and a predisposition to certain cancers, sometimes even in carrier family members of affected individuals. In fact, a direct genetic link between chromosome instability syndromes and cancer predisposition first came from the discovery that a biallelic inactivation of one of the genes mutated in familial breast cancer, *BRCA2*, causes Fanconi anemia [3].

Two levels of genetic instability have recently been characterized in human cancers: small sequence changes observed at the nucleotide level [Microsatellite Instability (MIN) and Nucleotide Instability (NIN)], and instability that is visible at the chromosome level [4]. The latter describes a variety of chromosome aberrations that can be numerical and structural. Deregulation of genes implicated in normal chromosome segregation or cell cycle checkpoint controls can cause numerical changes such as aneuploidy or polyploidy. Chromosome breaks and telomere dysfunction can result in various structural rearrangements, such as deletions, duplications, and inversions. Impairment of DNA repair, replication, or recombination, however, is responsible for causing sister chromatid exchanges, fragile sites, chromatid breaks, aberrant associations (such as radials), and mutagen sensitivity. Disorders that are associated with such impairments and are amenable to cytogenetic diagnoses are the subject of this chapter.

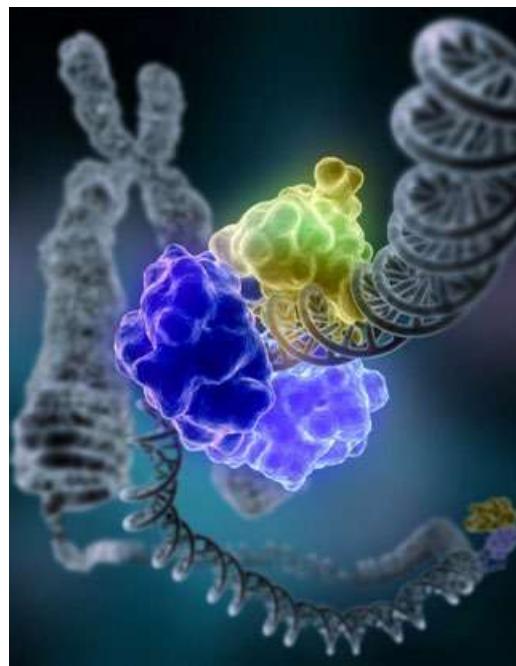


Figure 13.1 DNA repair. A special enzyme encircles the double helix to repair a broken strand of DNA. Without molecules that can mend such breaks, cells can malfunction, die, or become cancerous. Courtesy of Tom Ellenberger, Washington University School of Medicine. See insert for color representation of this figure.

In contrast to chromosome breakage, chromosomal fragile sites appear as nonstaining chromosome gaps, breaks, and endoreduplicated segments on metaphase preparations. With the exception of *FRAXA* and *FRAXE* (two well-known causes of familial intellectual disability associated with Fragile X syndrome) and possibly *FRA11B* associated with Jacobsen syndrome, fragile sites have not been shown to predispose to any heritable chromosome abnormality or malignancy [5]. Because of its diagnostic limitations, cytogenetic studies are no longer performed for the detection of clinically relevant fragile sites. Instead, diagnosis relies on molecular methodologies and will, therefore, not be discussed in this chapter.

13.1.1 Cytogenetics versus molecular diagnosis

Genes mutated in several chromosome breakage syndromes have been cloned, and their protein products characterized. For some breakage syndromes, molecular diagnosis of gene mutations has replaced cytogenetic analysis of chromosome breakage after damage induction for determining presence or absence of disease.

Several human chromosome breakage syndromes, including Fanconi anemia and Bloom syndrome, have long been known to cytogeneticists. The best way to diagnose these syndromes is dictated by many factors. First, it requires an understanding of the clinical aspect of any disease on the cellular or molecular level, in order to develop a diagnostic laboratory test. Second, the cost of such test should be amenable to the majority of health care providers. Third, the time by which the results of the test are available plays an important role in the choice of diagnostic method. Most of the time, the choice is well established and accepted amongst the different providers. For example, at the time of writing this chapter, there were 13 mutated genes identified in association with Fanconi anemia; yet at the cellular level, all complementation groups are sensitive to crosslinking agents. Therefore, a test to challenge cells with a crosslinking chemical could be used for any of the mutations. The algorithm currently agreed upon for Fanconi anemia testing relies on cytogenetic breakage testing of peripheral blood and/or skin fibroblasts to test for the cellular hallmarks of the disease. Subsequently, various molecular assays are employed to classify patients into the appropriate complementation groups, especially for potential prenatal testing.

In the following sections, chromosomal breakage syndromes that are relevant to the cytogenetics laboratory, and their subsequent diagnosis, are described (Table 13.1). A detailed description of the cellular and molecular aspect of each disease is also beyond the scope of this chapter. For further investigation, the interested reader can refer to the sources in the reference section for further reading.

Table 13.1 List of diseases discussed in this chapter: their genes, protein products, chromosomal locations, and cytogenetic manifestations

Genetic condition	Clinical manifestations	Gene(s) cloned	Protein product(s) and known function	Chromosomal location(s)	Cytogenetic diagnosis
Fanconi anemia	Multiple congenital anomalies, bone marrow failure, increased susceptibility to neoplasia	<i>FANCA</i> , <i>B</i> , <i>C</i> , <i>D1</i> , <i>D2</i> , <i>E</i> , <i>F</i> , <i>G</i> , <i>I</i> , <i>J</i> , <i>L</i> , <i>M</i> , <i>N</i>	<i>FANCA</i> , <i>B</i> , <i>C</i> , <i>D1</i> (<i>BRCA2</i>), <i>D2</i> , <i>E</i> , <i>F</i> , <i>G</i> , <i>I</i> , <i>J</i> , <i>L</i> , <i>M</i> , <i>N</i>	16q24, Xp22, 9q22, 13q12, 3p25, 6p22, 11p15, 9p13, 15q25, 17q22, 2p16, 14q21, 16p12 (respectively)	Breakage analysis: Increased incidence of chromosome breaks and radials following exposure to crosslinking agents
Ataxia telangiectasia	Cerebellar dysfunction, immunodeficiency, telangiectases, increased susceptibility to neoplasia	<i>ATM</i> (Ataxia-Telangiectasia Mutated)	ATM (kinase)	11q22	Chromosome 7 and 14 structural changes and increased chromosomal breakage following gamma irradiation
Nijmegen breakage syndrome	Microcephaly, recurrent infections, increased susceptibility to neoplasia	<i>NBN</i> (formerly <i>NBS1</i>)	Nibrin	8q21	Chromosome 7 and 14 structural changes and increased chromosomal breakage following gamma irradiation
Bloom syndrome	Short stature, skin erythema, sun sensitivity, increased susceptibility to neoplasia	<i>BLM</i>	BLM (DNA helicase RecQ protein-like-3)	15q26	Sister chromatid exchange
Immunodeficiency, centromeric instability, facial anomalies syndrome	Immunodeficiency, facial dysmorphisms	<i>DNMT3B</i>	DNMT3B (DNA methyltransferase)	20q11	Centromeric instability, especially chromosomes 1 and 16, and less often 9, 2, and 19
Roberts syndrome	Phocomelia, craniofacial anomalies, severe growth restriction	<i>ESCO2</i>	ESCO2 (sister chromatin cohesion protein)	8p21	Premature centromere separation
Werner syndrome	Scleroderma-like skin changes, subcutaneous calcifications, prematurely aged facies, increased susceptibility to neoplasia	<i>WRN</i> (also <i>RECQL2</i>)	WRN (RECQL DNA helicase)	8p12	“Variegated translocation mosaicism”
Rothmund-Thomson syndrome	Poikiloderma, skeletal anomalies, increased incidence of neoplasia	<i>RECQL4</i>	RECQL4 (DNA helicase)	8q24	Mosaicism for chromosomal abnormalities in fibroblasts and lymphoblasts

13.2 Fanconi anemia

Fanconi anemia (FA) is a rare recessive disorder (estimated incidence at 1 in 360,000 live births) that belongs to two classes: the diseases of DNA repair and the inherited bone marrow failure syndromes [6]. FA is characterized by developmental and hematological abnormalities and cancer predisposition. Clinical features are often heterogeneous, and several body systems may be affected. Approximately 25% have no congenital anomalies. Altered growth can be present, both in utero and postnatally, and is manifested by low birth weight and median height, often in the 5th percentile [7]. This may be due to growth hormone deficiency or hypothyroidism, which can further impair growth. In addition, microcephaly, microphthalmia, and global developmental delay are often present. Skeletal findings mostly include radial ray defects (hypoplasia of the thumbs and radial hypoplasia), and less commonly, congenital hip dislocation, scoliosis, and vertebral anomalies. Pigmentary changes are often present and include skin hyper- and hypopigmentation and typical café-au-lait spots. Conductive hearing loss can also occur, and may or may not be associated with external ear malformation. Renal defects are present in one third of FA patients, and may include unilateral renal aplasia, renal hypoplasia, horseshoe kidneys, and double ureters. Genital anomalies are common in FA, and include hypogenitalia, hypospadias, undescended testes and infertility in males. Females may also have underdeveloped genitalia, uterine abnormalities and irregular menses. The ability to reproduce, however, has been documented in several FA patients [8]. Cardiac defects have been documented in FA although to a lesser extent [9]. These include patent ductus arteriosus, ventricular septal defects, and pulmonary stenosis. Even less common are gastrointestinal defects such as atresia, and central nervous system abnormalities.

FA is the most common inherited bone marrow disorder. The ensuing hematological problems are by far the most serious clinical implications of FA. At birth, the blood count is usually normal, followed initially by macrocytosis. Often, the patient presents with pallor and recurrent infections, at which point hematological findings usually include thrombocytopenia and neutropenia, leading later on to pancytopenia [7]. This is usually followed by clonal cytogenetic abnormalities suggestive of myelodysplastic syndrome (MDS) or acute myelogenous leukemia (AML) in bone marrow specimens (see Chapter 11, section 11.3, Pediatric MDS). Although some FA patients are placed on androgen therapy to improve their anemia, bone marrow transplantation (BMT) from an HLA-matched donor is the only effective therapy. Unfortunately, rigid conditioning regimens for preparing the patient for the BMT are not well tolerated by FA patients and complications usually occur. It is, therefore, critical to make the diagnosis of FA before transplantation regimens are undertaken so that appropriate changes in the protocols can be made.

The cancer susceptibility aspect of FA is related to the inability to maintain the integrity of the genome (see later), leading to a high degree of chromosomal instability. The most common malignancy in FA is MDS, usually leading to AML. Common cytogenetic abnormalities seen in the bone marrow of these patients are monosomy 7 [10–12], 5q– [13–15], 7q– [16] 20q– [13], trisomy 8 [13, 17], and translocations and rearrangements involving chromosomes 1 [18, 19] and 3 [20].

FA has also been associated with nonhematologic tumors, particularly squamous cell carcinomas of the head and neck [21]. The most common sites of these tumors are the aerodigestive and anogenital tracts. Other tumors in FA include liver-related tumors such as hepatocellular carcinomas, hepatic adenomas, and focal nodular hyperplasia of the liver. Most of these tumors have been exclusively observed in FA patients on androgen therapy, are usually androgen dependent [22–25], and may regress after cessation of treatment. In addition, several groups have recently reported the occurrence of medulloblastoma and Wilms tumor (see Chapter 12, sections 12.3.6, Medulloblastoma and 12.3.12, Wilms tumor) in young children with FA [26, 27].

FA has proven to be a heterogeneous disorder both clinically and genetically (currently 13 FA genes have been identified: *A, B, C, D1/BRCA2, D2, E, F, G, I, J, L, M* and *N/PABL2* [28]). A unifying concept emerged several decades ago showing that cells from FA patients show a typical hypersensitivity to alkylating agents that cause DNA interstrand crosslinks (ICLs) [29]. These agents include mitomycin C (MMC), diepoxybutane (DEB), cisplatin, and photoactivated psoralens. Although their mode of action *in vivo* may vary [30–32], they all have the ability, under different conditions, to induce a covalent bond between the two DNA strands, causing an obstacle to DNA replication [33].

13.2.1 Cytogenetic diagnosis of Fanconi anemia

Historically, the FA breakage test has relied on the use of DEB for the induction of DNA interstrand crosslinks [34]. In this setting, 50 or 100 stained metaphases are scored for both chromosome number and increased spontaneous and DEB-induced breakage and rearrangements. Many laboratories, however, use both MMC and DEB in order to meet the need to confirm positive or negative findings, especially with the scarcity of positive controls [35]. Consistent results from two different clastogens allow a better confirmation of the diagnosis.

Both drugs, however, have several advantages and disadvantages. DEB has been recognized as a milder clastogen, yielding enough metaphases for analysis, but it has a very short shelf life and needs to be prepared daily. MMC, on the other hand, can

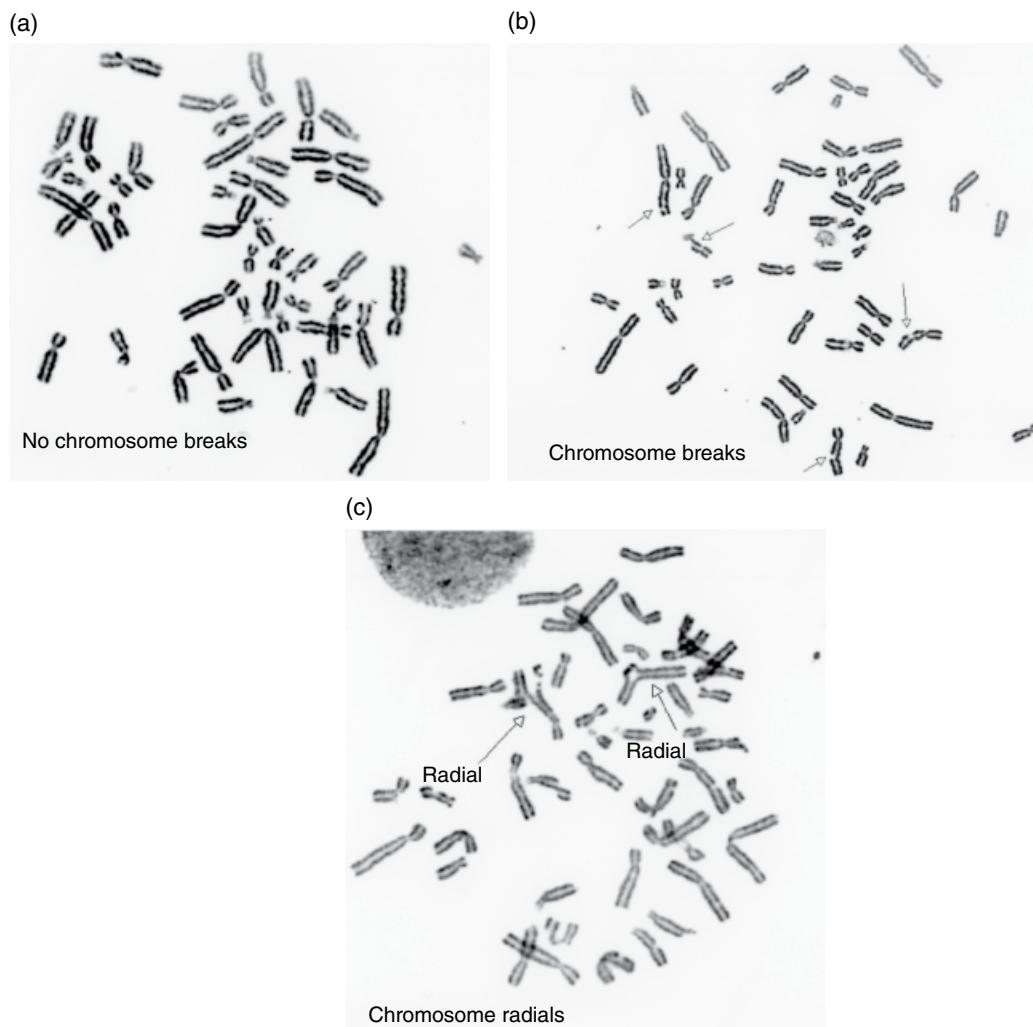


Figure 13.2 Cytogenetic diagnosis of FA. (a) Peripheral blood metaphase from a normal control pool in the presence of MMC. (b) Peripheral blood metaphase from an FA patient in the presence of MMC. (c) Peripheral blood metaphase from an FA patient in the presence of DEB. Arrows point to breaks and radials.

be stored for up to six months at 4°C [36], but is a stronger clastogen. Some protocols, therefore, use DEB and MMC on every patient, as well as a no-clastogen control culture. In addition, the use of two MMC concentrations allows the ability to retrieve information on the more sensitive cells (Figure 13.2).

Many chromosome instability syndromes may display extensive breakage when challenged with clastogens that induce double-strand breaks and hence, chromosomal damage. Therefore, there was a need to distinguish between these diseases, based upon variations in their cellular phenotype. For FA, it may be more accurate to score both the number of breaks and the number of radial formations per cell in order to reach the proper diagnosis. The term radial is used to describe any chromosomal figures with inter-chromatid interactions, most of the time involving two chromosomes. The ensuing aberrant joining of chromatids following breaks occurs almost exclusively between nonhomologous chromosomes [37]. Since the increased formation of radials seems to be fairly specific to FA cells exposed to MMC and/or DEB, these figures are adopted as the endpoint for the diagnosis of FA cells, in addition to the frequency of breaks per cell. However, with the emergence of data regarding increased chromosome breakage following treatment of cells from other chromosome instability syndromes with DNA crosslinking agents [38], it is crucial to document the clinical features and family history of all patients being tested for FA. It is also important to note that some laboratories use DEB only in their breakage testing, and document the average number of breaks/cell as their endpoint.

13.2.2 Somatic mosaicism in Fanconi anemia

The increased genomic instability in FA allows for reversion mutations to accumulate in a proportion of the patient's blood, leading in some cases to a milder phenotype or complete hematological remission [39]. The cloning of some of the FA genes has allowed the molecular characterization of the reversion event, and has revealed several mechanisms, including, among others, gene conversion, intragenic recombination, and accumulation of mutations in CpG hotspots [40–42]. Although somatic reversion in the blood cells of FA patients can correct the hematologic manifestations, it does not, unfortunately, allow the patient to escape predisposition to malignancies. Indeed, occasionally, these patients are diagnosed later in life because of the development of rare squamous cell carcinomas of the head and neck, a category of malignancies that is rare in the general population but common in FA, reviewed in [43]. In these instances, if breakage analysis of a blood sample is normal, despite a strong clinical suspicion for FA, a skin biopsy is recommended for breakage analysis of fibroblasts.

Early diagnosis of FA can allow regular monitoring for anemia, MDS/AML, and solid tumors. Because diagnosis of FA based solely on clinical features can be difficult, clinicians have relied on the cytogenetic test of blood, bone marrow, or skin cells from FA patients based on their hypersensitivity to DNA crosslinking agents. This hypersensitivity is illustrated by an inability to repair the DNA crosslinks resulting most likely in double-strand breaks. (A detailed protocol for breakage analysis is included in the Contributed methods section at the end of this chapter.)

The following is an algorithm for FA testing: First, the patient's clinical features are documented, and suspicion for FA is established. Second, a blood sample is sent to a cytogenetics laboratory for breakage testing. If positive, a skin biopsy is obtained to establish a fibroblast cell line to be used for complementation group assignment. Other alternative methods, such as protein analysis can also be used to assign complementation groups [44]. If the gene for the identified complementation group has been cloned, sequencing is undertaken to identify the deleterious mutations. This information is subsequently used for carrier testing and prenatal diagnosis. Prenatal diagnosis using breakage analysis of amniocytes or chorionic villi sampling is offered in many laboratories, but remains controversial, mainly due to the absence of appropriate controls for quality assurance.

13.3 Bloom syndrome

Bloom syndrome is a rare autosomal recessive disorder (incidence estimated at 1 in 1,000,000 live births) characterized by short stature, immunodeficiency, sun sensitivity, facial erythema, and increased predisposition to cancer. Cells from Bloom syndrome patients show chromosomal instability illustrated by chromosomal breaks, radial formation, and an increase in the incidence of sister chromatid exchange (SCE) [45]. This latter cellular manifestation is the most characteristic and consistent cytogenetic feature of Bloom syndrome, and is used for cellular diagnosis. SCEs appear as sharp demarcations in the intensity of staining along a chromatid, with the opposite staining pattern on the adjacent chromatid (see Chapter 6, Figure 6.24). In a laboratory setting, induction of SCEs requires two rounds of replication in the presence of BrdU. Normal controls will present with 5–10 SCEs per cell, whereas individuals with Bloom syndrome would have 100+ SCEs per cell.

The protein mutated in Bloom syndrome is BLM, a 3' to 5' RecQ helicase, that functions in homologous recombination or recombination during replication, and is likely to be able to bypass a DNA adduct during S phase [46, 47]. Although a polymerase chain reaction (PCR)-based molecular diagnostic test is available, SCE is still the most distinctive marker for this disease (see Protocol 12.4, Fanconi anemia chromosome breakage analysis policy for a detailed protocol on sister chromatid exchange analysis). Currently, clinical diagnosis relies on both cytogenetic and molecular genetic diagnoses. The latter offers a targeted mutation analysis to identify a disease-causing mutation found mainly in the Ashkenazi Jewish population (2281 delta 6ins7) [48]. Carrier testing relies on molecular techniques, whereas prenatal diagnosis may be performed cytogenetically.

13.4 Ataxia-telangiectasia

A-T is an autosomal recessive disease (incidence estimated at 1 in 100,000 live births) characterized by a progressive neurological degeneration secondary to cerebellar dysfunction. Symptoms typically present between one and four years of age, and include ataxia of the upper and lower limbs, oculomotor apraxia, deficiency in cell-mediated immunity rendering A-T patients susceptible to infection, and choreoathetosis. In addition, A-T patients also show thymic hypoplasia, high serum alpha-fetoprotein concentration, growth restriction, and telangiectases of different parts of the body, particularly the bulbar conjunctiva [49]. Another important feature of this disease is the sensitivity of A-T patients to ionizing radiation, and their very high risk to develop cancer, principally leukemia and lymphoid tumors [50, 51].

The diagnosis of A-T relies on clinical findings, including slurred speech, truncal ataxia, oculomotor apraxia, family history, neuroimaging, and the presence of telangiectases. Testing to support the diagnosis includes serum alpha-fetoprotein,

found to be elevated in ~95% of A-T patients, identification of balanced rearrangements involving chromosomes 7 and 14 on routine karyotype, and in vitro radiosensitivity. Molecular genetic testing is complicated by the large size of the *ATM* gene, together with the wide distribution and diversity of mutations found in A-T patients. However, sequence analysis of the *ATM* coding region is available on a clinical basis. Sequencing detects about 95% of *ATM* sequence alterations, but misses intronic mutations and heterozygous deletions. *ATM* functions as a kinase that is essential for the G1 to S cell cycle checkpoint and phosphorylates p53 and other proteins in response to ionizing radiation [52].

13.5 Nijmegen breakage syndrome

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disease, with an estimated prevalence of 1 in 100,000 live births (more common in Eastern Europeans/Slavic populations) [53] characterized by growth deficiency, “bird-like” facies, progressive microcephaly, and mild to moderate intellectual disability [54]. Patients with NBS are prone to respiratory infections, malignancies, especially B-cell lymphomas, and premature ovarian failure [55]. Previously thought to be a variant of A-T because of similar cytogenetic and cellular features, Nijmegen breakage syndrome (NBS) is now known to be caused by mutations in the Nibrin (*NBN*) gene, and to be a distinct clinical entity from A-T. Although NBS patients have neither ataxia nor telangiectases, both diseases have indistinguishable cytogenetic and cellular features. Moreover, several lines of evidence have shown that *NBN* and *ATM* function in the same pathway in response to DNA damage induced by ionizing radiation [56]. *NBN*, formerly known as *NBS1*, seems to be phosphorylated by *ATM* and forms nuclear foci with Mre11 and Rad50 to reverse ionizing radiation damage. This provided an explanation for the shared cellular and cytogenetic features of the two diseases. Indeed, similar to A-T, chromosomal instability is illustrated by high incidence of inversions and translocations involving chromosomes 7 and 14 (see Figure 13.3) [57]. The breakpoints most commonly involve the immunoglobulin and T-cell receptor genes. Definite diagnosis of NBS requires the demonstration of a disease-causing mutation in both alleles of the *NBN* gene. Such testing is available on a clinical basis. A truncating homozygous 5-bp deletion (657del5) was identified in ~100% of Slavic patients and in ~70% of North American patients. Therefore, molecular genetic diagnosis is usually first performed through testing for this common mutation [58]. Full gene sequencing is performed only after prior testing has revealed that the 657del5 mutation is absent, the nibrin protein is absent or truncated, and the cells are radiosensitive. This latter testing strategy is offered on a research basis only. Knowledge of the deleterious mutation is helpful for carrier testing and prenatal diagnosis.



Figure 13.3 t(7;14) in Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia (A-T). Chromosomal instability is illustrated by a high incidence of inversions and translocations involving chromosomes 7 and 14. The breakpoints most commonly involve the immunoglobulin and T-cell receptor genes.

13.6 Immunodeficiency, centromeric instability, and facial anomalies syndrome

Immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome is a rare autosomal recessive disorder with approximately 50 patients reported worldwide [59]. Affected individuals are mostly of European descent, with only two unrelated Japanese families reported. Clinical presentation includes prolonged immunodeficiency due to decreases in serum immunoglobulin levels in spite of the presence of B-cell lymphocytes. Agammaglobulinemia is often the result of a reduction in two to three classes of immunoglobulins to undetectable levels. The majority of patients have humoral immunodeficiency, but a considerable number display combined immunodeficiency. Most of the resulting infections affect the lungs or gastrointestinal tract. ICF patients can also present with facial anomalies that are not very remarkable. These include a broad, flat nasal bridge; hypertelorism; epicanthal folds; low set ears; high forehead; protrusion of the tongue; and growth restriction [60].

In addition to clinical findings, the diagnosis of this syndrome relies mostly on karyotypic abnormalities seen in peripheral blood samples stimulated with phytohemagglutinin (T-cell mitogen) or pokeweed (B-cell mitogen). These abnormalities include rearrangements in the juxtacentromeric heterochromatin mainly of chromosomes 1 and 16, with chromosomes 2, 9, and 10 sometimes involved [61]. The abnormalities include decondensation of the qh region of chromosomes 1 and 16, multibranching of many copies of chromosome arms joined in the pericentromeric region, and whole arm deletions in the aforementioned chromosomes (Figure 13.4) [62]. This centromeric instability has only been observed in stimulated ICF lymphocytes and very rarely in bone marrow or fibroblast cells [63]. The relationship between these chromosomal rearrangements and the deficient lymphoid system in this disorder is intriguing.

ICF syndrome is the only human disease associated with genomic DNA hypomethylation. The gene mutated in this disease is *DNMT3B* (DNA methyltransferase genes) located on the long arm of chromosome 20 at q11.2. The biallelic mutations usually reside in the C-terminal portion of the protein that contains the catalytic domain [64]. ICF patients have been shown to have residual protein activity from at least one of their *DNMT3B* alleles. *DNMT3B* is a de novo methyltransferase that is essential for development. It is a nuclear protein that colocalizes with pericentric heterochromatin in some, but not all, murine embryonic stem cells. Although no cancer incidence has been noted in ICF syndrome, research studies have demonstrated the relationship between reduced levels of genomic methylation and lymphoma development, as well as hypomethylation of satellite 2 and 3 DNA in various cancers [65–67].

The most current proposal is that hypomethylation of tandemly repeated, noncoding sequences in ICF lymphoid cells has a trans effect on expression of one or more genes involved in lymphocyte maturation or activation. This may happen through nuclear compartmentalization and sequestration of transcriptional regulators.

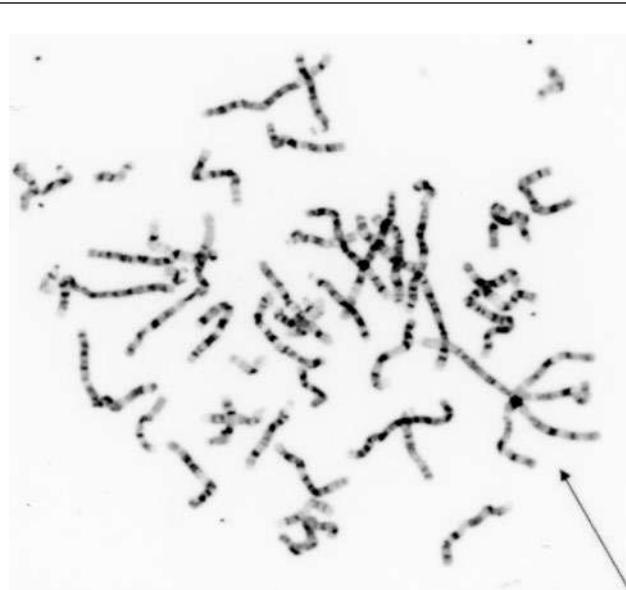


Figure 13.4 Peripheral blood metaphase from an ICF patient. Note the multibranching of four copies of the long arm of chromosome 1 (arrow).

13.7 Roberts syndrome

Also known as pseudothalidomide syndrome, Roberts-SC syndrome, and SC-phocomelia syndrome, Roberts syndrome is a rare autosomal recessive disorder with more than 100 individuals reported worldwide. No accurate estimates can be found for the prevalence of this disease, but parental consanguinity is known to be common. Clinical presentation includes severe growth restriction, limb and hand malformations, craniofacial abnormalities, and defects in the cardiac, renal, and genital systems (reviewed in [68]). Growth restriction is a consistent finding, and is found prenatally as well as postnatally. Newborns can present with growth curves below the 3rd percentile, and this finding is correlated with severity of the limb and craniofacial malformations. Tetraphocomelia is a prominent feature, with the phenotype varying from a complete absence of arms and legs with rudimentary digits (in stillbirths) to mild reduction in the limbs. The upper limbs are usually more severely affected than the lower limbs. Several cases have been reported with upper limb malformations, whereas no cases of lower limb without upper limb anomalies have been reported [69]. Hand malformations are also common, with the thumb affected by proximal positioning or digitalization, hypoplasia, or agenesis. The fifth finger is the next most affected with clinodactyly, and severe cases can have only one finger. Craniofacial abnormalities present with marked variability, ranging from mild hypoplastic nasal alae and hemangioma to cleft lip and palate and encephalocele. Hypertelorism is common and results from widely spaced orbits (reviewed in [68]).

Clinical diagnosis of Roberts syndrome relies on clinical presentation coupled with cytogenetic findings, namely premature centromere separation. This phenomenon consists of repulsion of the constitutive heterochromatin, most notably at the heterochromatic regions of chromosomes 1, 9, and 16, the short arms of the acrocentric chromosomes, and the long arm of the Y chromosome. Several other chromosomes may display a railroad track appearance [70, 71]. Heterochromatin repulsion is seen in mild and severe cases of the disease, and is used as a diagnostic criterion. It is not seen in blood samples from obligate heterozygotes. It is most easily detected by C-banding and regularly stained preparations, and less so with trypsin/Giemsa treatment.

The gene for Roberts syndrome was cloned in 2005, through the identification of seven families affected with the disease in two isolated villages near Bogota, Colombia [72]. A genome-wide search for the disease-associated locus by homozygosity mapping from a common ancestor identified the *ESCO2* gene to be mutated in this syndrome. Further characterization of the protein product revealed similarity with the yeast ECO1p, an essential protein for the establishment of sister chromatid cohesion. Defects in chromatid cohesion lead to mitotic checkpoint activation and impaired cell growth. During embryogenesis, the loss of progenitor cells could preclude the presence of a sufficient number of cells required for the development of structures affected in this syndrome [72]. Molecular diagnosis of this syndrome through sequencing of the *ESCO2* gene is available on a research basis only.

13.8 Werner syndrome

Werner syndrome (WS) is an autosomal recessive disease (incidence estimated at 1 in a million live births) clinically characterized by scleroderma-like skin changes, cataracts, subcutaneous calcification, premature arteriosclerosis, diabetes mellitus, and a wizened and prematurely aged facies [73]. Patients with WS have a high incidence of various types of cancer including sarcomas, meningiomas, and carcinomas.

The frequency of spontaneous chromosome damage is not very striking in WS cells. However, a variety of chromosome rearrangements was observed in WS skin fibroblasts. This phenomenon, called “variegated translocation mosaicism,” describes the pattern of chromosomal aberrations, including translocations, inversions, and deletions seen in WS fibroblast and lymphoblastoid cells [74]. Moreover, WS cells have an abbreviated lifespan in cultures, possibly due to accelerated telomere loss from individual sister chromatids, causing a DNA damage response that leads to genomic instability [75, 76].

The gene mutated in WS was identified in 1996 by positional cloning [77]. The protein, WRN, is a helicase and an exonuclease that functions in DNA repair, recombination, transcription, and replication. All WRN mutations found to date cause premature termination, causing the loss of the protein, which in turn could promote genomic instability and disease via recombination-mediated cellular events.

The diagnosis of WS relies mainly on clinical findings. In 1997, Goto [78] proposed the clinical diagnosis of WS if at least four of the following findings were present: consanguinity, characteristic facial appearance and body habitus, premature senescence, scleroderma-like skin changes, and endocrine-metabolic disorders. Testing for the increase in urinary or serum concentration of hyaluronic acid [79] or for the presence of variegated translocation mosaicism is not used for a definite diagnosis of WS. Clinical testing for Werner syndrome is now available as single gene testing or through Next Generation Sequencing of genes involved in progeroid syndromes.

13.9 Rothmund–Thomson syndrome

Rothmund–Thomson syndrome (RTS) is an autosomal recessive disease clinically characterized by early-onset poikiloderma, short stature, skeletal abnormalities, juvenile cataracts, and a high incidence of cutaneous or extracutaneous malignancies. Its incidence is unknown, but about 300 people with this condition have been reported in scientific studies.

Several chromosomal abnormalities have been observed in RTS fibroblasts, notably trisomy 8 mosaicism, and mosaic supernumerary chromosomes [80]. Likewise, other groups found similar chromosomal instability in lymphoblast cells from RTS patients, including aberrations involving chromosome 8, such as partial 8q duplication and tetrasomy 8q [81]. This suggested that RTS may be associated with clonal chromosomal rearrangements causing acquired somatic mosaicism.

Similar to Bloom syndrome and WS, RTS is also caused by a mutation in a helicase gene. Kitao et al. [82] identified mutations in the gene *RECQL4* in a subset of RTS patients. Molecular genetic testing is clinically available and involves sequence analysis as well as deletion/duplication analysis of the *RECQL4* gene.

13.10 Proficiency testing

Because the College of American Pathologists (CAP) does not provide proficiency testing for diagnosis of these rare diseases, each clinical laboratory must develop their own protocol, generally partnering with another laboratory for blinded exchange studies.

Diseases of the nucleotide excision repair pathway, such as xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy are not discussed in this chapter. These diseases do not have a cytogenetic diagnosis component, and the interested reader is referred to other reviews in the literature.

Glossary

Adenoma: Adenoma is a benign tumor of epithelial tissue, such as the mucosa of stomach, small intestine, and colon, in which tumor cells form glands or glandlike structures.

Retrieved 8/26/2011 <http://en.wikipedia.org/wiki/Adenoma>

Agammaglobulinemia: A condition of the blood, either congenital or acquired, in which there is near or complete absence of gamma globulin and a failure of the body to form antibodies, resulting in a frequent occurrence of infectious disease. Gamma globulins are a protein fraction of blood plasma that responds to stimulation of antigens, as bacteria or viruses, by forming antibodies: administered therapeutically in the treatment of some viral diseases.

Agammaglobulinemia. (n.d.). Dictionary.com Unabridged. Retrieved 8/27/2011 from Dictionary.com website: <http://dictionary.reference.com/browse/Agammaglobulinemia>

Agenesis: Absence or incomplete development of an organ or body part.

American Psychological Association (APA) agensis. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved October 16, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/agensis>

Alkylating agents that cause DNA interstrand crosslinks (ICLs): Alkylating agents are so named because of their ability to alkylate many nucleophilic functional groups under conditions present in cells. Alkylation is the transfer of an alkyl group from one molecule to another. It impairs cell function by forming covalent bonds with the amino, carboxyl, sulphydryl, and phosphate groups in biologically important molecules.

Retrieved 8/26/2011 http://en.wikipedia.org/wiki/Alkylating_agents

Alpha fetoprotein: An antigen produced in the fetal liver that can appear in certain diseases of adults, such as liver cancer, and whose level in amniotic fluid can be used to detect certain fetal abnormalities, including Down syndrome and spina bifida.

alpha fetoprotein. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 10/16/2011, from Dictionary.com website: http://dictionary.reference.com/browse/alpha_fetoprotein

Androgen therapy: Androgen replacement therapy is a hormone treatment often prescribed to counter the effects of male hypogonadism. It is also prescribed to lessen the effects or delay the onset of normal male aging. Additionally, androgen replacement therapy is used for men who have lost their testicular function to disease, cancer, or other causes.

http://en.wikipedia.org/wiki/Androgen_replacement_therapy

Anemia: A quantitative deficiency of the hemoglobin, often accompanied by a reduced number of red blood cells and causing pallor, weakness, and breathlessness.

Retrieved 8/26/2011 <http://dictionary.reference.com/browse/anemia>

Aplasia: A developmental failure resulting in the absence of an organ or tissue.

Mosby's Medical Dictionary, 8th edition. © 2009, Elsevier. 8/26/2011 <http://medical-dictionary.thefreedictionary.com/aplasia>

Apraxia: Apraxia is a disorder caused by damage to specific areas of the cerebrum. Apraxia is characterized by loss of the ability to execute or carry out learned purposeful movements, despite having the desire and the physical ability to perform the movements.

Retrieved 10/16/11 from <http://en.wikipedia.org/wiki/Apraxia>

Arteriosclerosis: Degenerative changes in the arteries, characterized by thickening of the vessel walls and accumulation of calcium with consequent loss of elasticity and lessened blood flow.

arteriosclerosis. (n.d.). Dictionary.com Unabridged. Retrieved October 16, 2011, from Dictionary.com website <http://dictionary.reference.com/browse/arteriosclerosis>

Ashkenazi Jewish population: Descendants from the medieval Jewish communities along the Rhine in Germany from Alsace in the south to the Rhineland in the north.

Ataxia: Loss of the ability to coordinate muscular movement. Also called dyssynergia, incoordination.

ataxia. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved October 16, 2011, from Dictionary.com website <http://dictionary.reference.com/browse/ataxia>

Atresia: The congenital absence or closure of a normal body orifice or tubular passage such as the anus, intestine, or external ear canal. The degeneration and resorption of one or more ovarian follicles before maturation.

atresia. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved August 26, 2011, from Dictionary.com website <http://dictionary.reference.com/browse/atresia>

Base pair (bp): Two DNA bases complementary to one another (A and T or G and C) that join the complementary strands of DNA to form the double helix characteristic of DNA.

Retrieved 8/27/2011 <http://www.medterms.com/script/main/art.asp?articlekey=2440>

Body habitus: The physique or body build. The term "body habitus" is somewhat redundant, since habitus by itself means "physique or body build."

Retrieved 8/27/2011 <http://www.medterms.com/script/main/art.asp?articlekey=21671>

Bulbar conjunctiva: The part of the conjunctiva (the delicate mucous membrane that covers the eyeball and the under surface of the eyelid) covering the anterior face of the sclera (The tough fibrous tunic forming the outer envelope of the eye and covering all of the eyeball except the cornea; the white of the eye. Also called sclerotic) and the surface epithelium of the cornea (the transparent, convex, anterior portion of the outer fibrous coat of the eyeball that covers the iris and the pupil and is continuous with the sclera).

bulbar conjunctiva. (n.d.). WordNet® 3.0. Retrieved October 16, 2011, from Dictionary.com website <http://dictionary.reference.com/browse/bulbar%20conjunctiva>; conjunctiva. (n.d.). Collins English Dictionary - Complete & Unabridged 10th Edition. Retrieved October 16, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/conjunctiva>; sclera. (n.d.). and cornea. (n.d.) The American Heritage® Stedman's Medical Dictionary. Retrieved October 16, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/sclera>

Café-au-lait spots: Light brown pigmented birthmarks. The name café au lait is French for "milky coffee" and refers to their light-brown color. They are also called "giraffe spots."

http://en.wikipedia.org/wiki/Caf%C3%A9_au_lait_spot. Retrieved 8-26-11.

Cerebellar dysfunction: The cerebellum is located in the lower back portion of the brain, just above the brain stem.

It is responsible for movement, balance and muscle tone. The vestibular region of the inner ear aids the cerebellum by providing information related to head position. The symptoms of cerebellar vestibular dysfunction primarily manifest as difficulty walking (ataxia), dizziness and abnormal eye movements (nystagmus).

Retrieved 8/27/2011 http://www.ehow.com/facts_5771848_cerebellar-vestibular-dysfunction_.html

Choreoathetosis: A condition marked by abnormal movements of the body that have a combined choreic (irregular, spastic, involuntary movements of the limbs or facial muscles) and athetoid (a condition characterized by uncontrolled rhythmic writhing movement, especially of fingers, hands, head, and tongue, caused by cerebral lesion pattern).

choreoathetosis. (n.d.). and choreic from The American Heritage® Stedman's Medical Dictionary. Retrieved October 16, 2011, from Dictionary.com website <http://dictionary.reference.com/browse/choreoathetosis>; athetoid. (n.d.). Collins English Dictionary - Complete & Unabridged 10th Edition. Retrieved October 16, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/athetoid>

Clastogen: Induce double-strand breaks.

Clinodactyly: Permanent deflection of one or more fingers.

clinodactyly. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved August 21, 2011, from Dictionary.com website <http://dictionary.reference.com/browse/clinodactyly>

Conductive hearing loss: Hearing loss due to problems with the bones of the middle ear Conductive hearing loss.

(n.d.). WordNet® 3.0. Retrieved August 26, 2011, from Dictionary.com website <http://dictionary.reference.com/browse/Conductive%20hearing%20loss>

Covalent bond: A covalent bond is a form of chemical bonding that is characterized by the sharing of pairs of electrons between atoms.

Retrieved 8/26/2011 http://en.wikipedia.org/wiki/Covalent_bond

Cretinism: A congenital disease due to absence or deficiency of normal thyroid secretion, characterized by physical deformity, dwarfism, and intellectual disability, and often by goiter.

Hypothyroidism. (n.d.). Dictionary.com Unabridged. Retrieved 8/25/2011 from Dictionary.com website: <http://dictionary.reference.com/browse/Hypothyroidism>

Cryptorchidism: Cryptorchidism is the absence of one or both testes from the scrotum. It is the most common birth defect regarding male genitalia.

Retrieved 8/26/2011 from <http://en.wikipedia.org/wiki/Cryptorchidism>

Diepoxybutane DEB: Diepoxybutane (DEB) is a bifunctional alkylating agent that induces a high incidence of chromosome breakage in lymphocyte cultures of FA patients. It is a known carcinogen, which can be rapidly inactivated by concentrated hydrochloric acid (HCl); disposable culture bottles and pipettes should be rinsed with HCl before being discarded.¹ DEB is rated T (toxic biohazard warning), and thus the following PPE (protective personal equipment) are recommended: Faceshields, full-face respirator (US), gloves, goggles, multipurpose combination respirator cartridge (US), and type ABEK (EN14387) respirator filter.²

1 Porto B, Chicchio L, Gaspar J, Faber A, Pinho L, Rueff J, Malheiros I. Role of haemoglobin in the protection of cultured lymphocytes against diepoxybutane (DEB), assessed by in vitro induced chromosome breakage. *Mutation Research* 2003;536:61–67.

2 Sigma Aldrich <http://www.sigmaaldrich.com/ghs-hazard>

DNMT3B: A de novo methyltransferase that is essential for development. It is a nuclear protein that colocalizes with pericentric heterochromatin in some, but not all, murine embryonic stem cells.

Double ureters: Existence of a second ureter on one side that may be a complete connection from the kidney to the bladder or a partial tube forming a blind pouch. Most are asymptomatic, but some are accompanied by ectopic ureterocele. Also called ureteral duplication.

Mosby's Medical Dictionary, 8th edition. © 2009, Elsevier. 8/26/2011 <http://medical-dictionary.thefreedictionary.com/double+ureters>

Encephalocele: A congenital gap in the skull that usually results in a protrusion of brain material. Also called bifid cranium, cephalocele, craniocoele.

encephalocele. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 10/16/2011 from Dictionary.com website: <http://dictionary.reference.com/browse/encephalocele>

Endoreduplication: A form of polyploidy or polysomy characterized by redoubling of chromosomes without centromere separation, giving rise to four-stranded chromosomes at prophase and metaphase.

Epicantal folds: An epicantic fold, epicantal fold, or epicanthus is a skin fold of the upper eyelid, covering the inner corner (medial canthus) of the eye.

http://en.wikipedia.org/wiki/Epicantal_fold

Erythema: Redness of the skin, caused by hyperemia (increase of blood flow) of the capillaries in the lower layers of the skin. It occurs with any skin injury, infection, or inflammation.

Retrieved 10/16/11 from <http://en.wikipedia.org/wiki/Erythema>

Exonuclease: Any of a group of enzymes that catalyze the hydrolysis of single nucleotides from the end of a DNA or RNA chain. exonuclease. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved October 16, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/exonuclease>

Fibroblast: A stellate or spindle-shaped cell with cytoplasmic processes present in connective tissue, capable of forming collagen fibers.

fibroblast. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 10/16/2011 from Dictionary.com website: <http://dictionary.reference.com/browse/fibroblast>

Focal nodular hyperplasia: Focal nodular hyperplasia (FNH) is a benign tumor of the liver (hepatic tumor), which is the second most prevalent tumor of the liver (the first is hepatic hemangioma).

Retrieved 8-26-2011 http://en.wikipedia.org/wiki/Focal_nodular_hyperplasia

Helicase: Any of various enzymes that catalyze the unwinding and separation of double-stranded DNA or RNA during its replication helicase.

(n.d.). Merriam-Webster's Medical Dictionary. Retrieved 10/16/2011 from Dictionary.com website: <http://dictionary.reference.com/browse/helicase>

Hemangioma: Angioma is a benign tumor consisting chiefly of dilated or newly formed blood vessels (hemangioma) or lymph vessels (lymphangioma).

hemangioma. (n.d.). Dictionary.com Unabridged. Retrieved 10/16/2011 from Dictionary.com website: <http://dictionary.reference.com/browse/hemangioma>

Hepatocellular carcinomas: Hepatocellular carcinoma (HCC, also called malignant hepatoma) is the most common type of liver cancer. Most cases of HCC are secondary to either a viral hepatitis infection (hepatitis B or C) or cirrhosis (alcoholism being the most common cause of hepatic cirrhosis).

Kumar V, Fausto N, Abbas A (eds). *Robbins & Cotran Pathologic Basis of Disease* (7th ed.) 2003; Saunders: 914–917.

Homologous recombination: Homologous recombination is a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA. It is most widely used by cells to accurately repair harmful breaks that occur on both strands of DNA, known as double-strand breaks. Homologous recombination also produces new combinations of DNA sequences during meiosis.

Retrieved 10/16/11 from http://en.wikipedia.org/wiki/Homologous_recombination

Horseshoe kidneys: An anomaly in which the right and left kidneys are linked at one end by tissue.

<http://medical-dictionary.thefreedictionary.com/horseshoe+kidneys>

Hypertelorism: Abnormal distance between two paired organs.

hypertelorism. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 10/16/2011, from Dictionary.com website: <http://dictionary.reference.com/browse/hypertelorism>

Hypogenitalia: Partial or complete failure of the genitalia to develop, often as a consequence of hypogonadism (inadequate functioning of the testes or ovaries as manifested by deficiencies in gametogenesis or the secretion of gonadal hormones).

hypogenitalia/hypogonadia. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 8/26/2011 from Dictionary.com website: <http://dictionary.reference.com/browse/hypogenitalia>

Hypopigmentation: Diminished pigmentation in a bodily part or tissue (as the skin) hypopigmentation.

(n.d.). Merriam-Webster's Medical Dictionary. Retrieved 8/26/ 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/hypopigmentation>

Hypoplasia: A condition of arrested development in which an organ or part remains below the normal size or in an immature state.

hypoplasia. (n.d.). Merriam-Webster's Medical Dictionary. Retrieved 8/25/2011 from Dictionary.com website: <http://dictionary.reference.com/browse/hypoplasia>

Hypospadias: A developmental anomaly of the urethra in which a part of the urethral canal is open on the undersurface of the penis or on the perineum. A similar anomaly in which the urethra opens into the vagina.

hypospadias. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved August 26, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/hypospadias>

Hypothyroidism: An abnormality of the thyroid gland characterized by insufficient production of thyroid hormone, which can result in a decreased basal metabolic rate, causing weight gain and fatigue.¹ The condition produced by a deficiency of thyroid secretion, resulting in goiter, myxedema (see later), and, in children, cretinism (see earlier).²

1 Hypothyroidism. (n.d.). The American Heritage® Science Dictionary. Retrieved August 25, 2011, from Dictionary.com website.

2 <http://dictionary.reference.com/browse/Hypothyroidism>

Intronic mutations: A mutation (usually a base substitution) within an intron that creates an alternative splice site that competes with the normal splice sites during RNA processing. Such a mutation results in a proportion of mature messenger RNA with improperly spliced intron sequences.

Retrieved 10/16/11 <http://ghr.nlm.nih.gov/glossary=intronicmutation>

Kinase: An enzyme that catalyzes the conversion of a proenzyme to an active enzyme. An enzyme that catalyzes the transfer of a phosphate group from a donor, such as ADP or ATP, to an acceptor. kinase.

(n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved October 16, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/kinase>

Macrocythemia: Unusually large numbers of macrocytes in the blood. Also called macrocytosis.

macrocythemia. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved August 26, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/macrocythemia>

Macrocytosis: see Macrocythemia

Medulloblastoma: Medulloblastoma is a highly malignant primary brain tumor that originates in the cerebellum or posterior fossa.

Retrieved 8/26/2011 <http://en.wikipedia.org/wiki/Medulloblastoma>

Methylation: The addition of a methyl group to cytosine and adenine residues in DNA that leads to the epigenetic modification of DNA and the reduction of gene expression and protein production.

Retrieved 10/16/11 from <http://en.wiktionary.org/wiki/methylation>

Methyltransferase: Any of several enzymes that catalyze the transfer of methyl groups from one compound to another. Also called transmethylase, methyltransferase.

(n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 10/ 16, 2011, from Dictionary.com website <http://dictionary.reference.com/browse/methyltransferase>

Microcephaly: Abnormal smallness of the head. Also called nanocephaly.

microcephaly. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved August 25, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/microcephaly>

Microphtalmia: Abnormal smallness of the eye. Also called microphthalmos, nanophthalmia, nanophtalmos.

Microphtalmia. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved August 25, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/Microphtalmia>

Myxedema: A condition characterized by thickening of the skin, blunting of the senses and intellect, and labored speech, associated with hypothyroidism.

myxedema. (n.d.). Dictionary.com Unabridged. Retrieved October 16, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/myxedema>

Neutropenia: The presence of abnormally small numbers of neutrophils (see later) in the blood. Also called neutrophilic leukopenia.

neutropenia. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 8/26/2011 from Dictionary.com website: <http://dictionary.reference.com/browse/neutropenia>

Neutrophils: A phagocytic white blood cell having a lobulate nucleus and neutrophil granules in the cytoplasm.

neutrophils. (n.d.). Dictionary.com Unabridged. Retrieved 8/26/2011, from Dictionary.com website: <http://dictionary.reference.com/browse/neutrophils>

Obligate heterozygotes: An individual in a family who is proven to carry one copy of a recessive allele by having had affected progeny who inherited two.

www.answers.com/topic/obligate-heterozygote

Oculomotor: Relating to or causing movements of the eyeball. Of or relating to the oculomotor nerve.

oculomotor. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 10/16/2011, from Dictionary.com website: <http://dictionary.reference.com/browse/oculomotor>

p53: A tumor suppressor gene that in a defective form tends to be associated with a high risk of certain cancers (as of the colon, lung, and breast).

p53. (n.d.). Merriam-Webster's Medical Dictionary. Retrieved October 16, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/p53>

Pallor: Paleness, as of the skin.

pallor. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved August 26, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/pallor>

Pancytopenia: An abnormal reduction in the number of red blood cells (RBS), white blood cells (WBC), and blood

platelets in the blood; also: a disorder (as aplastic anemia) characterized by such a reduction.

pancytopenia. (n.d.). Merriam-Webster's Medical Dictionary. Retrieved 8/26/2011, from Dictionary.com website: <http://dictionary.reference.com/browse/pancytopenia>

Patent ductus arteriosus: Patent ductus arteriosus (PDA) is a congenital disorder in the heart wherein a neonate's ductus arteriosus fails to close after birth. Early symptoms are uncommon, but in the first year of life include increased work of breathing and poor weight gain. With age, the PDA may lead to congestive heart failure if left uncorrected. A patent ductus arteriosus can be idiopathic (i.e., without an identifiable cause), or secondary to another condition.

http://en.wikipedia.org/wiki/Patent_ductus_arteriosus 8/26/2011

Phosphorylates: To add a phosphate group to (an organic molecule).

Retrieved 10/16/11 from <http://www.thefreedictionary.com/phosphorylates>

Poikiloderma: A variegated hyperpigmentation and telangiectasia of the skin, followed by atrophy.

poikiloderma. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 10/16/2011, from Dictionary.com website: <http://dictionary.reference.com/browse/poikiloderma>

Polymerase chain reaction (PCR): An in vitro technique for rapidly synthesizing large quantities of a given DNA segment that involves separating the DNA into its two complementary strands, binding a primer to each single strand at the end of the given DNA segment where synthesis will start, using DNA polymerase to synthesize two-stranded DNA from each single strand, and repeating the process – abbreviation PCR.

polymerase chain reaction. (n.d.). Merriam-Webster's Medical Dictionary. Retrieved 10/16/2011, from Dictionary.com website: [http://dictionary.reference.com/browse/polymerase chain reaction](http://dictionary.reference.com/browse/polymerase%20chain%20reaction)

Positional cloning: Positional cloning is a laboratory technique used to locate the position of a disease-associated gene along the chromosome. This approach works even when little or no information is available about the biochemical basis of the disease. Positional cloning is used in conjunction with linkage analysis. It involves the isolation of partially overlapping DNA segments that progress along the chromosome toward a candidate gene.

Retrieved 10/16/11 from <http://www.genome.gov/glossary/?id=162>

Progenitor cells: A progenitor cell is a biological cell that, like a stem cell, has a tendency to differentiate into a specific type of cell, but is already more specific than a stem cell and is pushed to differentiate into its "target" cell. The most important difference between stem cells and progenitor cells is that stem cells can replicate indefinitely, whereas progenitor cells can only divide a limited number of times. Controversy about the exact definition remains and the concept is still evolving.

Retrieved 10/16/11 from http://en.wikipedia.org/wiki/Progenitor_cell

Pulmonary stenosis: Abnormal narrowing of the orifice between the pulmonary artery and the right ventricle called also pulmonic stenosis.

pulmonary stenosis. (n.d.). Merriam-Webster's Medical Dictionary. Retrieved 8/26/2011, from Dictionary.com website: <http://dictionary.reference.com/browse/pulmonary stenosis>

Radial ray defects: Duane anomaly results from the improper development of certain nerves that control eye movement. This condition limits outward eye movement (toward the ear), and in some cases may limit inward eye movement (toward the nose). As the eye moves inward, the eye opening becomes narrower and the eyeball may pull back (retract) into its socket. Bone abnormalities in the hands include malformed or absent thumbs, an extra thumb, or a thumb that looks like a finger. Partial or complete absence of bones in the forearm is also common. Together, these hand and arm abnormalities are called radial ray malformations.

http://en.wikipedia.org/wiki/Duane-radial_ray_syndrome 8/26/11

Renal hypoplasia: An abnormally small kidney that is morphologically normal but has either a reduced number of nephrons or smaller nephrons.

The American Heritage® Medical Dictionary © 2007, 2004 by Houghton Mifflin Company. Published by Houghton Mifflin Company. All rights reserved. <http://medical-dictionary.thefreedictionary.com/renal+hypoplasia>

Scleroderma: A pathological thickening and hardening of the skin caused by swelling and thickening of fibrous tissue. Also called dermatosclerosis.

scleroderma. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 10/16/2011, from Dictionary.com website <http://dictionary.reference.com/browse/scleroderma>

Scoliosis: A condition of lateral curvature of the spine, which may have just one curve or primary and secondary compensatory curves and be fixed or mobile.

scoliosis. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 8/26/2011, from Dictionary.com website: <http://dictionary.reference.com/browse/scoliosis>

Tetraphocomelia: Tetraphocomelia-cleft lip-palate syndrome: Symmetrical phocomelia-like limb defects similar to those seen in thalidomide embryopathy (hence the synonym SC-pseudothalidomide syndrome), flexion contractures of the joints, facial anomalies, micrognathia, scanty blond hair, cloudy cornea, delayed growth, and occasional intellectual disability, SC phocomelia and Roberts syndrome are considered by some as the same entity termed Roberts-SC phocomelia syndrome. The syndrome was first observed in a family with surname beginning with S and another with surname beginning with C.

http://www.rightdiagnosis.com/medical/tetraphocomelia_cleft_lip_palate_syndrome.htm

Thrombocytopenia: An abnormal decrease in the number of platelets in the blood. Also called thrombopenia.

thrombocytopenia. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved 8/26/2011, from Dictionary.com website <http://dictionary.reference.com/browse/thrombocytopenia>

Undescended testes: see Cryptorchidism

Variegated translocation mosaicism: The repeated occurrence, within cultures of human skin fibroblasts, of a multiplicity of chromosomal rearrangements.

Retrieved 10/16/11 from <http://www.ncbi.nlm.nih.gov/pubmed/1222585>

Ventricular septal defect: A ventricular septal defect (VSD) is a defect in the ventricular septum, the wall dividing the left and right ventricles of the heart.

http://en.wikipedia.org/wiki/Ventricular_septal_defect

Wilms tumor: Wilms tumor or nephroblastoma is cancer of the kidneys that typically occurs in children, rarely in adults.

Its common name is an eponym, referring to Dr. Max Wilms, the German surgeon (1867–1918) who first described this kind of tumor.

<http://www.whonamedit.com/doctor.cfm/2109.html> retrieved 8/26/2011 http://en.wikipedia.org/wiki/Wilms_tumor#cite_note-1

References

1. Kanaar R, Wyman C, Rothstein R. Quality control of DNA break metabolism: in the “end”, it is a good thing. *EMBO J* 2008; 27: 581–588.
2. Eyfjord JE, Bodvarsdottir SK. Genomic instability and cancer: networks involved in response to DNA damage. *Mutat Res* 2005; 592(1–2): 18–28.
3. Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, De Die-Smulders C, Persky N, Grompe M, Joenje H, Pals G, Ikeda H, Fox EA, D'Andrea AD. Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* 2002; 297(5581): 606–609.

4. Boland CR, Sato J, Saito K, Carethers J, Marra G, Laghi L, Chauhan D. Genetic instability and chromosomal aberrations in colorectal cancer: a review of the current models. *Cancer Detect Prev* 1998; 22(5): 377–382.
5. Gersen SL, Keagle MB, eds. *The Principles of Clinical Cytogenetics*. Humana Press Inc., 2005.
6. Alter BP. Cancer in Fanconi anemia, 1927–2001. *Cancer* 2003; 97(2): 425–440.
7. Tischkowitz MD, Hodgson SV. Fanconi anaemia. *J Med Genet* 2003; 40(1): 1–10.
8. Alter BP, Frisso CL, Halperin DS, Freedman MH, Chitkara U, Alvarez E, Lynch L, Adler-Brecher B, Auerbach AD. Fanconi's anaemia and pregnancy. *Br J Haematol* 1991; 77(3): 410–418.
9. Rossbach HC, Sutcliffe MJ, Haag MM, Grana NH, Rossi AR, Barbosa JL. Fanconi anemia in brothers initially diagnosed with VACTERL association with hydrocephalus, and subsequently with Baller-Gerold syndrome. *Am J Med Genet* 1996; 61(1): 65–67.
10. Stivrins TJ, Davis RB, Sanger W, Fritz J, Purtilo DT. Transformation of Fanconi's anemia to acute nonlymphocytic leukemia associated with emergence of monosomy 7. *Blood* 1984; 64(1): 173–176.
11. Maarek O, Jonveaux P, Le Coniat M, Derré J, Berger R. Fanconi anemia and bone marrow clonal chromosome abnormalities. *Leukemia* 1996; 10(11): 1700–1704.
12. Ortega M, Caballin MR, Ortega JJ, Olive T, Coll MD. Follow-up by cytogenetic and fluorescence in situ hybridization analysis of allogeneic bone marrow transplantation in two children with Fanconi's anaemia in transformation. *Br J Haematol* 2000; 111(1): 329–333.
13. Berger R, Jonveaux P. Clonal chromosome abnormalities in Fanconi anemia. *Hematol Cell Ther* 1996; 38(4): 291–296.
14. Davies SM, Khan S, Wagner JE, Arthur DC, Auerbach AD, Ramsay NK, Weisdorf DJ. Unrelated donor bone marrow transplantation for Fanconi anemia. *Bone Marrow Transplant* 1996; 17(1): 43–47.
15. Huret JL, Tanzer J, Guilhot F, Frocrain-Herchkovitch C, Savage JR. Karyotype evolution in the bone marrow of a patient with Fanconi anemia: breakpoints in clonal anomalies of this disease. *Cytogenet Cell Genet* 1988; 48(4): 224–227.
16. Barton JC, Parmley RT, Carroll AJ, Huang ST, Goodnough LT, Findley HW Jr, Ragab AH. Preleukemia in Fanconi's anemia: hematopoietic cell multinuclearity, membrane duplication, and dysgranulogenesis. *J Submicrosc Cytol* 1987; 19(2): 355–364.
17. Standen GR, Hughes IA, Geddes AD, Jones BM, Wardrop CA. Myelodysplastic syndrome with trisomy 8 in an adolescent with Fanconi anaemia and selective IgA deficiency. *Am J Hematol* 1989; 31(4): 280–283.
18. Ferti A, Panani A, Dervenoulas J, Raptis SA. Cytogenetic findings in a Fanconi anemia patient with AML. *Cancer Genet Cytogenet* 1996; 90(2): 182–183.
19. Oliveira NI, Ribeiro EM, Raimondi SC, Bittencourt MA, Pasquini R, Cavalli IJ. Two different karyotypes with 1q abnormalities in a patient with Fanconi anemia. *Leuk Res* 2002; 26(11): 1047–1049.
20. Tonnes H, Huber S, Kuhl JS, Gerlach A, Ebelt W, Neitzel H. Clonal chromosomal aberrations in bone marrow cells of Fanconi anemia patients: gains of the chromosomal segment 3q26q29 as an adverse risk factor. *Blood* 2003; 101(10): 3872–3874.
21. Kutler DI, Auerbach AD, Satagopan J, Giampietro PF, Batish SD, Huvos AG, Goberdhan A, Shah JP, Singh B. High incidence of head and neck squamous cell carcinoma in patients with Fanconi anemia. *Arch Otolaryngol Head Neck Surg* 2003; 129(1): 106–112.
22. Mulvihill JJ, Ridolfi RL, Schultz FR, Borzy MS, Haughton PB. Hepatic adenoma in Fanconi anemia treated with oxymetholone. *J Pediatr* 1975; 87(1): 122–124.
23. Farrell GC. Fanconi's familial hypoplastic anaemia with some unusual features. *Med J Aust* 1976; 1(5): 116–118.
24. Garel L, Kalifa G, Buriot D, Sauvageain J. Multiple adenomas of the liver and Fanconi's anaemia. *Ann Radiol (Paris)* 1981; 24(1): 53–54.
25. Cap J, Ondrus B, Danihel L. [Focal nodular hyperplasia of the liver and hepatocellular carcinoma of the liver in children with Fanconi's anemia after long-term treatment with androgens]. *Bratisl Lek Listy* 1983; 79(1): 73–81.
26. Tischkowitz MD, Chisholm J, Gaze M, Michalski A, Rosser EM. Medulloblastoma as a first presentation of Fanconi anemia. *J Pediatr Hematol Oncol* 2004; 26(1): 52–55.
27. Hirsch B, Shimamura A, Moreau L, Baldinger S, Hag-alshiekh M, Bostrom B, Sencer S, D'Andrea AD. Association of biallelic BRCA2/FANCD1 mutations with spontaneous chromosomal instability and solid tumors of childhood. *Blood* 2004; 103(7): 2554–2559.

28. Kee Y, D'Andrea AD. Expanded roles of the Fanconi anemia pathway in preserving genomic stability. *Gene Dev* 2010; 24(16): 1680–1694.
29. Sasaki MS. Is Fanconi's anaemia defective in a process essential to the repair of DNA cross links? *Nature* 1975; 257(5526): 501–503.
30. Tomasz M, Lipman R, Chowdary D, Pawlak J, Verdine GL, Nakanishi K. Solution and structure of a covalent cross-link adduct between mitomycin C and DNA. *Science* 1987; 235(4793): 1204–1208.
31. Cummings J, Spanswick VJ, Tomasz M, Smyth JF. Enzymology of mitomycin C metabolic activation in tumour tissue: implications for enzyme-directed bioreductive drug development. *Biochem Pharmacol* 1998; 56(4): 405–414.
32. Millard JT, Wilkes EE. Diepoxybutane and diepoxyoctane interstrand cross-linking of the 5S DNA nucleosomal core particle. *Biochemistry* 2001; 40(35): 10677–10685.
33. McCabe KM, Olson SB, Moses RE. DNA interstrand crosslink repair in mammalian cells. *J. Cell Physiol* 2009; 220(3): 569–573.
34. Auerbach AD, Adler B, Chaganti RS. Prenatal and postnatal diagnosis and carrier detection of Fanconi anemia by a cytogenetic method. *Pediatrics* 1981; 67(1): 128–135.
35. Akkari Y, Olson S, unpublished data.
36. Cervenka J, Arthur D, Yasis C. Mitomycin C test for diagnostic differentiation of idiopathic aplastic anemia and Fanconi anemia. *Pediatrics* 1981; 67(1): 119–127.
37. Newell AE, Akkari YM, Torimaru Y, Rosenthal A, Reifsteck CA, Cox B, Grompe M, Olson SB. Interstrand crosslink-induced radials form between nonhomologous chromosomes, but are absent in sex chromosomes. *DNA Repair (Amst)* 2004; 3(5): 535–542.
38. Hemphill AW, Bruun D, Thrun L, Akkari Y, Torimaru Y, Hejna K, Jakobs PM, Hejna J, Jones S, Olson SB, Moses RE. Mammalian SNM1 is required for genome stability. *Mol. Genet. Metab* 2008; 94(1): 38–45.
39. Hirschhorn R. In vivo reversion to normal of inherited mutations in humans. *J Med Genet* 2003; 40(10): 721–728.
40. Waisfisz Q, Morgan NV, Savino M, de Winter JP, van Berkel CG, Hoatlin ME, Ianzano L, Gibson RA, Arwert F, Savoia A, Mathew CG, Pronk JC, Joenje H. Spontaneous functional correction of homozygous Fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. *Nat Genet* 1999; 22(4): 379–383.
41. Lo Ten Foe JR, Kwee ML, Rooimans MA, Oostra AB, Veerman AJ, van Weel M, Pauli RM, Shahidi NT, Dokal I, Roberts I, Altay C, Gluckman E, Gibson RA, Mathew CG, Arwert F, Joenje H. Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. *Eur J Hum Genet* 1997; 5(3): 137–148.
42. Gross M, Hanenberg H, Lobitz S, Friedl R, Herterich S, Dietrich R, Gruhn B, Schindler D, Hoehn H. Reverse mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction. *Cytogenet Genome Res* 2002; 98(2–3): 126–135.
43. Van Waes C. Head and neck squamous cell carcinoma in patients with Fanconi anemia. *Arch. Otolaryngol Head Neck Surg* 2005; 131(7): 640–641.
44. Shimamura A, Montes de Oca R, Svenson JL, Haining N, Moreau LA, Nathan DG, D'Andrea AD. A novel diagnostic screen for defects in the Fanconi anemia pathway. *Blood* 2002; 100(13): 4649–4654.
45. Schroeder TM, German J. Bloom's syndrome and Fanconi's anemia: demonstration of two distinctive patterns of chromosome disruption and rearrangement. *Humangenetik* 1974; 25(4): 299–306.
46. Ellis NA, Groden J, Ye TZ, Straughen J, Lennon DJ, Ciocci S, Proytcheva M, German J. The Bloom's syndrome gene product is homologous to RecQ helicases. *Cell* 1995; 83(4): 655–666.
47. Ellis NA, German J. Molecular genetics of Bloom's syndrome. *Hum Mol Genet* 1996; 5 Spec No: 1457–1463.
48. Roa BB, Savino CV, Richards CS. Ashkenazi Jewish population frequency of the Bloom syndrome gene 2281 delta 6ins7 mutation. *Genet Test* 1999; 3(2): 219–221.
49. Taylor AM, Byrd PJ. Molecular pathology of ataxia telangiectasia. *J Clin Pathol* 2005; 58(10): 1009–1015.
50. Jaspers NG, Gatti RA, Baan C, Linssen PC, Bootsma D. Genetic complementation analysis of ataxia telangiectasia and Nijmegen breakage syndrome: a survey of 50 patients. *Cytogenet Cell Genet* 1988; 49(4): 259–263.
51. Hecht F, Hecht BK. *Cancer Genet Cytogenet* 1990; 46(1): 9–19.
52. Brown KD, Barlow C, Wynshaw-Boris A. Multiple ATM-dependent pathways: an explanation for pleiotropy. *Am J Hum Genet* 1999; 64(1): 46–50.

53. Seemanova E, Passarge E, Beneskova D, Houstek J, Kasal P, Sevcikova M. Familial microcephaly with normal intelligence, immunodeficiency, and risk for lymphoreticular malignancies: a new autosomal recessive disorder. *Am J Med Genet* 1985; 20: 639–648.
54. Weemaes CM, Hustinx TW, Scheres JM, van Munster PJ, Bakkeren JA, Taalman RD. A new chromosomal instability disorder: the Nijmegen breakage syndrome. *Acta Paediatr Scand* 1981; 70(4): 557–564.
55. Wegner RD, Chrzanowska KH, Sperling K, Stumm M. Ataxia-telangiectasia variants (Nijmegen breakage syndrome). In: Ochs HD, Smith CIE, Puck JM, eds. *Primary Immunodeficiency Diseases, a Molecular and Genetic Approach*. 1999; Oxford, UK: Oxford University Press, 324–334.
56. Zhao S, Weng YC, Yuan SS, Lin YT, Hsu HC, Lin SC, Gerbino E, Song MH, Zdzienicka MZ, Gatti RA, Shay JW, Ziv Y, Shiloh Y, Lee EY. Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. *Nature* 2000; 405(6785): 473–477.
57. Maraschio P, Peretti D, Lambiase S, Lo Curto F, Caufin D, Gargantini L, Minoli L, Zuffardi O. A new chromosome instability disorder. *Clin Genet* 1986; 30(5): 353–365.
58. Varon R, Vissinga C, Platzer M, Cerosaletti KM, Chrzanowska KH, Saar K, Beckmann G, Seemanová E, Cooper PR, Nowak NJ, Stumm M, Weemaes CM, Gatti RA, Wilson RK, Digweed M, Rosenthal A, Sperling K, Concannon P, Reis A. Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell* 1998; 93(3): 467–476.
59. Ehrlich M, Jackson K, Weemaes C. Immunodeficiency, centromeric instability, facial anomalies syndrome (ICF). *Orphanet J Rare Dis* 2006; 1: 2.
60. Maraschio P, Zuffardi O, Dalla Fior T, Tiepolo L. Immunodeficiency, centromeric heterochromatin instability of chromosomes 1, 9, and 16, and facial anomalies: the ICF syndrome. *J Med Genet* 1988; 25(3): 173–180.
61. Tiepolo L, Maraschio P, Gimelli G, Cuoco C, Gargani GF, Romano C. Multibranched chromosomes 1, 9, and 16 in a patient with combined IgA and IgE deficiency. *Hum Genet* 1979; 51(2): 127–137.
62. Fryns JP, Azou M, Jaeken J, Eggermont E, Pedersen JC, Van den Berghe H. Centromeric instability of chromosomes 1, 9, and 16 associated with combined immunodeficiency. *Hum Genet* 1981; 57(1): 108–110.
63. Maraschio P, Tupler R, Dainotti E, Piantanida M, Cazzola G, Tiepolo L. Differential expression of the ICF (Immunodeficiency, Centromeric heterochromatin, facial anomalies) mutation in lymphocytes and fibroblasts. *J Med Genet* 1989; 26(7): 452–456.
64. Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X, Russo JJ, Viegas-Péquignot E. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* 1999; 402(6758): 187–191.
65. Narayan A, Ji W, Zhang XY, Marrogi A, Graff JR, Baylin SB, Ehrlich M. Hypomethylation of pericentromeric DNA in breast adenocarcinomas. *Int J Cancer* 1998; 77(6): 833–838.
66. Qu GZ, Grundy PE, Narayan A, Ehrlich M. Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. *Cancer Genet Cytogenet* 1999; 109(1): 34–39.
67. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Castillejo JA, Navarro G, San Jose-Eneriz E, Garate L, Cordeu L, Cervantes F, Prosper F, Heiniger A, Torres A. Repetitive DNA hypomethylation in the advanced phase of chronic myeloid leukemia. *Leuk Res* 2008; 32(3): 487–490.
68. Van Den Berg DJ, Francke U. Roberts syndrome: a review of 100 cases and a new rating system for severity. *Am J Med Genet* 1993; 47(7): 1104–1123.
69. Levy M, Sacrez R, Luckel JC, Warter S, Stoll C, Szwarcberg R. Severe malformations of the limbs and oligophrenia in a family with chromosome studies. *Ann Pediatr* 1972; 19(4): 313–320.
70. Judge C. A sibship with the pseudothalidomide syndrome and an association with Rh incompatibility. *Med J Aust* 1973; 2(6): 280–281.
71. Freeman MV, Williams DW, Schimke RN, Temtamy SA, Vachier E, German J. The Roberts syndrome. *Clin Genet* 1974; 5(1): 1–16.
72. Vega H, Waisfisz Q, Gordillo M, Sakai N, Yanagihara I, Yamada M, van Gosliga D, Kayserili H, Xu C, Ozono K, Jabs EW, Inui K, Joenje H. Roberts syndrome is caused by mutations in ESCO2, a human homolog of yeast ECO1 that is essential for the establishment of sister chromatid cohesion. *Nat Genet* 2005; 37(5): 468–470.

73. McKusick VA, Abbey H, Bartalos M, Bowen P, Boyer SH IV, Cohen BH, Danks DM, Duchastel Y, Emery AEH, Epstein EJ, Fainer DC, Finn R, et al. Medical genetics 1962. *J Chronic Dis* 1963; 16: 457–634.
74. Salk D. Can we learn about aging from a study of Werner's syndrome? *J Am Geriatr Soc* 1982; 30(5): 334–339.
75. Shonberg S, Niermeijer MF, Bootsma D, Henderson E, German J. Werner's syndrome: proliferation in vitro of clones of cells bearing chromosome translocations. *Am J Hum Genet* 1984; 36(2): 387–397.
76. Crabbe L, Jauch A, Naeger CM, Holtgreve-Grez H, Karlseder J. Telomere dysfunction as a cause of genomic instability in Werner syndrome. *Proc Natl Acad Sci USA* 2007; 104(7): 2205–2210.
77. Yu CE, Oshima J, Fu YH, Wijsman EM, Hisama F, Alisch R, Matthews S, Nakura J, Miki T, Ouais S, Martin GM, Mulligan J, Schellenberg GD. Positional cloning of the Werner's syndrome gene. *Science* 1996; 272(5259): 258–262.
78. Goto M. Hierarchical deterioration of body systems in Werner's syndrome: implications for normal aging. *Mech Ageing Dev* 1997; 98(3): 239–254.
79. Tanabe M, Goto M. Elevation of serum hyaluronan level in Werner's syndrome. *Gerontology* 2001; 47(2): 77–81.
80. Ying KL, Oizumi J, Curry CJ. Rothmund-Thomson syndrome associated with trisomy 8 mosaicism. *J Med Genet* 1990; 27(4): 258–260.
81. Lindor NM, Devries EM, Michels W, Schad CR, Jalal SM, Donovan KM, Smithson WA, Kvols LK, Thibodeau SN, Dewald GW. Rothmund-Thomson syndrome in siblings: evidence for acquired in vivo mosaicism. *Clin Genet* 1996; 49(3): 124–129.
82. Kitao S, Shimamoto A, Goto M, Miller RW, Smithson WA, Lindor NM, Furuichi Y. Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. *Nat Genet* 1999; 22(1): 82–84.

Contributed protocol section

IMPORTANT: No protocol included in this manual should be used for clinical testing unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements for all chemicals used in the laboratory.

Protocol 13.1 Fanconi anemia chromosome breakage procedure for whole blood

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

For the diagnosis of the recessive genetic disorder Fanconi anemia by detection of the increased chromosome breakage caused by specific chromosome clastogens. Mitomycin C (MMC) and diepoxybutane (DEB) are known chromosome clastogens. Individuals with a normal chromosome repair mechanism when exposed to low levels of MMC for 96 hours or DEB for 48 hours show low level breakage (<6% of cells with radials as a rule) while cells from subjects with Fanconi anemia exposed to comparable concentrations of clastogens show markedly increased and multiple radial forms.

Safety warnings

All tissue specimens should be handled as biohazardous, using Universal Precautions. Use the laminar flow hood for all steps up to harvest spin. Wear a laboratory coat and protective gloves for all steps through slide-making. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with fresh Sanimaster 4 (made weekly). Cover cuts with protective bandages even when gloves are worn. Dispose of Pasteur pipettes in sharps container. Wash hands thoroughly after removing gloves.

Mitomycin C, Colcemid, and diepoxybutane are toxic chemicals and should be handled accordingly. Avoid contact with skin.

Diepoxybutane (DEB) is a CARCINOGEN, toxic, mutagenic, irritant. Flush for 15 minutes in case of contact. Readily absorbed through skin, wear gloves and lab coat at all times.

Mitomycin C (MMC) is a CARCINOGEN, toxic, mutagenic, irritant; may be fatal if swallowed or absorbed through the skin. Overexposure may cause reproductive disorders. Flush skin or eyes for 15 minutes in case of contact. Seek medical attention if swallowed. Wear gloves and lab coat at all times when handling.

Colcemid® is mutagenic, tumorigenic, embryotoxic and teratogenic with acute overexposure. Wear gloves and lab coat at all times when handling. Avoid contact with skin or inhalation, flush for 15 minutes if accidental contact.

Specimen

Lymphocytes are obtained through procurement of anticoagulated peripheral blood. Three to five mL sodium heparinized whole blood are required for optimum results. However, other anticoagulants, though not recommended, may be accepted. Samples should be kept at room temperature and sent to the laboratory as soon as possible for optimum viability. Samples sent by overnight mail generally yield adequate results.

II. Materials

Supplies

1. Micropipettes, 200 µL, 20 µL
2. ART 200 pipette tips with plugs
3. Corning polypropylene tube, 15 mL (Lab Stores #64.2005)
4. Pasteur pipettes, 5¾ inches (Lab Stores #63.1614)
5. Pipette bulbs, 2 mL (Lab Stores #63.0202)
6. Sterile pipettes, 5 mL (Falcon #7543)
7. Sterile pipettes, 10 mL (Falcon #7551)
8. Sterile pipettes, 25 mL (Falcon #7525)
9. Sterile pipettes, 1 mL (Falcon #7521)

Culture media and stock reagents

10. RPMI 1640 (Gibco® #11875-051)
11. Fetal Bovine Serum (Irvine #3000)
12. Glutamine, 200 mM (Gibco® #250030-016)
13. Gentamicin 50 mg/mL (BioWhittaker #17-5282)
14. Incomplete media

50 mL of Fetal Bovine Serum

10 mL Glutamine

0.5 mL Gentamicin

439.5 mL RPMI 1640

Total = 500 mL; aliquot into 250 mL bottle and label as incomplete media (also known as bone marrow media).

15. Phytohemagglutinin M-Form (Gibco® #10576-023)

As needed, add 0.5 mL of PHA-M to 5 mL of incomplete media. This is now complete medium used to grow peripheral blood lymphocytes.

16. KCl (Mallinckpost (MCB) #6838)

Hypotonic KCl = 5.59 g/L of deionized H₂O

Aliquot into 100-mL bottles and refrigerate. Warm to 37 °C before using.

17. Methanol, Absolute (Mallinckpost #3016)

Methanol is EXTREMELY flammable, may cause flash fires. May be fatal or cause blindness if swallowed. Harmful if inhaled or absorbed through skin. Cannot be made nonpoisonous. In case of contact, flush skin or eyes for 15 minutes, remove to fresh air in case of inhalation. Seek medical attention if swallowed. Use gloves, lab coat, and goggles at all times.

18. Glacial Acetic Acid (EM Science #AX0073-9)

Acetic acid is flammable as a liquid and a vapor. Avoid contact with skin, inhalation or ingestion. May be fatal if swallowed. Causes severe eye and skin burns. In case of contact, flush skin or eyes for 15 minutes, remove to fresh air in case of inhalation. Do not induce vomiting if swallowed. Use gloves, lab coat, and goggles at all times.

Fixative = 3 parts methanol to 1 part glacial acetic acid. Make fresh before each use.

19. Hanks' 10× (Gibco® #14180-020)
Hanks' (1×) = 9 parts double distilled sterile water to 1 part Hanks' (10×)
20. Colcemid® 10 µg/mL (Gibco® #15210-016)
21. Mitomycin C (MMC) – Sigma C #M-0503

Stock solution

Add 5 mL sterile distilled water to vial containing 2 mg mitomycin C.

Working solution

Add 0.05 mL (50 µL) of stock to 9.95 mL of RPMI 1640.

When 0.1 mL (100 µL) of working solution is added to 5 mL of complete medium, the final concentration will be 40 ng/mL.

Make mitomycin stock and working solution every 6 months.

22. Diepoxybutane (DEB) – 1,3-butadiene diepoxide (Sigma #20,253-3, 5 g)

Stock solution

Add 10 µL stock DEB to 10 mL RPMI 1640.

Working solution

Add 50 µL of diluted to 10 mL RPMI 1640.

When 0.1 mL (100 µL) of working solution is added to 5 mL of complete medium, the final concentration will be 100 ng/mL.

Make DEB dilutions and working solution fresh each time (4-day half life).

23. Wright stain (MCB #WX005-3)

Working solution is made by adding 0.3 g of Wright powder to 200 mL of methanol. Agitate for 1 minute then let stand another 9 minutes. Filter through #1 Whatman filter paper into a brown bottle.

III. Blood culture and setup procedure

1. Allow blood in vacutainer to settle for 1 hour. If hematocrit is approximately 50%, proceed to #2. If it is less than 50%, remove top and pipet serum off until you have a 50 : 50 ratio of cells to serum. Mix thoroughly by inverting vacutainer.
2. Determine the amount of blood to add to each culture tube, according to the white blood cell count and hematocrit of the patient.
 - For patients with normal counts and normal hematocrits, add 0.35-0.4 mL of heparinized whole blood per culture using a 1 mL pipette.
 - For anemic patients, add 0.45–0.5 mL of the 50 : 50 cell-serum mixture.
 - For severely anemic patients, buffy coat is recommended. The entire buffy coat from 5 mL whole blood may be needed per culture in severely anemic patients.
3. Add the blood to each of four 15-mL polypropylene centrifuge tubes containing 5 mL of RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 2% glutamine, 1% Gibco® phytohemagglutinin-M form (PHA) and 0.1% gentamicin (50 mg/mL).
4. Label and treat each culture as follows (see Note 1, Use of two clastogens):
 - Culture B = no clastogen (internal control) (use white tape)
 - Culture MMC1 = 40 ng/mL MMC, final concentration (use orange tape)
 - Culture MMC2 = 20 ng/mL MMC, final concentration (use orange tape)
 - Culture DEB3 = 100 ng/mL DEB, final concentration (use blue tape)
 - Culture MMC3 = 40 ng/mL MMC, final concentration (set up for NB or children) [use orange tape]

A normal control group should be set up for each new lot of clastogens. Setup three cultures as follows:

Culture B = no clastogen [use white tape]

Culture MMC1 = 40 ng/mL MMC, final concentration [use orange tape]

Culture DEB3 = 100 ng/mL DEB, final concentration [use blue tape]

Whenever possible, positive FA controls should be tested. If none are available, testing a positive FA lymphoblast cell line would be advisable.

5. Wrap all cultures, except Bs, with aluminum foil to protect against light degradation of the clastogens, especially MMC.
6. Incubate MMC cultures for 96 hours, adding MMC at T=0; DEB cultures are grown for 72 hours, adding DEB at T=24 hr.
7. Harvest by standard procedure.
8. Stain slides with Wright stain for 2 minutes with no pre-treatment (unbanded).
9. Score 50 to 100 cells from MMC1 and DEB3 cultures for RADIAL formations and breakage. If adequate numbers of metaphases are not obtained from MMC1 cultures, use MMC2 cultures. Score the B culture for spontaneous breakage only if MMC is positive.
10. Photographically document RADIALS and increased breakage in all scored cultures.
11. Prepare grid as shown. Use data from controls and FA patients.

Patient name	Clastogen	Conc (ng/mL)	Number of cells with:										% Cells with radials	Total no. of cells
			0 brk	1 brk	2 brk	3 brk	4 brk	5 brk	6 brk	7 brk	>8 brk	Radials 1 >1		
	MMC	40	0	0	0	0	0	0	0	0	0	0	0	50
	DEB	100	0	0	0	0	0	0	0	0	0	0	0	50
Normal control average (N=10)	MMC	40	33	11	1	4	0	0	0	0	0	1	2	50
Normal control average (N=10)	DEB	100	46	3	1	0	0	0	0	0	0	0	0	50
Fanconi control average (N=11)	MMC	40	2	2	1	1	1	1	0	0	0	42	84	50
Fanconi control average (N=11)	DEB	100	15	10	8	5	2	1	1	1	1	6	12	50

¹ Neither mitomycin C (MMC) nor diepoxybutane (DEB)-treated cultures showed increased breaks or radial forms. There is no evidence for the diagnosis of Fanconi anemia (FA). If FA is of strong clinical suspicion, a fibroblast breakage study may be useful in addressing potential somatic mosaicism.

² Both mitomycin C (MMC) and diepoxybutane (DEB)-treated cultures showed excess breakage and radial forms. This is consistent with the clinical diagnosis of Fanconi anemia.

IV. Notes

1. Use of two clastogens: The rationale for the dual clastogen system is to provide internal controls, MMC for DEB and vice versa, as they are added at different time points. Using two concentrations of MMC further insures addition of clastogen. In addition, overly sensitive cells may do better at the lower concentration.

V. Additional readings

1. Cervenka J, Hirsch BA. Cytogenetic Differentiation of Fanconi Anemia, "Idiopathic" Aplastic Anemia, and Fanconi Anemia Heterozygotes. *Am J Med Genet* 1983;15:211-223.
2. Auerbach A, Rogatko A, Schroeder-Kurth TM. International Fanconi Anemia Registry: Relation of Clinical Symptoms to Diepoxybutane Sensitivity. *Blood* 1989(Feb);73(2):391-396.

Protocol 13.2 Supplemental procedure; Ficoll separation of whole blood

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

The specific gravities for the individual cell types found within whole blood are not uniform. Blood lymphocytes from a whole blood sample with low white blood cell counts (WBC) and/or low lymphocyte percentages (% L) may require concentration for culture and eventual cytogenetic analysis. Centrifugation of whole blood against a high-density fluid (Ficoll–Paque) is successful in separating lymphocytes from the other components found within the blood mixture (i.e., serum, red blood cells, platelets, etc.). With this procedure, lymphocytes can be extracted from relatively large volumes of whole blood (5–25 mL) that would prove otherwise insufficient in lymphocyte counts for successful culturing.

Safety warnings

All tissue specimens should be considered hazardous. Use Biohazard handling procedures and follow Universal Precautions.

1. Use the laminar flow hood for all steps up to harvest spin.
2. Wear a laboratory coat and protective gloves for all steps through slide-making. Cover cuts with protective bandages even when gloves are worn. Wash hands thoroughly after removing gloves.
3. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with fresh Sanimaster 4 (made fresh weekly).
4. Pasteur pipettes, needles, and syringes should be disposed in the sharps container. Do not bend, recap, break, or remove needles from disposable syringe. Dispose all needles in labeled red sharps container.
5. Ficoll–Paque is a potential irritant to eyes, respiratory system, and skin. This product may also be harmful if ingested. Complete toxicological properties have yet to be determined.

Specimen

Lymphocytes are obtained through procurement of anticoagulated peripheral blood. For optimum results, 5–25 mL of sodium-heparinized whole blood is required. Samples should be kept at room temperature and sent to the laboratory as soon as possible for optimum viability. Samples sent by overnight mail generally yield adequate results.

II. Materials

1. Corning polypropylene tube, 15 mL (Lab Stores #64.2005)
2. Sterile pipettes: 1 mL (Falcon #7521), 5 mL (Falcon #7543), 10 mL (Falcon #7551), 25 mL (Falcon #7525)
3. RPMI 1640 “Complete Media” (See Protocol 3.1, Blood culture and harvest procedure for recipe and instructions)
4. Ficoll-Paque PLUS (Amerisham GE Health #171440002)
5. Hypotonic Solution: 0.075 M KCl (Mallinckrodt #6838)
Add 5.59 g of KCl to 1 liter of deionized H₂O. Aliquot into 100-mL bottles and store at 4 °C. Shelf life is 4 weeks. Warm to 37 °C before using.
6. Fixative: Add 3 parts absolute methanol (Mallinckrodt #3016) to 1 part glacial acetic acid (EM Science #AX0073-9). Make fresh before each use. WARNING: Methanol is EXTREMELY flammable and may cause flash fires. *May be fatal or cause blindness if swallowed.* Harmful if inhaled or absorbed through skin. Cannot be made nonpoisonous. In case of contact, flush skin or eyes for 15 minutes, remove to fresh air in case of inhalation. Seek medical attention if swallowed. Use gloves, lab coat, and goggles at all times. Acetic acid is flammable as a liquid and a vapor. Avoid contact with skin, inhalation or ingestion. *May be fatal if swallowed.* Causes severe eye and skin burns. In case of contact, flush skin or eyes for 15 minutes, remove to fresh air in case of inhalation. Do not induce vomiting if swallowed. Use gloves, lab coat, and goggles at all times.

III. Method

A. Ficoll separation procedure

1. Assess the total volume of blood that has arrived for culturing. You will need *at least* an equal volume of Ficoll to perform the separation.

2. Calculate how many 15 mL Corning polypropylene tubes you will need to perform the separation based on the requirement that each tube to be spun will need between 2–6 mL of blood. Please note: Every tube used should go toward only one culture. However, if the counts are extremely low, multiple tubes may be needed for a single culture to work. Do not divide one tube into multiple cultures.
 3. For each tube, add the Ficoll syrup in at least an equal volume to the blood volume to be used.
 4. GENTLY, add the whole blood to the tube so that it is layered on top of the Ficoll syrup. Be careful not to invert, shake, or bump the tubes in any way that will call the Ficoll and blood layers to mix. KEEP THEM LAYERED! You will note that in little time the effects of gravity will begin to pull small amounts of red blood cells into the Ficoll layer. That is expected.
 5. Centrifuge the tubes at 3000 RPM for 30 minutes. Use a centrifuge with internal climate control, if available. Older centrifuges may get hot.
 6. After centrifugation, the samples should be layered as follows: red blood cells on the bottom, Ficoll syrup above that, a small and sometimes imperceptible layer of white cells (buffy coat) next, and then the blood serum on top.
 7. Using sterile technique, aspirate off some of the serum down to near the buffy coat. Take care not to disturb the buffy coat. Next, aspirate out the remaining serum and buffy coat. It is ok to also aspirate out some of the Ficoll below the buffy coat, but try to keep it to a minimum.
 8. Add this buffy coat layer to 5 mL Complete RPMI in a new 15 mL polypropylene centrifuge tube. Mix the contents of tube well to dilute out and rinse any remaining Ficoll from buffy coat.
 9. Spin the tube at 1000 RPM for 10 minutes.
 10. Once spinning is complete, there should be a pellet of lymphocytes at the bottom of the tube. Use this pellet to culture as needed in place of whole blood, following either Protocol 3.1, Blood culture and harvest procedure in Chapter 3, or Protocol 13.1, Fanconi anemia chromosome breakage procedure.
- **PLEASE NOTE**: Ficoll-treated cultures have been observed as having a slightly larger number of breaks/radial formations in the MMC cultures of normal patients than typical whole blood cultures. A half dose of MMC (20 ng/mL) is strongly recommended). These cultures can be treated the same as any nonFicolled cultures until the first centrifugation step in the harvest procedure. The Ficolled cultures do not have any red blood cells, so the hypotonic and fixative steps are reduced.

B. Harvest of Ficolled culture

1. After the first spin in the harvest procedure, remove supernatant. Add 4 mL warm (37 °C) 0.075M KCl to each tube and resuspend cell pellet.
2. Cap the sample and let stand for 10–15 minutes without centrifugation. Usually, you can proceed with the other non-Ficolled tubes in the harvest and spin them while this sample sits.
3. After the wait, add 5 drops fresh fixative to each tube and mix well. Centrifuge for 10 minutes at 1000 RPM. This can be done along with the other non-Ficolled tubes in the harvest after they receive their second “hypo.”
4. **Each tube must be treated individually for this step.** Remove supernatant and resuspend cell pellet. Add 2 mL fresh fixative and mix well. Add an additional 2 mL fixative and mix well. Repeat for each individual tube. Centrifuge for 10 minutes at 1000 RPM.
5. The harvest for the Ficoll separated material is now complete, and ready for slide-making.

IV. Reference

1. Ficoll-Paque PLUS. For *in vitro* isolation of lymphocytes. Amerisham Biosciences (Handbook); 2008. <http://fachschaft.biochemtech.uni-halle.de/downloads/chromatography/ficoll.pdf>

Protocol 13.3 Fanconi anemia fibroblast set up, culture, subculture, and harvest procedure

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

For the diagnosis of the recessive genetic disorder Fanconi Anemia by detection of the increased chromosome breakage caused by specific chromosome clastogens. Mitomycin C (MMC) and diepoxybutane (DEB) are known chromosome clastogens. Fibroblasts of individuals with a normal chromosome repair mechanism when exposed for 48 hours to either MMC or DEB

show low-level breakage (<6% of cells with radials as a guide), while subjects with Fanconi Anemia show markedly increased breakage and multiple radial forms in both DEB- and MMC-treated cultures.

Safety warnings

All tissue specimens should be handled as biohazardous, using Universal Precautions. Use the laminar flow hood for all steps up to harvest spin. Wear a laboratory coat and protective gloves for all steps through slide-making. Avoid spills and contact of any biological materials with skin or mucous membranes. Clean up spills immediately with fresh Sanimaster 4 (made weekly). Cover cuts with protective bandages even when gloves are worn. Dispose of Pasteur pipettes in sharps container. Wash hands thoroughly after removing gloves.

Mitomycin C, Colcemid®, and diepoxybutane are toxic chemicals and should be handled accordingly. Avoid contact with skin.

Diepoxybutane (DEB) is a CARCINOGEN, toxic, mutagenic, irritant. Flush for 15 minutes in case of contact. Readily absorbed through skin, wear gloves and lab coat at all times.

Mitomycin C (MMC) is a CARCINOGEN, toxic, mutagenic, irritant; may be fatal if swallowed or absorbed through the skin. Overexposure may cause reproductive disorders. Flush skin or eyes for 15 minutes in case of contact. Seek medical attention if swallowed. Wear gloves and lab coat at all times when handling.

Colcemid® is mutagenic, tumorigenic, embryotoxic and teratogenic with acute overexposure. Wear gloves and lab coat at all times when handling. Avoid contact with skin or inhalation, flush for 15 minutes, if accidental contact.

Specimen

Ideally, tissue specimens of at least 0.5–1 cm³ are collected with sterile methods into closable containers with sterile culture medium supplemented with serum and antibiotics (transport medium). If transport medium is unavailable, sterile Ringer's solution (lactated or nonlactated) or sterile isotonic saline are acceptable. Specimens should be delivered to the laboratory as soon as possible (up to 2 days after collection), and should be well protected from temperature extremes. Samples sent by overnight mail generally yield adequate results. Skin samples should be deep enough to include the dermis layer.

Unacceptable specimens include dried-out, frozen, formalin-fixed, and over-heated specimens, each of which causes cell death and thus precludes culturing. In case of receipt of unacceptable specimens, notify referring physician immediately.

II. Materials

Supplies

1. Sterile Corning plastic Petri dishes, 35 cm²
2. Sterile forceps
3. Sterile scissors
4. Sterile scalpel handles with sterile disposable blades
5. Sterile T-25 Corning tissue culture flasks (#25100)
6. Sterile cotton-plugged Pasteur pipettes
7. Pipette bulbs, 2 mL (Lab Stores #63.0202)
8. Sterile graduated pipettes, 1 mL, 5 mL, and 10 mL
9. Inverted microscope
10. CO₂ controlled incubator at 37 °C

Reagents

1. Worthington Type I Collagenase, (#LS004196), 210 units/mg
Dilute collagenase with incomplete medium to a final concentration of about 1 unit per mL, rounding up to the closest multiple of 10 (e.g., for 142 U/mg, use 150 mL medium). Filter through a vacuum filtration unit with 0.2 µ pore size. Aliquot into 4 mL snap cap sterile tubes and freeze. Shelf life is one year.
2. MEM Alpha Complete
 - a. MEM Alpha (Gibco #12561)
 - 20% Fetal Bovine Serum (Irvine #3000)
 - 0.1% Gentamicin (BioWhittaker #17-5282)
 - 1% L-glutamine. (Gibco #250030-016) (Shelf life 14 days. Store at 4 °C.)

3. Hypotonic solution (warm to 37 °C before using). To prepare:
 - a. 9 mL 0.075 M hypotonic KCl
 - 5.59 g of KCl (Mallinckpost #6838)
 - 1 L of deionized H₂O
 - Aliquot into 100-mL bottles and refrigerate.
 - b. 2.4 mL sterile H₂O
 - c. 0.6 mL of fetal bovine serum
4. Fixative = 3 parts methanol to 1 part glacial acetic acid. Make fresh before each use.

Methanol, Absolute (Mallinckpost #3016)

Methanol is EXTREMELY flammable, may cause flash fires. May be fatal or cause blindness if swallowed. Harmful if inhaled or absorbed through skin. Cannot be made nonpoisonous. In case of contact, flush skin or eyes for 15 minutes, remove to fresh air in case of inhalation. Seek medical attention if swallowed. Use gloves, lab coat, and goggles at all times.

Glacial acetic acid (EM Science #AX0073-9)

Acetic acid is flammable as a liquid and a vapor. Avoid contact with skin, inhalation or ingestion. May be fatal if swallowed. Causes severe eye and skin burns. In case of contact, flush skin or eyes for 15 minutes, remove to fresh air in case of inhalation. Do not induce vomiting if swallowed. Use gloves, lab coat, and goggles at all times.

5. HBSS 1× (Hanks' Buffered Saline Solution)
 - 50 mL 10× HBSS (Irvine Scientific #9230)
 - 450 mL of sterile distilled water
6. Colcemid® 10 µg/mL (Gibco #15210-016)
7. Mitomycin C (MMC) (Sigma #M-0503)

Stock MMC Solution: Add 5 mL sterile distilled water to vial containing 2 mg of Mitomycin C.
Working MMC Solution: Add 0.05 mL (50 µL) stock MMC to 9.95 mL MEM Alpha.
 When 38 µL of working solution is added to 5 mL complete medium, the final concentration will be 15 ng/mL.
 Make Mitomycin Stock and Working Solution every 6 months.
8. Diepoxybutane (DEB) (Sigma #20,253-3, 5 g of 1,3-Butadiene diepoxide)

Stock DEB Solution: Add 10 µL of DEB to 10 mL MEM Alpha.
Working DEB Solution: Add 50 µL of stock DEB to 10 mL MEM Alpha.
 When 150 L working DEB solution is added to 5 mL complete medium, the final concentration will be 100 ng/mL.
 Make DEB dilutions and working solution fresh each time (4-day half life).
9. Wright stain (MCB #WX005-3)

Working solution is made by adding 0.3 g of Wright powder to 200 mL of methanol. Agitate for 1 minute then let stand another 9 minutes. Filter through #1 Whatman filter paper into a brown bottle.
10. Trypsin-EDTA 1×
 - 2 mL of Trypsin-EDTA 10× (Gibco #610-5400AG)
 - 18 mL HBSS 1×.
11. Gas: 5% CO₂, 5% O₂, 90% N₂

III. Methods

FA fibroblast setup and culture procedure

There are two methods for establishing solid-tissue cultures: the collagenase method which dissolves the collagen and elastin fiber matrix of the sample, thereby releasing the cells from the tissue; and the explant method in which very small, minced-up fragments of tissue are allowed to attach to the flask and grow from the edges out. The collagenase method is always preferred.

A. Tissue preparation

1. Rinse tissue in HBSS 1×. Remove any excess adipose tissue.
2. Go to either the **B. Collagenase method** or **C. Explant method**.

B. Collagenase method

1. Place tissue in one sterile 35 mm² Petri dish. Use about 5 mm² of tissue per dish. Add 4 mL of collagenase to dish. Mince the tissue as much as possible to increase the total surface area of tissue being digested by the collagenase. Note time.
2. Incubate at 37 °C with 5% CO₂; check the sample every half hour. The smaller the pieces, the less time is required. Check tissue dissociation on an inverted scanning microscope. Tissues should appear translucent and cellular. If not, incubate further.
3. Add cell-medium suspension to a sterile centrifuge tube and pipet vigorously to dissociate any clumps. Cap tubes and centrifuge at 1000 RPM for 10 minutes @ RT.
4. Label flasks with GL#, flask #, date, tissue type, and patient name.
5. Decant supernatant and resuspend cell pellet in 1-2 mL of MEM Alpha complete medium, depending on the pellet size. Seed 2 mL of suspension in a T-25 flask. Gas with 5% CO₂, 5% O₂, and 90% N₂ for 5–10 seconds, close tightly, and incubate at 37 °C. Check for growth, and feed with 2 mL media at 3–5 days.
6. Skip to step D. Subculturing FA fibroblast cultures from solid tissues.

C. Explant method

1. Transfer cleaned, appropriate tissue into Petri dish. Moisten with 1 mL medium, and mince each tissue with scissors and/or scalpel into fragments as small as possible.
2. Transfer fragments with the 1 mL medium into T-25 flasks and rotate flask to allow cell suspension to cover the surface of the growth area. If there is more than 1 mL of medium, cells will not be in contact with growth surface, and may not attach. Remove excess medium, if necessary.
3. Label flasks with GL#, flask #, date, tissue type, and patient name.
4. Gas with 5% CO₂, 5% O₂, 90% N₂ for 5–10 seconds; tighten cap.
5. Leave undisturbed in incubator for 3–5 days. Then check cultures for growth and feed as follows: if good growth is observed, pour off medium and feed with 3 mL of fresh medium. If no growth is observed, pipet off the medium and gently replace with 1 mL fresh medium. Re-gas cultures which are not growing.
6. Cultures will yield better harvests if subcultured first. Subculture when three or more colonies are seen per flask that fill the lower-power field during microscopy and appear mitotic.

D. Subculturing FA fibroblast cultures from solid tissues

Fibroblast cultures are subcultured when growth is such that when transferred, the subcultured flask reaches confluence in 3–5 days.

1. Pour off medium into biohazard waste container.
2. Rinse cells in flask twice with 2 mL HBSS 1× solution and pipet off into waste container. Add 1 mL trypsin-EDTA 1× solution and incubate on the warming plate or in the 37 °C incubator for 1–5 minutes, until cells are rounded up and floating. Rap flask sharply on the countertop to encourage cell detachment.
3. Depending upon the mitotic activity of the cultures, select a T-25 flask for rapidly-dividing cells.
4. Pipet cells a few times against the culture flask to encourage single cell suspension. Pipet an appropriate volume of cells into the new T-25 flask. Volume to be transferred depends upon cell density and activity, and varies from about 0.1 mL to 0.9 mL.
5. Label flask with culture designation (A, B, C, etc.), GL#, patient's name, date, tissue of origin. Feed both flasks with fresh medium to bring volume to a total of 5 mL in the T-25 flasks. Gas cultures and incubate @ 37 °C.
6. Record subcultures in FA log book.
7. After the study has been reported out and no further clinical work is necessary, freeze down the cultured cells (in MEM Alpha complete with DMSO @ 10%) for long term storage in liquid nitrogen (see Chapter 4, Protocol 4.28, Freezing Tissue Cultures (Cryopreservation)).

FA fibroblasts treatment and harvest

1. Three fibroblast cultures (5 mL complete MEM Alpha in T-25 flask), each at 25–40% confluency, are needed to perform the full range of testing:
 - a. C culture = no clastogen (internal control)
 - b. M15 culture = MMC @ 15 ng/mL (38 µL of working solution to 5 mL of culture)
 - c. D150 culture = DEB @ 150 ng/mL (150 µL of working solution to 5 mL of culture)

2. All treated cultures are wrapped in aluminum foil to protect against light induced degradation of the clastogens.
3. All cultures are grown for 48 hours at 37 °C.
4. Colcemid® (15 µL per culture) is added ~6 hours prior to harvest. Continue to incubate @ 37 °C.
5. After a sufficient number of cells are rounded up and floating (visible with an inverted microscope), begin harvest.
6. Label one 15 mL centrifuge per culture: C, M15, D150.
7. Decant medium from each culture into its own labeled 15 mL centrifuge tube.
8. Rinse each culture flask with 2 mL HBSS 1x twice. Decant each rinse to its corresponding 15 mL centrifuge tube.
9. Add 1 mL trypsin-EDTA solution to each culture flask. Incubate 3-5 minutes @ 37 °C. Tap culture flasks to help loosen cells, if necessary.
10. Aspirate trypsinized cell suspension with pipette and transfer to corresponding 15 mL centrifuge tube.
11. Rinse each culture flask with 2 mL HBSS 1x and transfer to corresponding 15 mL centrifuge tube.
12. Centrifuge tubes at 1000 RPM for 10 minutes @ RT.
13. Aspirate supernatant down to approximately 0.1 mL above cell pellet.
14. Resuspend cells by flicking bottom of tube with your finger.
15. Slowly add 10 drops of hypotonic solution and mix, flicking the bottom of the tube with your finger. Slowly bring volume of hypotonic solution to 2-4 mL (depending upon the original size of the cell pellet), mix as before and let sit at RT for 15 minutes.
16. Add 1 mL fresh fixative dropwise and mix as before.
17. Centrifuge as before.
18. Aspirate supernatant down to approximately 0.1 mL above cell pellet.
19. Resuspend cells as before taking care to leave no clumps. Slowly add 1 mL fresh fixative dropwise, and mix. Bring volume to 2-4 mL (depending upon pellet size) and let sit at RT for 20 minutes.
20. Centrifuge as before.
21. Aspirate supernatant avoiding pellet. Add fresh fix and make slides for breakage analysis.
22. Score 50 metaphases per culture for radials/breaks.
23. Record results using the following chart format.

NOTE: The rationale for the dual clastogen system is to provide internal controls, MMC for DEB and vice versa, as they are added at different time points. Two concentrations of MMC further insures addition of clastogen. In addition, overly sensitive cells may do better at the lower concentration.

Patient Name	Clastogen	Conc (ng/mL)	Number of cells with:										% Cells with radials	Total no. of cells
			0 brk	1 brk	2 brk	3 brk	4 brk	5 brk	6 brk	7 brk	>8 brk	Radials		
			1	>1										
	MMC	15	0	0	0	0	0	0	0	0	0	0	0	50
	DEB	150	0	0	0	0	0	0	0	0	0	0	0	50
Normal controls	MMC	15	45	3	1	0	0	0	0	0	0	1	2 ¹ Range: 0-4	50
Normal controls	DEB	150	44	4	1	0	0	0	0	0	0	1	2 ¹ Range: 0-6	50
Fanconi controls	MMC	15	11	4	3	2	1	1	0	0	1	27	54 ² Range: 14-92	50
Fanconi controls	DEB	150	14	5	1	2	1	1	1	0	1	24	48 ² Range: 22-72	50

¹ Neither mitomycin C (MMC) nor diepoxybutane (DEB) treated cultures showed increased breaks or radial forms. There is no evidence for the diagnosis of Fanconi anemia (FA).

² Both mitomycin C (MMC) and diepoxybutane (DEB) treated cultures showed excess breakage and radial forms. This is consistent with the clinical diagnosis of Fanconi anemia.

IV. Additional readings

1. Cervenka J, Hirsch BA. Cytogenetic Differentiation of Fanconi Anemia, "Idiopathic" Aplastic Anemia, and Fanconi Anemia Heterozygotes. *Am J Med Genet* 1983;15:211–223.
2. Auerbach A, Rogatko A, Schroeder-Kurth TM. International Fanconi Anemia Registry: Relation of Clinical Symptoms to Diepoxybutane Sensitivity. *Blood* 1989(Feb);73(2):391–396.

Protocol 13.4 Fanconi anemia chromosome breakage analysis policy

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

Chromosome breakage analysis testing is used in the diagnosis of the recessive genetic disorder Fanconi anemia. Cultures from an individual suspected for this syndrome are treated with specific chromosome clastogens to induce chromosome breakage and radial formations. Cells from these cultures are analyzed and scored for the presence of these breaks and radials. Individuals with Fanconi anemia show markedly increased levels of breakage and radial formations compared to normal individuals with proper functioning chromosome repair mechanisms. This protocol establishes guidelines for chromosome breakage analysis of suspected Fanconi anemia syndrome patients.

A standard (see Note 1, Standard analysis) chromosome breakage analysis derived from a blood sample will have cultures treated with the following clastogens: 40 ng/mL Mitomycin C (MMC), 20 ng/mL MMC, and 100 ng/mL diepoxybutane (DEB). There is also one untreated culture.

Chromosome breakage analysis for fibroblast samples will have cultures treated with 15 ng/mL of MMC and 150 ng/mL of DEB. There is also one untreated culture.

II. Method

1. All standard* analysis cases should have 50 metaphase cells scored for chromosome breakage and radial formations from the MMC- and DEB-treated cultures. The untreated culture should be scored on all cases where the MMC- and DEB-treated cultures yield positive or "gray zone" (see Note 2, Gray zone analysis) results. The untreated culture may also be scored upon request of the referring physician.
2. Cells that contain zero to seven breaks should be scored in the appropriate column for breaks on the analysis sheet I (see Protocol 13.5, Table for breakage studies result interpretation). Each cell that contains eight or more breaks, and/or contains radial formations, should be scored individually on the analysis sheet for:
 Number of radials (**A**)
 Number of breaks that are not involved in radial formation (**B**)
 Total number of breaks, including breaks from radial formations (**C**)
 The nomenclature for reporting these cells is: **A/B/C** (see Note 3, Analysis examples)
3. To calculate the number of breaks, the following structural abnormalities are scored as such:
 chromosome break = 1 break
 chromatid break wider than chromatid width = 1 break
 radial = 2 breaks
 ring chromosome = 2 breaks
 dicentric chromosome = 2 breaks
4. A minimum of four cells should be captured from each culture scored. Cases that include cells with complex results may require more cells to be captured. If the breakage and radial formation score for a cell is uncertain, capturing that cell for director review is recommended.
5. Once the cultures have been read, the relevant data should be reported in the chromosome breakage worksheet, which should document specimen type and culture information. For each culture, the total number of breaks for all cells combined is reported within the table. This total number of breaks is divided by the total number of cells scored for each culture to get the ratio of breaks per cell. The chromosome report should include the appropriate Fanconi anemia breakage table and interpretation.

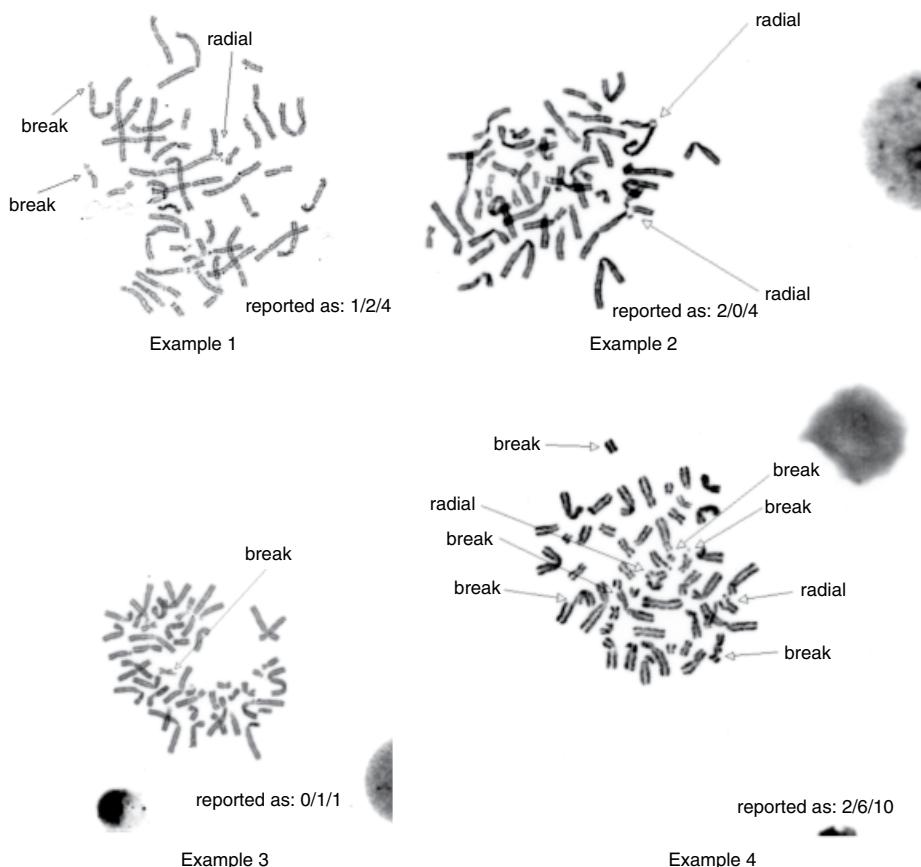


Figure 13.5 Reported breakage findings.

III. Notes

1. **Standard Analysis.** Standard analysis requires that samples have a sufficient WBC and lymphocyte percentage to allow for all four cultures. Samples with decreased WBC or lymphocyte percentages may require a reduction in the number of cultures due to inoculums requiring increased white cell densities.
 2. **Gray Zone Analysis.** “Gray zone” results are those where the 40 ng/mL MMC-treated blood culture yields $\geq 10\%-20\%$ radial formations.
 3. **Analysis Examples.** Examples of reported breakage findings are shown in Figure 13.5.

Protocol 13.5 Table for breakage studies result interpretation

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Method

A. Table for blood results

Normal controls	MMC	40	33	11	1	4	0	0	0	0	0	1	2	Range: 0–4 ¹	50	
Normal controls	DEB	100	46	3	1	0	0	0	0	0	0	0	0	Range: 0–1 ¹	50	
Fanconi controls	MMC	40	2	2	1	1	1	1	0	0	0	42	84	Range: 30–100 ²	50	
Fanconi controls	DEB	100	15	10	8	5	2	1	1	1	1	1	6	12	Range: 0–32 ²	50

B. Table for Dana-Farber results QA/QC

Patient name	Clastogen	Conc (ng/ml)	Number of cells with:											% Cells with Radials	Total # Cells	
			0 brk	1 brk	2 brk	3 brk	4 brk	5 brk	6 brk	7 brk	>8 brk	Radials 1	>1			
A	??	0	0	0	0	0	0	0	0	0	0	0	0	0	50	
B	??	0	0	0	0	0	0	0	0	0	0	0	0	0	50	
C	??	0	0	0	0	0	0	0	0	0	0	0	0	0	50	
Normal controls	MMC	40	33	11	1	4	0	0	0	0	0	1	2	Range: 0–4 ¹	50	
Normal controls	DEB	100	46	3	1	0	0	0	0	0	0	0	0	0	Range: 0–1 ¹	50
Fanconi controls	MMC	40	2	2	1	1	1	1	0	0	0	42	84	Range: 30–100 ²	50	
Fanconi controls	DEB	100	15	10	8	5	2	1	1	1	1	6	12	Range: 0–32 ²	50	

C. Table for fibroblast results

Patient Name	Clastogen	Conc (ng/mL)	Number of cells with:											% Cells with Radials	Total # Cells
			0 brk	1 brk	2 brk	3 brk	4 brk	5 brk	6 brk	7 brk	>8 brk	Radials 1	>1		
MMC	15	0	0	0	0	0	0	0	0	0	0	0	0	0	50
DEB	150	0	0	0	0	0	0	0	0	0	0	0	0	0	50
Normal controls	MMC	15	45	3	1	0	0	0	0	0	0	1	2	Range: 0–4 ¹	50
Normal controls	DEB	150	44	4	1	0	0	0	0	0	0	1	2	Range: 0–6 ¹	50
Fanconi controls	MMC	15	11	4	3	2	1	1	0	0	1	27	54	Range: 14–92 ²	50
Fanconi controls	DEB	150	14	5	1	2	1	1	1	0	1	24	48	Range: 22–72 ²	50

¹ Neither mitomycin C (MMC) nor diepoxybutane (DEB) treated cultures showed increased breaks or radial forms. There is no evidence for the diagnosis of Fanconi anemia (FA).

² Both mitomycin C (MMC) and diepoxybutane (DEB) treated cultures showed excess breakage and radial forms. This is consistent with the clinical diagnosis of Fanconi anemia.

Protocol 13.6 Fanconi anemia

Contributed by Weisskopf Child Evaluation Center, Department of Pediatrics, School of Medicine, University of Louisville, Louisville, Kentucky

I. Principle

Fanconi anemia is a hematopoietic disorder caused by an autosomal recessive condition. Although many patients exhibit a high proportion of spontaneous chromosomal breakages, a number do not. The alkylating agent, (\pm)-1,2:3,4-diepoxybutane (DEB), is employed in this test to ascertain patients with FA who may not express spontaneous breakage and to differentiate between patients with true FA and those with idiopathic aplastic anemia.

II. Materials

Reagents

1. (\pm)-1,2:3,4-diepoxybutane (DEB). Aldrich #20253-3, 1 g. Store at 0–5 °C. Protect from light.
Caution: diepoxybutane (DEB) is poison. It is readily absorbed through the skin and may be harmful if inhaled. Wear gloves and open only under laminar flow hood when using.
2. DEB, stock solution. Add 1 mL RPMI 1640 medium to vial containing 1 gram DEB. Allow to dissolve completely. Withdraw 0.1 mL DEB from vial. Dilute with 9.9 mL RPMI 1640. Store in refrigerator. Protect from light.
3. DEB, working solution. Dilute 2 mL of stock DEB with 24 mL RPMI 1640. Store in refrigerator; protect from light.
4. McIlvane's buffer. Mix 200 mL 0.1 M citric acid and 800 mL 0.2 M sodium phosphate. Measure pH using pH meter. Adjust to pH 7.0 using the component needed, that is, if the pH is above 7.0, add citric acid solution. If below 7.0, add more sodium phosphate solution.
5. Giemsa stain (Harleco #620 or Gurr #R66). Store in refrigerator. Discard if staining is inadequate.
6. Giemsa staining solution: 46 mL distilled water, 4 mL McIlvane's buffer, 3 drops 0.1 M citric acid, 2 mL Giemsa stain.
7. Water, distilled.

III. Method

1. Set up three 12 mL blood cultures on the patient being tested. Mark one “no addition”, and two “DEB.”
2. Set up two 12 mL blood cultures on a normal control that is the same sex as the person being tested. Label one “no addition” and the other “DEB.”
3. After 24 hours in culture, add 2 or the indicated number of drops of DEB working solution using a 1 mL syringe, fitted with a 25-gauge needle, to the patient and the control “DEB” cultures.
4. **Caution:** diepoxybutane (DEB) is poison. It is readily absorbed through the skin and may be harmful if inhaled. Wear gloves and open only under laminar flow hood when using.
5. Harvest all six cultures after a minimum of 72 hours of culture (a minimum of 48 hours after addition of agents) using the regular blood harvest procedure.
6. Prepare slides as follows:

	Patient	Control
No addition of chemicals	4 slides	3 slides
DEB	10 slides	5 slides

7. After slides are made, give them to the laboratory director or designee for coding.
8. After coding, rinse in McIlvane's Buffer.
9. Stain slides in Giemsa as indicated by the coding. Usually one or two slides are GTG banded and the others stained without banding.
10. Rinse in distilled water.

IV. Notes

1. The laboratory technical director makes up new batches of DEB and tests them in culture to determine the number of drops to be used. The amount added should be sufficient to induce breaks without depressing the culture growth.

V. Additional reading

1. Cervenka J, Hirsch BA. Cytogenetic differentiation of Fanconi anemia "ideopathic" aplastic anemia, and Fanconi anemia heterozygotes. *American Journal of Medical Genetics* 1983;15:211–223.

CHAPTER 14

Microscopy and imaging

Margaret J. Barch¹ and Helen J. Lawce²

¹*(deceased) formerly, Frank F Yen Cytogenetics Laboratory, Weisskopf Child Evaluation Center, University of Louisville, Louisville, KY, USA

²Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA

14.1 The standard microscope

The microscope is an indispensable tool in cytogenetics. Technologists spend many hours using this remarkable instrument in the analysis of chromosomes and FISH. Therefore, an informed working knowledge of microscope theory and photomicrography is an asset bordering on necessity.

The simplest and the most complex microscopes all contain the following components (see Figure 14.1):

1. Eyepiece or ocular lenses. The oculars further magnify the image of the specimen, either to the eyes or to the camera.
2. Microscope tube or body tube. The body of the microscope may not actually resemble a tube in today's microscope, but it is the section that connects the objective and eyepieces.
3. Nosepiece. Most microscopes have a revolving nosepiece that holds several objective lenses.
4. Objective lenses. The objectives focus and magnify the image of the specimen. Some are equipped with a correction collar to correct for aberrations in the thickness of coverslips for best contrast and image quality.
5. Stand or limb. The stand is the backbone of the microscope, holding all of the mechanisms of the stage, substage, and tube.
6. Specimen stage. The stage holds the slide.
7. Substage condenser. The condenser focuses light on the specimen.
8. Iris or aperture stop diaphragm. The aperture stop is found in the substage condenser.
9. Coarse adjustment. This focus mechanism moves the lenses or the stage up and down, depending upon the model of microscope.
10. Fine adjustment. This knob moves the lens or stage a short distance for fine focusing.
11. Condenser adjustment. There is usually a rack and pinion mechanism that moves the condenser up and down relative to the stage.
12. Field stop diaphragm. The field stop is found below the condenser, usually in the base of the microscope. A filter may be placed here to increase contrast.
13. Base of microscope.
14. Light source.

In the early days of microscopy, daylight was the light source, and a mirror simply reflected light from a nearby window into the condenser. With time, artificial light became the preferred source because it is brighter and does not depend on the time of day and the weather. When the advantages of Köhler illumination (improved illumination by adjustment of the substage condenser) were discovered, the trend was to build the light source into the microscope so that the lamp could be easily aligned. Modern light microscopes generally contain a built-in tungsten filament electric lamp. Because this lamp has a slightly

* Editors' note: We lost Margaret in the final stages of producing this book. May her spirit shine through and the reader be touched by her love of science, and her passion for passing it on.

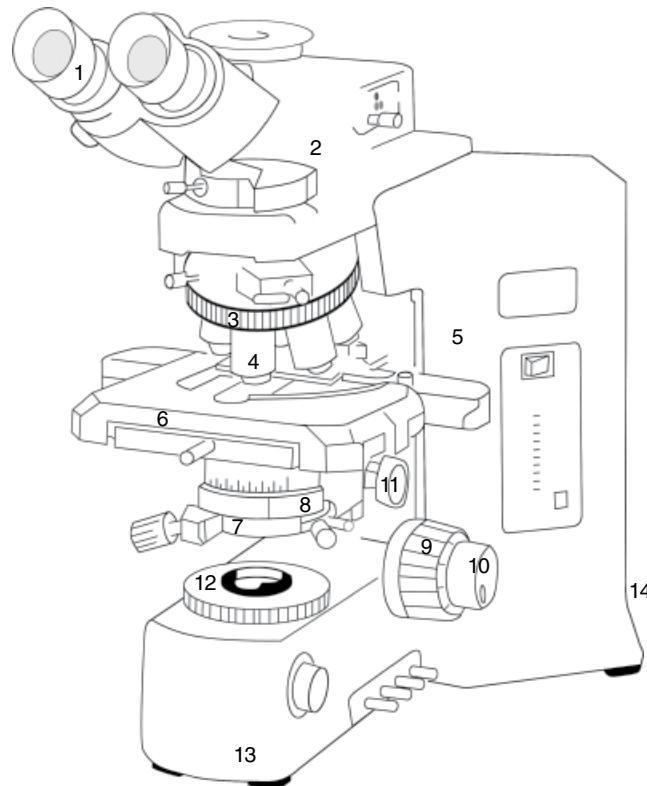


Figure 14.1 Parts of the microscope. (1) Eyepiece or ocular (2) Body tube (connects objectives and eyepieces) (3) Revolving nosepiece (4) Objective lens (5) Stand or limb (6) Specimen stage (7) Condenser with centering screws for Köhler illumination (8) Iris or aperture stop diaphragm (9) Coarse adjustment (10) Fine adjustment (11) Condenser adjustment (12) Field stop diaphragm (controls flare) (13) Base of microscope (14) Light source with centering screws is in the back (not shown).

yellow tint, a thin blue filter is often used to correct the color and may be found in the base of many brands of microscopes. Uneven illumination of the filament in the bulb is usually corrected by the use of a ground-glass disk in front of the lamp or by the use of frosted bulbs. A built-in mirror reflects the light up to the condenser. All the other modifications of the microscope are adjuncts to these basic parts and help them work more efficiently in a wide variety of conditions.

Both diaphragms mentioned above are important components of a microscope. All microscopes have at least two diaphragms, the field stop diaphragm and the aperture stop diaphragm. The proper use of these two stops is the key to producing sharp images and providing optimal clarity when using the microscope. The field stop and aperture stop diaphragms are similar, in that they are both leaves of metal that open and close to change the size of an opening through which light passes, but they serve entirely different functions. The field stop is used to view the light beam for centering and to control glare during Köhler illumination adjustments; it should not be used to change the intensity of illumination. This diaphragm limits the diameter of the light beam, allowing its edges to be imaged (focused) in the field of view at the same time the object is in focus. For the optimum light level, the field stop should be opened just slightly more than is necessary to illuminate the field of view. The aperture stop, on the other hand, can be imaged only in the back lens of the objective. To see its image, remove one eyepiece so that the back lens of the objective can be seen, and close the aperture stop until its edges become visible. The aperture stop changes the angle of the cone of light inside the objective and is used to adjust resolution and contrast. This adjustment also affects depth of field. Like the field stop, the aperture stop should not be used to change the brightness. Brightness should be adjusted by the regulating transformer or by a change of filters.

14.1.1 The light path

The light beam originates in the lamp, is deflected toward the object by a mirror, travels through the field diaphragm where its diameter is adjusted, and then moves to the substage condenser, which focuses (condenses) the beam of light on the object. The substage condenser includes the aperture stop, or iris diaphragm, which changes the angle of the cone of light inside the

objective. The objective lens accepts the light and focuses an image of the object inside the body tube (this is called the primary image), and the ocular eyepiece focuses on and magnifies the primary image for the eye to perceive through its own lens. Microscope manufacturers put devices to alter this light at various points along the path, such as in front of the light source, just before the field diaphragm, in the objective, or elsewhere, depending on individual design [1–3].

14.1.2 Magnification, numerical aperture, and resolution

Novice microscopists often ask how much magnification the microscope can give. Although it may be capable of magnifying the object by 2000 times, so much detail and sharpness will be lost at that magnification that the image will be useless. Magnification that does not reveal further detail is called empty magnification. To understand this concept, use a magnifying lens to observe detail in a newspaper photograph; instead of showing more detail, the lens shows a lot of black dots.

This applies to the microscope because of the wave nature of light. Consider a point source P, which sends out waves in a sphere (see Figure 14.2). When this spherical wave front hits the front lens of the objective, only part of it is allowed to pass through the aperture, so the waves of light that the objective focuses are not a perfect replica of P. A larger lens opening (aperture) allows a larger fraction of light from P to be admitted and more detail of P to be accurately rendered. Thus, the ability to discern detail (resolution) can be expressed by the fraction of the wave front that is admitted by the lens. This fraction of light is expressed by a value called the numerical aperture (NA). This number is engraved on each lens and holds fundamental importance to the microscopist. It determines the ability to discern detail, and it has a marked effect upon the intensity of illumination. The NA of a lens [1] is equal to the sine of the angle μ multiplied by the refractive index of the medium between the object and lens (n), where 2μ is the angle of the cone of light admitted by the lens (see Figure 14.3). (Recall that the sine of an angle is the ratio of the side opposite an acute angle and the hypotenuse, so that as the sine increases, the angle increases.)

$$\text{NA} = n \sin \mu$$

The sine of an angle cannot exceed 1, so the maximum NA is equal to n , the refractive index of the immersion medium. Thus, no dry objective can have an NA greater than 1 (the refractive index of air), and no oil lens can exceed an NA of 1.52 (the refractive index of oil). If we let $n = 1$, we find that as the NA increases, the angle of light admitted by the lens increases.

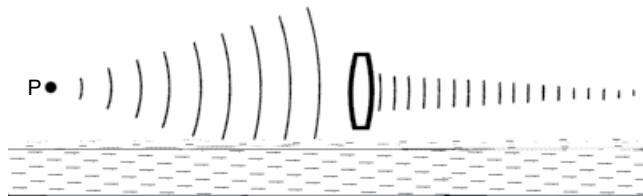


Figure 14.2 Empty magnification due to the wave nature of light. Point source P sends out waves of light in a sphere. The larger the lens used to observe P, the more detail of P is rendered until the fraction of the wave front that is admitted to the lens is no longer sufficient to provide a replica of P. Thus, resolution depends on the fraction of the wave front admitted by the lens. This fraction of light is expressed as the numerical aperture of the lens.

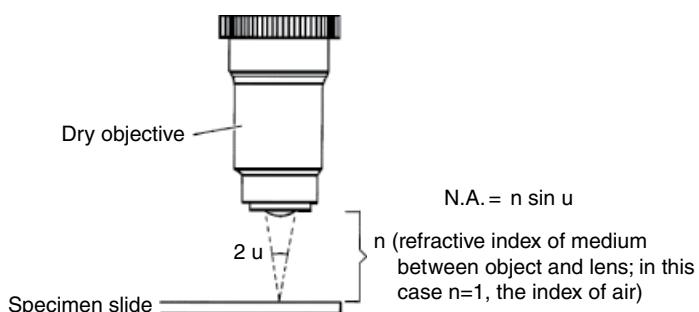


Figure 14.3 Numerical aperture. The numerical aperture of a lens is an index of its light-gathering capacity.

The useful magnification limit is determined by the resolving power of the lens (r). Resolution is the ability to distinguish two close points as separate entities, and the minimum resolvable distance is determined by the NA and the wavelength of the light:

$$r = \lambda / 2NA$$

Here λ is the wavelength of light, and r is the minimum distance at which the objects can be seen as two points. Thus, as the NA increases, r decreases. Also, as the wavelength is shortened, the resolution increases.

Diffraction is the bending of light rays that occurs whenever the free passage of a wavefront is impeded by an object (the specimen). As the distance between diffracting structures decreases (fine detail), the angles of diffraction increase. To recreate the image of the object faithfully, the lens must collect all the diffracted rays as well as the undiffracted rays of light. Only a lens with a large aperture can admit the highly diffracted rays, so a lens with high NA is able to give better images.

Equally important is the numerical aperture of the condenser. The NA of the condenser should be equal to or greater than the NA of the objective. For the critical work of cytogenetics, the condenser should be apochromatic and planatic (corrected for color and flatness of field). If the NA of the condenser falls below that of the objective, the light waves become less independent, and the resolution suffers. If the NA of the condenser is much too low or if the condenser is missing, rings and halos appear around the object, caused by wave interference. Thus, the resolving power of the microscope depends on the NA and quality of the objective, the refractive index of the mounting medium, and the NA of the condenser.

Resolution is not the only important quality to be achieved, because contrast (the relative difference in intensity between the object and its immediate surroundings) is also necessary; resolution without contrast would have little use. For example, under the best possible conditions for resolution, a transparent object would be invisible. A good balance of resolution and contrast is ideal.

14.1.3 Lenses

Objective lenses

High-quality objective lenses are an important investment in the cytogenetics laboratory. They may be expensive, but they are surprisingly intricate, and their cost is justified by the benefits. The following are the types of objectives most commonly found in the cytogenetics laboratory.

Planapochromatic objectives

The planapochromatic lens is the ideal lens for cytogenetics and is essential for imaging chromosomes. This objective is corrected to visualize a flat (plan) field so that the middle of the field and the edges are in focus at the same time. The apochromatic designation indicates that they are corrected for four wavelengths of light. These objectives usually have the highest numerical apertures.

Apochromatic objectives

Apochromatic objectives also bring four wavelengths to a common focus rather than just two [4], but lack flatness of field. These lenses usually have a high NA and hence tend to give better resolution than do achromats.

Achromatic objectives

Achromatic objectives, which correct for two colors, are usually the least expensive. A simple lens will not bring all wavelengths of light (colors of light) to focus in the same plane because each wavelength is diffracted differently. Halos, for example, will result from blue rays focusing at a lower point than red rays through a simple lens. In achromats, lenses that have different refractive indexes are combined in the objective, thereby bringing rays of two colors, blue and red, to a common focus. Achromatic lenses are designed to perform best with green light, which helps filter out violet light, which is not corrected by achromats.

Semi-apochromatic or fluorite objectives

Semi-apochromatic, or fluorite, objectives have optical corrections intermediate between achromats and apochromats. Neofluar (fluorite type) objectives have a high numerical aperture with accurate color rendition and are available with a flat field. These lenses are well suited for work in fluorescence microscopy because of their low-dispersion glass and high NA.

Quartz lenses and lenses for fluorescence

Unlike glass lenses that stop much of the UV spectrum, quartz lenses allow ultraviolet light to pass through easily, making them more ideal for ultraviolet microscopy. Do not confuse ultraviolet microscopy with fluorescence microscopy, because many lenses used for fluorescent microscopy have a built-in iris to control flare.

Lens characteristics

Curvature of field

Lenses may be corrected for curvature of field. Without any correction, lenses give better image quality in the center of the field, and when the center is in focus, the edges of the field are out of focus. This is acceptable for visual purposes, because the microscopist can refocus on any part of the field that he or she wishes. However, for photomicroscopy, the situation is much different. The camera demands an entirely focused field of view. Developments in correction for curvature of field have resulted in very high-quality flat-field lenses, or planapo objectives. In the cytogenetics laboratory, the oil immersion lens used for photomicroscopy must be a plano lens, preferably a planapo, which is corrected for curvature of field and three colors.

Depth of field

Any lens will focus at a given distance plus or minus a certain range. This range of distances on which an objective can focus reasonably well without any adjustment is called the depth of field of that lens. The larger the NA, the smaller the depth of field. Depth of field is almost nonexistent in high-power oil lenses. It can be increased by closing the condenser iris diaphragm, effectively reducing the working NA, but only at the expense of resolution.

Working distance

The working distance of a lens is the distance between the lowest point of the objective and the object in focus. As the magnification power of the lens increases, the working distance decreases. Great care must be taken with a lens of high magnification not to hit the slide. Also, the working distance of a low-power dry lens used for scanning must not be so small that it is in danger of getting oil on it when rotating the nosepiece; in such a case, the oil would have to be wiped off the slide after each observation with high power. In very short working distances, if the thickness of the cover glass exceeds the working distance, it is impossible to focus on the object.

Lens magnification

Lens magnification is determined by tube length (the distance from the rear focal plane of the objective to the position of the primary image) and focal length (the distance from the surface of the lens to the point of convergence of the rays). Their interrelation is expressed by the following formula:

$$\text{Magnification} = \text{tube length/focal length}$$

The tube length of most modern microscopes is 160 mm. Thus, the focal length of a standard 10× objective would be 160/10, or 16 mm, and the magnification of a 4 mm lens would be 160/4 or 40×. Most achromatic oil immersion lenses have focal lengths of 1.8 to 2.0 mm. Do not interchange objectives between microscopes unless their tube lengths match or they are infinity corrected because the image quality will be decreased. In infinity corrected systems the image distance is set to infinity and a tube lens is placed within the body tube between the objectives and the oculars to form the intermediate image.

Parfocal lenses

Modern lenses usually do not require any more than a small adjustment of the fine focus knob when switching from one lens to another. Lenses that are so closely matched are called parfocal lenses, and lenses that are not parfocal can often be adjusted by a microscope company sales representative.

Oil immersion lenses

It has already been shown that the resolution of a lens increases as the NA increases and that $\text{NA} = n \sin \mu$. The value of n in air is 1. If one were able to increase n , the NA of the lens would be effectively increased also. This can be done by placing a drop of fluid, such as water or oil (with an n value higher than air), between the cover glass and the lens.

Light is diffracted when it passes from one medium to a medium that has a different refractive index. This means that light that passes through a glass slide or coverslip into air is refracted away from the lens. Recall that the NA is an expression of the fraction of a wavefront admitted by a lens. If one could effectively increase the wavefront admitted by the lens, the NA would increase, and the resolution would improve. This can be done by putting something with the same refractive index as glass between the cover glass and lens. Crown glass is a type of optical glass used in lenses because of its low refractive index and low dispersion. The refractive index of crown glass is 1.51 to 1.56, so oils used for immersion microscopy usually have a refractive index of 1.515 to 1.520 at 23 °C. In practical usage, it is obvious that an oil immersion objective loses resolution if it is used dry; in theory, this is due to the low refractive index of air. Similarly, if the refractive index of the oil is too high or too low, much resolution is lost. Also important is the refractive index of the medium used to mount the coverslip. One should follow

the lens markings (see Lens markings) on the objective to determine if a coverglass should be used. This will alleviate any deviation from the desired refractive index. The condenser lens may be oiled, also, for critical work demanding full performance of the system.

Lens markings

The following information is engraved on most objective lenses (Figure 14.4):

Magnification, either as a whole number or as a ratio (e.g., 100 or 100:1)

Tube length in mm (the distance from the rear focal plane of the objective to the position of the primary image inside the body tube)

Coverslip designations, which can be seen after the tube length ("0.17" means that a coverglass of $0.17\text{ mm} \pm 0.01$ should be used, "0" should be used with no coverslip, and “-“ can be used with or without a coverslip with no loss of resolution).

Numerical aperture, usually under or next to the magnification marking (e.g., 1.30). For example, a lens might be engraved 170/0.17 Pl Apo Oel 100/1.32. This signifies that the lens is to be used with a 170 mm tube and a cover glass with a thickness of 0.17 mm, that it is a plano (Pl) lens (flat field), and an apochromat (Apo) oil (Oel) immersion lens (corrected for three colors) that magnifies 100 times and has an NA of 1.32 when immersed in oil. Other markings are the brand name, the serial number of the lens, and the focal length of the lens in inches or millimeters.

Other markings may also be seen on common microscope lenses for cytogenetics laboratories. For example, HI on a lens means homogenous immersion (in oil), and WI means water immersion. Ph followed by a number means that the lens can be used for phase contrast; the number refers to the phase annulus on the condenser that matches the phase ring in the lens. Ultrafluar (Zeiss) means that the lens is corrected for UV wavelengths. Fluotar (Leitz) and Neofluar (Zeiss) mean fluorite objectives. Lenses that do not specify whether they are achromats or apochromats are usually achromats. There are too many symbols to be able to do them justice in this chapter; the reader is encouraged to read their microscope's handbook for any additional markings or specific operational characteristics.

14.1.4 Condensers

Condensers act to gather light from the microscope's light source and concentrate it into a cone of light that illuminates the specimen. Its ability to focus the full range of light on the object so that the light waves act independently functions to increase resolving power. The iris diaphragm of the condenser adjusts the working aperture of the condenser to that of the objective. It is never used to control the intensity of illumination, and it should rarely be racked down very far from its highest position. The lens of the condenser is marked with a numerical aperture value and may also be marked achromatic or



Figure 14.4 Lens markings. The lens in the foreground is a planapochromatic lens with 100 \times magnification and numerical aperture of 1.35. It is an infinity corrected lens which should be used with 0.17 thickness coverslip.

aplanatic. In addition, there are different condensers for each type of illumination (brightfield, darkfield, phase contrast). Brightfield condensers usually consist of a main section and a swing-in lens, located either above the main section or below it. This swing-in lens corrects the NA of the condenser for use with lower power, because oil immersion lenses require a different aperture to fill the lens with light. Some condensers are designed for use with oil immersion (between the condenser and the slide). This has the effect of increasing the NA, but in practice, the difference is insignificant except for the most exacting photographic work.

The adjustment of the aperture stop of the condenser is an important consideration. Using the maximum aperture gives the best resolution, but it decreases the contrast; a compromise is usually best. The most contrast is obtained by closing the aperture stop down all of the way; however, this causes a decrease in resolution. Resolution, it will be recalled, is best when the light rays are made to act independently by using a condenser lens with a numerical aperture equal to that of the objective. If the object has high contrast, the aperture can be left wide open, but if the contrast is low, as with a poorly stained or unstained object, the aperture should be closed down in spite of the resulting loss of resolution. With the aperture closed down all the way, unstained chromosomes may be viewed (without phase contrast) to check spreading and mitotic index, but the resolution will be too poor to assess chromosome quality. Similarly, with the condenser racked down low, unstained preparations can be viewed; although contrast is enhanced, resolution is decreased.

14.1.5 Eyepieces (or oculars)

The primary (or virtual) image that is formed by the objective inside of the microscope tube is further magnified by the eyepiece, or ocular, which is made up of two lenses. The upper lens closer to the observer's eye is called the eye lens and the lower one is the field lens. The field lens is responsible for reducing the oblique angle of the light coming into the eye lens so that the eye lens does not have to be very large. Between these two lenses is a fixed field stop, which can be observed by unscrewing the eye lens and looking down the eyepiece. This field stop is in focus simultaneously with the object, and, like the field stop in the base of the microscope, it limits the field of view. The simplest eyepieces, with two plano-convex lenses and a field diaphragm between them, are called Huygens eyepieces. More complex eyepieces, which are specially designed for use with apochromatic lenses, are called compensating eyepieces. (These compensate for astigmatisms and lateral chromatic aberrations.) There are eyepieces made to focus at a high point for those who wear glasses during microscopy.

Those with certain eye defects must wear eyeglasses while using the microscope. Zeiss [3, 4] recommends a simple test to determine if the microscopist must wear his or her eyeglasses during microscopy: The person should observe an object with the eyeglasses held at arm's length while rotating the eyeglass lens around its center. If the shape of the object does not change, then corrective eyeglasses are not needed for microscopy; the eyepiece will compensate for the defect. If, however, the length and width of the object appear to change during rotation, the eyeglasses have toric lenses, and the wearer should use them while using the microscope. Eyepieces all have an eyepoint, or place where the light rays converge, and the eye should be positioned at this point. The eyepoint can be determined by holding a piece of white paper above the eyepiece and moving it up and down; a white circle of light will appear on the paper. It will become smaller and then larger as the paper is moved. The position at which the circle appears the smallest is the eyepoint. If the microscopist is astigmatic, he or she must wear glasses for photomicrography, because this visual defect cannot be corrected by the microscope.

Eyepieces come in several magnifications, but those most commonly seen in cytogenetics laboratories are the 8 \times , 10 \times , and 12.5 \times versions. The total magnification of the object is determined by multiplying the magnification of the ocular and the objective. For example, a 100 \times objective and a 10 \times eyepiece magnify the object 1000 times. Some microscopes have another lens system that further magnifies the object, called the optovar magnification changer, or tube factor; the number on it (e.g., 1.0, 1.25, 1.6, 2.0) must also be multiplied into the total magnification. To avoid empty magnification, remember that the total magnification of the object must not exceed 1000 times the NA of the objective being used. For example, for an oil immersion lens (100 \times) with a numerical aperture of 1.25, eyepieces that bring the magnification to greater than 1,250 (i.e., eyepieces greater than 12.5 \times magnification) should not be used, because the magnification gained will not reveal any further detail and may in fact cause artifacts.

Markings found on eyepieces include magnification (8 \times , 12.5 \times , etc.); C, K, or comp (compensating eyepieces for flat-field objectives); a symbol appearing like a pair of eyeglasses (signifying high-point eyepieces that are designed for glasses wearers, but which can be used by anyone); pl (flat field); W (wide field – very helpful when scanning slides with a low mitotic index); and field of view number (the diameter of the visible field). In the past, oculars were corrected for aberrations in the objectives, making it important to use oculars and objectives from the same manufacturer. Exceptions to this rule are the infinity corrected lenses that are now available.

14.1.6 Homogenous system

In addition to the lenses of the objective, condenser, and ocular systems, the often-overlooked system of the slide, mounting medium, coverslip, and immersion fluid must be considered part of the lens system. As has already been pointed out, refraction and reflection of light occur when rays pass from a dense medium into a less dense one, effectively limiting the NA of a lens. Because $NA = n \sin u$, and because the sine of an angle cannot exceed 1, the maximum possible NA is equal to n , the refractive index of the immersion medium. For cedarwood oil, this is 1.515, for water 1.33, and for air 1.0; thus, if an oil immersion lens is used in air or in the wrong immersion fluid, its NA is not fully usable, and its resolution and clarity suffer. One should usually use the immersion oil recommended by the microscope company for use with particular lenses. It should have an index of refraction of 1.515 to 1.520. The refractive index of the mounting medium ranges from 1.44 to 1.515. The reason that this index is sometimes lower than glass is that sectioned tissues tend to disappear in media with an index close to their own, which is usually about 1.530–1.540. Stained chromosomes, however, unlike tissues, are dense enough that the mounting medium can be nearer to that of glass.

14.1.7 Mechanical stages

The mechanical stage is a necessity for cytogenetic work. It holds the specimen and moves it smoothly, horizontally or vertically, allowing a systematic search over the slide for metaphases. The mechanical stage should have graduated markings so that a given metaphase can be relocated and possibly re-photographed or analyzed. A rotating stage can be useful in photography if the camera is stationary, because the metaphase can be better positioned to fit the rectangular film format. If the camera can be rotated, the same end is achieved.

Vernier scales are usually present on mechanical stages and are read as follows: If the zero on the vernier scale coincides with a mark on the stage scale, read the whole number indicated. If, however, the zero lies between two marks on the stage scale, read the next lower whole number, and read the tenths by determining which reading on the vernier is opposite a division on the stage scale.

Some stages are removable, and the scale should not be moved when the stage is removed, as when it is professionally cleaned. This will cause old vernier scale records to be useless for finding cells. Some microscope verniers are adjustable, and newly purchased microscopes may be calibrated to read the same as others in the laboratory.

14.1.8 Practical microscopy

The microscope is usually set upon a table, preferably independent of other countertops, at a convenient height so that the user can sit in a chair and see comfortably into the eyepieces. This means that the microscopist neither strains to keep an erect posture nor slouches over the scope. The height of the microscope can be adjusted by setting rubber pads under the base of the scope or by adding special mounts made for the purpose of cutting down vibration. (These mounts are often listed in general laboratory supply house catalogs under the section on weighing scales.) The chair should also be adjustable for different heights.

The room in which the microscope is kept should be as free as possible from dust and vibrations. Windows that open to the outside of the building are a source of dust; the microscope will need less cleaning if it is in a room without windows that open. The room should be well lit; however, the light should be adjustable with a switch (preferably with a rheostat) near the microscope for fluorescent work and critical photography. If possible, the microscope room should not be used for chromosome preparation, because glacial acetic acid is corrosive and will ruin the lenses and metal parts of the microscope.

To avoid scratching high magnification objective lens, focus the slide image on low power before switching.

14.1.9 Cleaning the microscope

The microscope should be stored under a dustcover of some sort when it is not in use, because dust may get inside the microscope parts or mix with the lubricant used in the stage and condenser rack mechanism, which will require expensive professional cleaning. Dust usually does not cause a problem with the objectives unless it gets inside them; they should always be stored in the special plastic tubes supplied by the manufacturer. If an empty hole is left in the revolving nosepiece, it should be covered by a plug, which is also available from the manufacturer. The eyepiece should be kept free of dust and grease for the most comfortable viewing, and the field diaphragm should be kept clean because the aperture shows in the field of view and will be in focus along with the object. Dust on the other parts of the microscope does not directly affect the quality of the image, but the microscope should be regularly dusted as a matter of routine. The working space around the microscope should be kept clean with a cloth moistened with distilled water or alcohol to prevent raising the dust as it is wiped away.

Cleaning objectives is usually not recommended unless they become covered with a film or fingerprints. A bad cleaning job is often worse than no cleaning at all. Dirt on the objective will cause severe deterioration of the image; cleaning should be done with a bit of lens paper moistened, if necessary, with commercial lens cleaner or human breath. Use as little solvent as possible, and avoid other solvents such as alcohol, because the cement holding the lens in place is subject to damage. Use a magnifier (such as the objective lens held upside down) to examine the lens surface. Do not scrub hard on a lens with tissues not designed for lenses; though they may feel soft, they may still be capable of scratching. Never rub a dry lens with dirt on it because the dirt can scratch the lens.

The eyepieces can be cleaned in a similar fashion and need constant attention to keep them clear of natural oils from the eyes. When it is necessary to locate dirt in the field of view, the best method is as follows: rotate the eyepieces. If the dirt rotates, clean the eyepieces. Similarly, rotate the filters on the field diaphragm. If the dirt rotates, clean the filters and glass over the field diaphragm. Next move the condenser up and down. If the dirt moves in and out of view, clean the condenser lenses. If the spots cannot be removed by cleaning, unfocus the condenser slightly to omit seeing the spots in the field of view. Less-than-perfect Köhler illumination is preferable to seeing a spot constantly. Some spots (e.g., blood and glycerine) may not be soluble in the usual cleaning agents, in which case a little distilled water (not tap water, which may have damaging salts or acids in it) may be tried. Also, be sure to move the slide to ensure that the dirt is not on the preparation. If the dirt is not on the filters or glass covering the field diaphragm, eyepieces, condenser, or slide, it may be inside the microscope, in which case professional help should be sought.

Never attempt to clean inside the microscope tube or other parts that require dissembling the microscope. Never clean inside an eyepiece or objective. Laboratory microscopes should be professionally cleaned and serviced yearly or at least every two years.

14.2 Brightfield microscopy

14.2.1 Köhler illumination

The best way to view or photograph objects with brightfield-transmitted light is with Köhler illumination, which fulfills the need for the best image quality and the highest resolution [1]. It provides an even distribution of light across the field of view and makes the object act like a self-luminous object by imaging the light source in the condenser. The following are the steps for proper illumination (Figure 14.5):

1. Center and focus the bulb according to the manufacturer's directions. If done properly it should not have to be done again until the bulb is replaced. It may be necessary on some microscopes to remove a ground-glass filter from in front of the bulb in order to focus on the filament. Some microscopes have flip-in focusing screens for ease of use in lamp centering for even illumination.
2. Focus on the specimen with a low-power objective. Close down the field diaphragm completely, and rack the condenser up until its edges are in focus. If the field diaphragm image seems off-center, correct it with the two screws on the condenser. Otherwise, it may be very difficult to center the light on high power.
3. Switch to oil immersion, and refocus on the object. (It is assumed that the object must be viewed on oil immersion. Köhler illumination can also be used on low power.) The object must remain in sharp focus during the adjustment of the light. Close the field diaphragm down again until its edges are visible, and focus the edges as sharply as possible by racking the condenser up and down. There may be colored edges on the fringes of the diaphragm; the green/blue fringe will allow for the best resolution. Center the light with the center screws as in step 2. This step is important for evenly exposed photographs.
4. When the condenser is at the right height, open the field diaphragm until its edges just disappear. This gives the best setting to prevent glare.
5. Set the aperture stop. This can be done in two ways: First, you can remove one eyepiece and adjust the aperture diaphragm so that 2/3 to 3/4 of the circular illuminated area (which represents the objective aperture) is covered. This adjusts for maximum contrast without sacrificing resolution; if the diaphragm is left open, poor image quality can result. Second, you can set the iris diaphragm visually. With chromosomes, there is already quite a bit of contrast, owing to the staining properties of the chromosomes. Setting the aperture stop by closing it until the chromosomes appear to have just enough contrast without sacrificing resolution may be preferable to the first method. Once steps 1 and 2 have been performed, only steps 3, 4, and 5 need to be repeated for each lens change or for each new photograph to be taken.

14.2.2 Filters

For observing and imaging Giemsa-stained chromosomes, a green filter is recommended; this increases contrast and improves the recording of the stain on panchromatic films. There are several types of green filters. Aside from the ordinary Wratten green filters, other useful filters are the 546-nm interference band filters and the barrier filters, which are used for fluorescence microscopy, such as the 530-nm or 500-nm filters. These may be used singly or in combination.

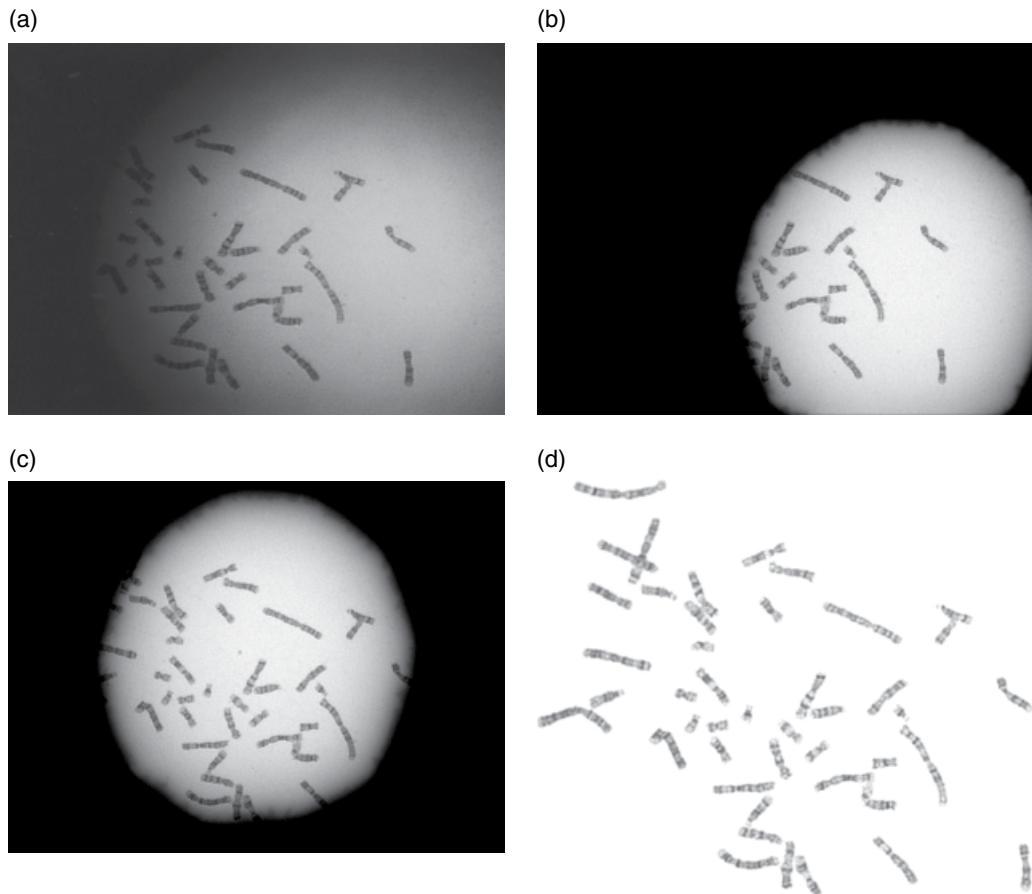


Figure 14.5 Centration of light source for Köhler illumination. (a) The field diaphragm must be closed, and the condenser must be (b) focused, (c) centered, and (d) opened just past the field of view. Courtesy of Patricia Mouchrani.

14.2.3 Immersion oil

The proper oil is critical to resolution in microscopy. In general, use the oil supplied with the microscope. An exception to this is when the oil accelerates fading of the stain on uncoverslipped slides. To address this, many laboratories use the Cargille type A or type B oils or a mixture of the two. Type A is thin and has a very low fluorescence, making it suitable for fluorescent work. Type B is very thick and has a slightly higher fluorescence, but it is still within reasonable limits for use in fluorescent microscopy. Mixing the two types of oil gives an oil of intermediate thickness. Resolve is another oil found not to induce fading. Please note, care should be taken to check before using immersion oils produced before the mid-1970s, as they may contain PCBs (polychlorinated biphenyls), a known carcinogen.

14.2.4 Coverglass

Most objectives used for close working distances are marked with the number 0.17 (see Figure 14.4). This number indicates that the coverslip plus mounting medium should be 0.17 mm thick. Microscope cover glass No. 1 ½ provides this appropriate range, between 0.16 and 0.19 mm, and is thus the recommended choice with a lens designating 0.17. A minus (–) sign can be used with or without cover glass.

Mounting medium should form the thinnest layer possible. Laboratories that prefer to analyze and photograph slides without coverslips should verify that their objectives are designed for the best resolution without a coverslip.

14.2.5 Slides

Just as the cover glass thickness is important optically for the objective, so is the slide thickness important for the condenser. High NA achromatic condensers usually require slides of 1 mm plus or minus 0.05 thickness.

14.2.6 Eyepiece adjustment

Eyepieces have three potential adjustments for the user. First, with feet on the ground, adjust the chair's height so that your eyes are parallel with the eyepieces, without unnecessary stretching or bending. Newer microscopes offer an ergonomic feature by which oculars can be raised or lowered at various angles in order to accommodate the reader's torso height rather than relying on chair adjustment.

Second, adjust the interpupillary distance so that one field is observed when viewing with both eyes, by moving the eyepieces closer together or further apart. Finally, if the focus is not equally sharp in both eyes, find out which eyepiece has a revolving focus adjustment. Using the other eyepiece, focus the specimen with the fine adjustment knob on the microscope. Once the nonadjustable eyepiece is focused, return to the first eye and turn the revolving adjuster in the eyepiece until the image is crisp. This will allow the use of both eyes with minimal strain for chromosome analyses.

For photographing, many microscopes provide fine lines, called reticles, in the adjustable eyepiece, which when properly aligned, will ensure proper focus of the object on the imaging plane. Focus the lines in the reticle so that the camera is adjusted to your eye. This is most easily done without a specimen on the stage.

Modern microscopes also have many ergonomic options which can be considered when purchase of a new microscope is considered.

14.3 Fluorescence microscopy

Many modern chromosome staining and probe detection techniques take advantage of fluorochromes, which are dyes that release energy in the form of fluorescent light when struck by light of short wavelength, such as ultraviolet. Light sources used with fluorescence [1, 5] (usually a high-pressure mercury vapor lamp, a tungsten halogen lamp, or a xenon lamp) all excite the fluorochrome (or fluorophore) to produce fluorescent light in the visible spectrum. (Ultraviolet microscopy is different from fluorescent microscopy and will not be considered here.) The actual reaction is as follows: An atom of fluorochrome (e.g., quinacrine or acridine orange), when struck by a quantum of light, undergoes an electron change. One or more electrons jump from one energy shell to another of a higher energy level, then return to the original ground state and emit energy in the form of light and heat. Because some of its energy is lost as heat, the energy emitted by the light is lower and has a longer wavelength than the light that energized it, making it visible to the eye. The main difference between standard microscopy and fluorescence microscopy is the illumination system; fluorescence microscopy uses an illumination source with shorter wavelengths and a receiving system with longer wavelengths. In addition to the special light source, the fluorescent microscope requires excitation and barrier filters. The excitation filter transmits only the desired short wavelength light to the specimen for excitation; the barrier filter then transmits only the desired visible light for the eye or camera and filters out the ultraviolet light and blue light that detract from the image. Each fluorochrome has its own combination of exciter and barrier filters for optimal viewing conditions. See Figure 14.6, which shows the light path and filters used in *in situ* hybridization (FISH) microscopy. Fluorescence microscopes can use either transmitted light or incident light [5]. Transmitted light follows the same light path as the brightfield microscope light, but the brightfield condenser is replaced with a darkfield condenser. This condenser causes the light rays to hit the slide at such an acute angle that they illuminate the specimen but do not pass into the objective, thus separating both the exciting and emitted light.

Incident light, also called epi-illumination or Ploem illumination, uses light that originates above the objective, passes through the objective to the object, and excites the fluorochrome; the emitted light then bounces back up through the objective to the eye or camera. The exciting light does not bounce back, but rather passes out through the specimen. The advantages of epi-illumination are less loss of light (resulting in shorter exposure times and better photographs), ease of alignment (the condenser is eliminated and therefore does not have to be refocused constantly), and the ability to use transmitted light and incident light without changing condensers.

14.3.1 Light sources for fluorescence

There are several light sources for fluorescence microscopy. These may or may not emit true ultraviolet light; many substances will excite just as well with blue or green radiation. Ultraviolet light is in the 300 to 400 nm range. One nm is 0.001 mm, or 10^{-9} m. Violet light starts at 400 nm and is at the lower end of the visible spectrum. As the wavelengths get longer, the light appears blue, green, yellow, orange, and red (700 nm); above red, which is the upper end of the visible spectrum, is infrared light. Quinacrine stain has its highest fluorescence in the 460–500-nm range, so blue light, rather than ultraviolet, is used to excite quinacrine.

Light sources most commonly used for fluorescence are the high-wattage halogen tungsten, mercury vapor, and xenon lamps [1]. Each of these comes in different wattages—some use alternating current (AC), and others take direct current (DC). Those using DC tend to have a longer life. The following is a list of available fluorescent light sources: Xenon

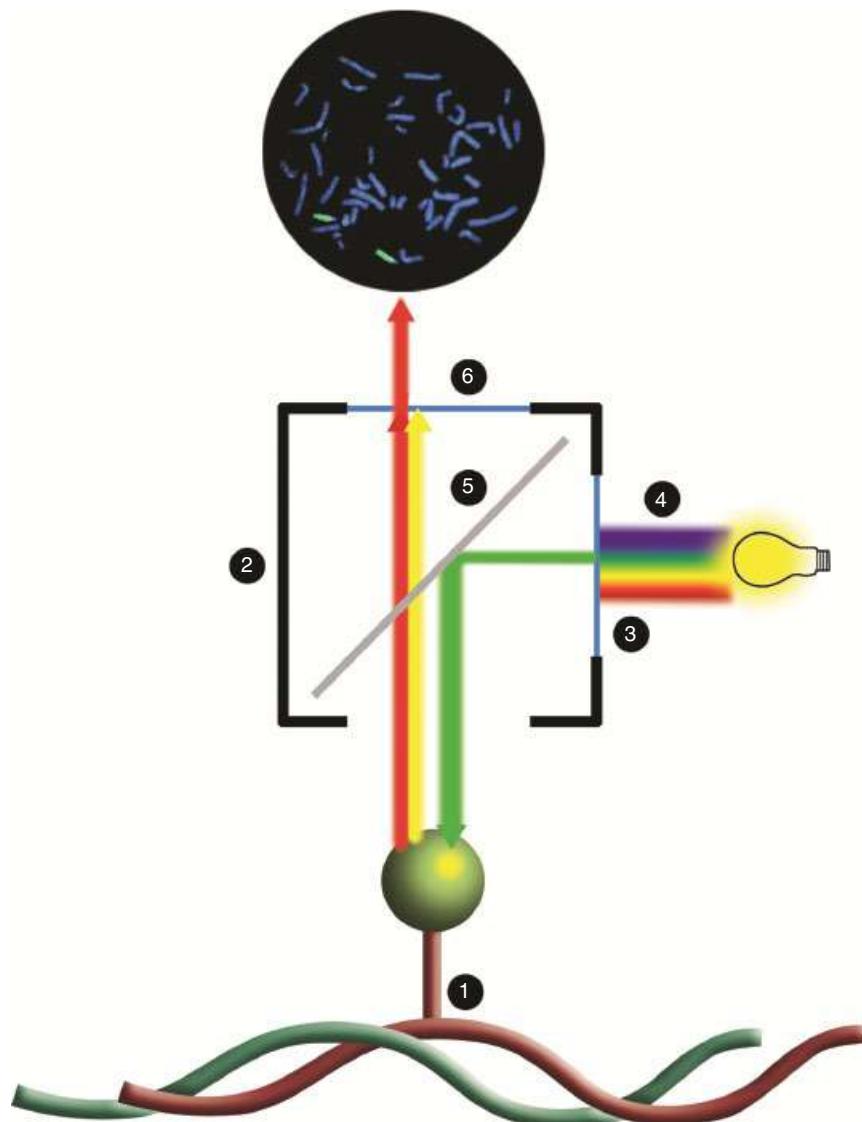


Figure 14.6 Fluorescence *in situ* hybridization microscopy: light path and filters. (1) DNA from a cell on a slide that has been hybridized with a probe. The red DNA strand is the patient's and the green strand is the probe that has bound to sequences that match its own. The green fluorophore is directly labeled on the probe. (2) Filter sets (boxed area) used to detect probes by fluorescence microscopy. (3) Exciter filter – full-spectrum light is reduced to light of a defined wavelength that specifically excites the fluorophore of the probe. (4) Full-spectrum light emitted by microscope lamp. (5) Dichroic mirror reflects defined wavelength (excitation) onto the specimen. (6) Barrier filter – reduces the emitted light to a defined wavelength that can be detected. Reproduced with permission from Zytovision GmbH, Bremerhaven, Germany. See insert for color representation of this figure.

XBO 75, 150, and 450 W; Mercury HBO 50, 100, and 200; and Halogen Tungsten 12 V, 100 W. The 100-watt mercury lamp is the usual light source for fluorescence microscopy. The 50-watt mercury lamp is also available. Though these light sources are strong in the UV region, the resultant fading can be slowed by a heat-reflection mirror. The tungsten halogen bulb, which is standard for transmitted light, can also be used. Xenon light sources are now rare, and some consider them obsolete.

A relatively recent development in light sources for fluorescence is the availability of a pre-centered 120-watt light source with a uniformly illuminated field of view. They are advertised as having 1500–2000 hours of life and will give off less heat

than other bulb types. Although expensive, their improved quality and long life may justify the cost in some laboratories. Most major microscope manufacturers offer their version of this lamp.

One common problem with fluorescent bulbs is flickering; this is usually caused by insufficient burning the first time the bulb is turned on. New bulbs should be burned a minimum of 2 hours. When putting a new bulb into the microscope, file the corrosion off the contacts and keep the bulb scrupulously clean. Never touch the bulb with the fingers; the bulb can be cleaned with alcohol if it appears to have grease on it. Fluorescent lamps are under high pressure and should be treated with caution. Mercury spills should be cleaned up immediately according to laboratory safety protocols.

14.3.2 Filters for fluorescence

The success of fluorescence microscopy and photography depends on the proper choice of filters [1, 5]. The choice of exciter filter and barrier filter depends on the wavelengths necessary to cause the particular stain to fluoresce (excitation wavelength) and on the wavelength emitted by the stain. For example, quinacrine is best excited to fluoresce by wavelengths between 460 nm and 500 nm and emits a yellow-green light between 500 nm and 550 nm. For excitation, then, a filter that passes either all wavelengths between 460 nm and 500 nm or specific wavelengths inside the range (a band-pass filter) should be used. The light emitted by the specimen can be separated from unwanted short-wave excitation light by a barrier filter that passes in the yellow-green range (510–550 nm) but completely cuts off all of the radiation below 500 nm. Acridine orange requires excitation at 500 nm and emits at 530 nm. Hoechst is excited at 365 nm and emits at 480 nm.

Filters are described by their transmission curves, which show what portions of the spectrum are absorbed or partially absorbed. Wide-band filters, such as the VG9 (Zeiss), transmit large portions of the spectrum [5]. Band-pass filters are designed to cut off wavelengths above and below a given wavelength, such as the interference filter for 546 nm. Short-wave pass filters (cut-off filters) allow wavelengths below a certain point to pass but completely cut off everything higher. Likewise, a long wavelength pass filter (also a cut-off filter) emits wavelengths above a certain point and completely cuts off everything below it. Band-pass filters are more commonly used as exciter filters, and long-wave pass filters are used for barriers. Triple bandpass filters allow simultaneous visualization of three different wavelengths, as when viewing dual color probes and counterstain.

Epifluorescence requires a third type of filter, the dichroic reflector, in addition to the excitation and barrier filters. The dichroic reflector (also called chromatic beam splitter, reflector, or dichromatic beam splitter) is an interference mirror, similar to the interference filter in that it reflects most of a given spectral range and almost completely transmits others. This reflector is found above the lens, where it reflects the desired light down to the specimen and allows unwanted wavelengths to pass through it unreflected.

14.3.3 Practical advice for fluorescence

Making a few adjustments can improve the fluorescent visibility and intensity.

1. Work in a darkened room.
2. Use objectives with a high numerical aperture (such as apochromats or neofluars). The fluorescent intensity increases exponentially with the increase in numerical aperture.
3. Similarly, use eyepieces of a low magnification, because fluorescent intensity decreases exponentially with an increase in total magnification.
4. Use appropriate mounting medium and oil. Because fluorescence is an emitted light rather than a refracted light, the refractive index of the mounting medium and oil is not nearly as critical as with brightfield microscopy. However, the total thickness of these media is important because the weak emitted light will not penetrate much glass or fluid.
5. Using a diaphragm on the oil immersion lens will reduce flare.

14.4 Specialized microscopy

14.4.1 Phase contrast microscopy

Thin, unstained, or poorly stained objects can be better viewed with a system called phase contrast microscopy [1, 2, 6, 7]. This can be installed on most existing microscopes and is of great value in the cytogenetics laboratory. It can be used on the inverted microscope in the tissue culture lab for viewing cultures as they are growing and for looking at slides as they are being made to check for spreading and mitotic index. Phase is also useful for photographing very lightly stained objects, such as overtreated C-bands and lightly stained R-bands obtained with Giemsa.

Phase contrast microscopy makes use of the facts that transparent objects cause a change in the phase of transmitted light, depending on differences in thickness, and that these changes can be converted to visible differences in intensity when part of the transmitted light has its optical path changed by about 1/4 wavelength. Phase contrast requires only a phase annulus in the condenser, which resembles a transparent ring in a dark glass insert, and a phase plate in the objective lens, a glass disk that has a ring-shaped area of a different thickness from the rest of the disk (see Figure 14.7). Phase lenses will have the marking "PH" to designate that they contain the phase ring.

The ring in the lens must be aligned to coincide with the annulus in the condenser; this is done with a special telescope provided for the eyepiece (e.g., Olympus) or with a special setting of the Zeiss optovar, both of which are used to focus on the ring and the annulus. Once they are focused, screws on the condenser or special wrenches that fit into sockets in the condenser are used to align them. Each phase lens requires a different annulus in the condenser, and the lens is usually marked (e.g., PH 2),

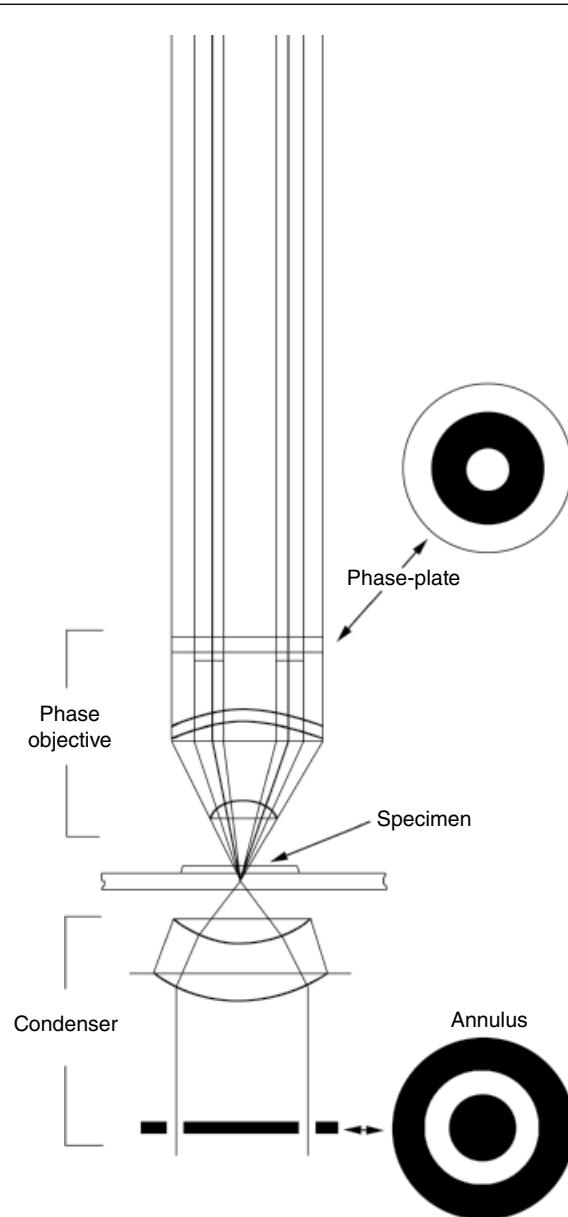


Figure 14.7 Transmitted light phase contrast. Light passes through the annular (ring-shaped) diaphragm in the condenser, forming a hollow cone of light that passes through the specimen and through the phase plate in the objective up to the ocular.

indicating the position of the proper annulus on the condenser. The addition of phase to a microscope involves the purchase of a special condenser and one or more phase lenses.

14.4.2 Inverted microscope

The inverted microscope has the advantage of placing the objective lens below the object to be viewed so that tissues in a Petri dish or flask can be viewed in spite of the distance between the cells and the top of the container. The lens focuses on the bottom of the culture flask rather than trying to focus through the top. (Closed flasks may be viewed on a conventional microscope, however, by inverting the flask and viewing the cells directly through the bottom. This may dry the cells out and should be done only for short periods of time.)

An inexpensive inverted microscope is a handy item for the cytogenetics laboratory, especially if long-term cultures are maintained. A low-power (3 \times to 10 \times) objective on the inverted tissue culture microscope can be used for scanning cultures to determine health and mitotic activity and for checking unstained slides. (The student can watch chromosomes spread in fixative on an inverted scope without interference from the heat of the light source.)

14.5 Capturing the microscopic image

Resolving the banded details of chromosomes is the usual goal of imaging in cytogenetics, whether accomplished by photographic or digital imaging. Because the highest resolution of microscope objectives is about 0.2 μm , it is necessary to employ the greatest care in every detail of microscopy and imaging to produce a karyogram with sufficient detail in the chromosomes.

All the conditions recommended above are especially critical in recording the image. In particular, the following steps in Köhler illumination should be repeated for each exposure or capture:

1. Close down the field diaphragm and check the focusing and centering of the substage condenser.
2. Open the field diaphragm until the edges just disappear from view, except in digital imaging. For digital imaging open the field diaphragm fully. The last step in the Köhler process (contrast) is performed digitally.

These two steps control flare, glare, and uneven exposure. Other important elements are correct setting of the substage diaphragm (iris), correct focus of the eyepiece reticles, use of immersion oil and mounting medium whose refractive index matches that of the objective, coverglasses of the proper thickness, proper tube length, and suitable lenses.

An oil immersion objective with the highest possible resolution is necessary for recording the detailed information found in well banded chromosomes.

14.5.1 Brightfield photography

Filters

Filters are used in brightfield photomicrography for two purposes: to change the color temperature of the light source when using color film and to increase the contrast of banded chromosomes when using black and white film. For color photomicrography, check the color temperature of the light source and the appropriate filters to use [1]. Note that this does not apply to color photography of fluorescent chromosomes, because the specimen emits the light.

Davidson [8] suggests a yellow-green filter (560 nm) for average-stained G-bands and a deeper green filter (510 nm) for lightly stained ones. Interference filters in this range work well, because they provide a narrow transmission band, but they also require longer exposures. For phase contrast photomicrography, Kodak Wratten filter numbers 13, 58, and 61 are recommended. They are all green, with number 61 being the deepest green. Phase objectives are often achromatic and are corrected optimally for green light; therefore, a green filter is the best choice.

Cameras

With a built-in camera, only a camera back is needed because the objective of the microscope becomes the lens of the camera. In cameras with integral lenses, the lens must be positioned at the eyepoint so that it brings to focus the image formed by the objective. This distance is critical, and it varies with the camera and the microscope. Many camera manufacturers make special adaptive devices as accessories so that their cameras can be correctly positioned. The camera is then set at infinity using the largest f-stop aperture. Magnification will usually be reduced in such systems.

If the camera has a removable lens, the lens should be removed and the camera attached directly to the microscope. This improves the image because it does not have to pass through as many surfaces before reaching the film plane. Appropriate

beam-splitters, photographic eyepieces, and auxiliary focusing telescopes are available for use with the attached camera and will give sharper pictures.

Historically, most cytogenetics laboratories have used 35-mm cameras, either integrated into the microscope or added onto it. Cameras with adjustable bellows are available [1] to accept 4 × 5 inch sheet film for high-quality photomicrography. Cameras with adjustable bellows are capable of a complete range of magnification. An additional advantage to the 4 × 5 format is the ability to take Polaroid photographs, which are immediately available for documentation and identification of chromosomes without the need for a darkroom or an image analysis system. Many bellows systems allow for the attachment of a 35-mm camera, as well. However, currently most cameras used in cytogenetics are digital.

14.5.2 Digital imaging

Over the last decades, most laboratories have adopted computer imaging to replace 35 mm photography and to assist in karyotyping. Metaphase finders are very useful in some applications and computerized data management is more commonly used in laboratories than ever before.

Most image analysis systems are built on the Apple Macintosh, IBM, and UNIX platforms. Software is the major difference between the systems. Software packages may provide ideograms, and the ability to add arrows and text annotation. Chromosome counting and analysis as well as accumulation of statistical data may be possible. For hardware, memory and storage capacity are important considerations. In choosing systems, laboratories usually consider image quality, ease of use, and operating costs. Other factors are laboratory management databases and compatibility with existing computers. Advantages of imaging systems are thus the elimination of the darkroom and the consequent time savings which can reduce turnaround time.

For image acquisition, a sensing device (camera) uses voltage to form an analog image in which brightness and sometimes color vary continuously with position. This analog image is a matrix of picture elements or pixels. The image is digitized by an analog to digital converter for storage in the computer as a gray-scale image. Video cameras use a picture tube or multiple tubes. Another type of camera uses a solid state sensor, such as a charge coupled device, hence, CCD camera. These devices can collect a charge over time without significant loss when cooled and are therefore useful for recording dim images [9, 10] such as fluorescent probe signals. Color cameras may be used or pseudocolor may be used to accentuate small brightness variations. Analog enhancement [11] can optimize the quality of the image to be digitized by adjusting the gain and offset so that light and dark areas are balanced.

Once an image is digitized (snapped, captured, grabbed, or frozen), it can be identified and stored in the database, which will further allow for the manipulated image to be displayed on a monitor or printed as a hard copy. The image can also be stored long term on an optical disk.

Image processing is used to eliminate irrelevant detail (reduce noise) by digital filtering or averaging, to subtract background, and to enhance contrast by spreading out pixel values over a wider range of gray shading. Sharpening the image will often increase contrast at edges while suppressing contrast in the rest of the image. This edge sharpening or crispening of images produces a result that appears sharper than the original [12].

In practical terms, the use of digital imaging in cytogenetics calls for a concerted effort when the system is set up to ensure that images are obtained that are at least as good as photographic results. If precautions are not taken at the outset, lower standards of quality may become entrenched. Given that digital imaging is capable of producing representations of banded chromosomes as good as, or better than photography, every effort should be made to use this capability to its full advantage.

In general, one should trypsinize less and stain lighter than for photography to get good results with imaging. Care must be taken to ensure that fine bands are imaged and that pale bands at the ends of chromosomes are not lost. As with photography, an essential first step is to adjust the microscope correctly for the best resolution and contrast.

To image a metaphase

1. Adjust the light so that it is as bright as possible, with the palest areas viewed in the monitor just beginning to disappear; set the light just under this point to get the most light without losing detail. Some systems give a histogram on the monitor for adjusting the light level.
2. Adjust the microscope image to its sharpest image on the monitor. Some systems have a numerical representation to aid in sharply focusing the microscope image.
3. Adjust gain and offset to balance light and dark areas so that band contrast is comparable to that seen through the microscope.
4. Capture the image. Identify it and store it as a file within an organized, retrievable system in the computer.
5. Manipulate the image as desired, for example, enhance, karyotype.
6. If required, print the image so that it reproduces the detail with which it had been seen on the monitor.

For specific detailed information on digital imaging, refer to Chapter 15, Computer imaging.

14.5.3 Printer

Printers are an integral part of an imaging set-up. It is important that the printer quality is sufficient to render in the print all the image detail recorded digitally.

14.5.4 Modern advances in microscopy tools

Metaphase finders and analyzers

Metaphase finders have found a permanent home in many cytogenetics laboratories, not just because of its usefulness in cases where metaphases are sparse, such as in post-treatment bone marrows or direct preparations of chorionic villus samples [13], but also in reducing the technologists' time in scanning and photographing, especially in neoplastic specimens where every cell must be analyzed. It also allows directors to review both the linear array of chromosomes and to be able to review additional cells from a remote site if the need were to arise, without requiring a microscope or the patient's slide. Automated chromosome analysis, on the other hand, has not met with the same success and still requires the human mind to accurately identify chromosomes variance caused by inconsistencies due to differential degrees of condensation or its position within the cell at the time it was fixed. Some systems have also been challenged with the excessive file size of scanned images, which can affect both computer efficiency and storage capacity.

Digital imaging of FISH probes

Digital imaging has been especially useful in recording pale signals that are sometimes produced using FISH techniques. Image processing can increase contrast and filtering systems can be used to improve the signal to noise ratio.

CCD cameras

Charge-coupled device technology is used in CCD cameras making it possible to detect signals not visible to the microscopist and to use probes smaller than 2 kb.

Confocal microscopes

Laser scanning microscopes greatly reduce out of focus fluorescence and are often used to image three-dimensional structures. Though not as sensitive as the CCD camera, the confocal microscope is able to image probe signals quite well. One limitation, however, is in its use with fluorochromes, usually green and red, which can be excited by the existing lasers.

References

1. Delly JG. *Photography through the Microscope*, 9th ed. Rochester, NY: Eastman Kodak; 1988.
2. Determan H, Lepusch F. *The Microscope and its Application*. Wetzlar, Germany: Leitz.
3. Mollring FK. *Microscopy from the Very Beginning*. Oberkochen, West Germany: Carl Zeiss; 1981.
4. Kapitza HG. *Microscopy from the Very Beginning*. Oberkochen, West Germany: Carl Zeiss; 1994.
5. Holz HM. *Worthwhile Facts about Fluorescence Microscopy*. Oberkochen, West Germany: Carl Zeiss; 1975.
6. Culling CFA. *Modern Microscopy--Elementary Theory and Practice*. London: Butterworths; 1974.
7. Lawson D. *Photomicrography*. New York: Academic Press; 1972.
8. Davidson NR. Photographic techniques for recording chromosome banding patterns. *J Med Genet* 1973;10:122.
9. Green WB. *Digital Image Processing: A Systems Approach*. New York: Van Nostrand Reinhold; 1989.
10. Russ JC. *Computer-Assisted Microscopy*. New York: Plenum Press; 1991.
11. Lacey AJ. *Light Microscopy in Biology: A Practical Approach*. Oxford, UK: IRL Press; 1989.
12. Piper J, Lundsteen C. Human chromosome analysis by machine. *TIG* 1987;3:309–313.
13. Ji L. Fully automatic chromosome segmentation. *Cytometry* 1995;17:196–208.

CHAPTER 15

Computer imaging

Christine E. Haessig

(retired), Cytogenetics Laboratory, Vancouver General Hospital, Vancouver, BC, Canada

15.1 Introduction

With the development of imaging technology in the field of Cytogenetics, computer-assisted karyotyping systems producing digitized images have all but replaced traditional photographic prints. Technological advances in microprocessors, video cameras, monitors, and printers all contribute to a higher quality image.

Computer imaging in cytogenetic terms is the process of transforming a metaphase image from a microscope into digital components for display and manipulation on a monitor. By applying the computer enhancement tools (sharpen and contrast), the resulting laser print produces a high-resolution image. A benefit of this technology includes the ability to link microscope workstations to one another via local area network and satellite capture systems. Technologists are able to digitize an image from any microscope, store it on a central computer, and later recall and karyotype the image from any workstation. This networking capability increases case accessibility from any one station, thereby reducing any “bottle-neck” effect.

Computer imaging systems offer several advantages over conventional photography. Turn-around-time, for example, is significantly reduced as patient results with completed karyograms can be reported more quickly. Printing costs are considerably less with laser printers. Long-term archival storage is also easily retrieved from in-house computer network systems or from disks that require minimal storage space.

15.2 Techniques to improve karyogram image quality

In any technology, problems can occur and adjustments to equipment and/or procedures may be needed to achieve the desired quality of prints. Laboratories may sometimes find that their prints are poorly contrasted or look “muddy,” have diminished resolution or loss of telomeres and/or satellites. Multiple factors can affect the quality of results provided by your imaging system, and not all of these involve imaging software. Metaphase preparation, microscope adjustments, image capture settings and the computer’s enhancement capabilities all contribute to the quality of metaphase and karyogram prints. To attain better image quality, the following topics will be discussed:

- Metaphase preparation
- Microscopy
- Image capture
- Enhancement
- Advanced contrast
- Macro programming.

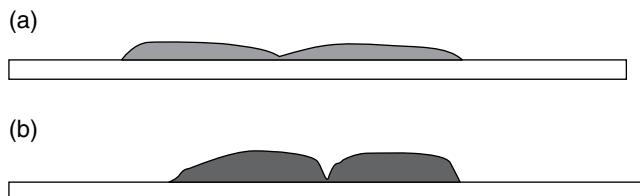


Figure 15.1 Side view illustrations of chromosomes under phase contrast. (a) Long, medium-gray, well-spread chromosome. (b) Short, dark, refractile chromosome.

15.3 Metaphase preparation

Understanding the relationships between the uses of phase contrast, chromosome length, staining, and band resolution may be helpful in resolving image quality problems.

15.3.1 Use of phase contrast

During initial slide preparation, the technologist will find it useful to view unstained slides under the phase microscope. Metaphase chromosomes should be long, well spread and appear medium-gray under phase contrast (Figure 15.1a). Very dark or refractile chromosomes under phase contrast tend to be shorter, not as flat and are more difficult to band (Figure 15.1b). Adjustments to relative humidity, room temperature, cell concentration, dropping cell suspensions from different heights, and slide drying all contribute to obtaining optimal metaphase spreads (see Chapter 2, section 2.4, Slide-making).

15.3.2 Chromosome length and staining

Chromosome length can affect the way stain is absorbed. For example, shorter chromosomes are more compact than longer chromosomes and will stain darker. When these dark, dense chromosomes are enhanced, they will yield less resolution or less differentiation of the finer bands than longer chromosomes. Overstaining can also decrease band resolution; this can be influenced by the type and strength of stain and the length of staining time. Standard laboratory staining protocols may need to be adjusted to prevent images that appear too dark, look muddy, and have decreased resolution on the final prints.

15.3.3 Band resolution

When optimizing metaphase staining techniques, it is useful to quantify resolution as it is affected by chromosome length and/or staining time. One method to accurately evaluate the resolution or band level of a metaphase is with the assistance of a banding resolution system [1]. Use this system to establish the resolution of banded metaphases of similar length. Band additional metaphases with a decreased stain time and compare the resolution of these cells to those previously stained of similar length. The presence of more bands in the more lightly stained preparation would indicate that poor resolution was initially due to overstaining. If resolution is not improved by decreasing the staining time, then increasing the actual length of the chromosomes may be required. This can be accomplished by using prophase or high resolution techniques (see Chapter 2, section 2.3.9, Chromosome anticontraction methods). It should be remembered, however, that unless banding conditions are optimal, longer chromosomes will not necessarily increase overall resolution.

15.4 Microscopy

15.4.1 Köhler illumination

Proper Köhler illumination directs the light optimally up from its source in the microscope through the metaphase image. To achieve proper Köhler illumination for image capture (see also Chapter 14, section 14.2.1, Köhler illumination):

1. Select the metaphase and high power magnification objective that will be used to digitize the image under brightfield. Avoid using phase contrast.
2. Close down the field diaphragm at the base of the microscope and focus the condenser until the outline of the diaphragm is sharp.

3. Open the diaphragm slightly so that the outline almost fills the field of view and center it accordingly. Once the diaphragm is in the correct position, open it to its maximum setting.
4. Set the light intensity to the highest possible setting without eroding the palest segments of the chromosomes on the screen.

15.4.2 Magnification

Higher magnification of a metaphase is sometimes used in an attempt to increase chromosome resolution. Adding magnification by the use of an optivar or a camera adapter with a “zoom” feature can, in fact, diminish resolution. This empty magnification will increase the actual size of the chromosomes but will not increase resolution. To produce sharp images with good resolution, use the highest quality 100 \times oil objective corrected to infinity.

15.4.3 Focus

Sharpness of an image is greatly affected by focus. It may be difficult to obtain a sharp image of all chromosomes within a metaphase to be captured. Some of the chromosomes may appear sharp while others are out of focus. This can occur if the entire metaphase is not lying within the same plane of focus on the slide. It is important to take the time to refocus those chromosomes which are out of focus and capture additional images, as is done with conventional photography. With computer imaging, these chromosomes can then be fused to produce a single metaphase with all chromosomes in focus.

15.5 Image capture

15.5.1 Computer gray levels

When proper Köhler illumination is attained and the image is sharply focused, the metaphase is ready to be captured. The metaphase image seen on the monitor is captured when the digitizing system within the computer takes the signal from the microscope’s video camera and converts it into a numerical matrix. This matrix is composed of row and column indices creating a grid-like system. The intersecting points of this grid are called pixels. Each pixel or “picture element” is attributed a gray value. In the CytoVision® imaging system there are 256 computer gray levels in an image (Figure 15.2); black is 0 and 255 is white [2]. The gray values represent the entire field of view, including chromosomes, debris, cytoplasm, and interphase nuclei. The gray levels or gray scale values represent whatever is being looked at on the monitor.

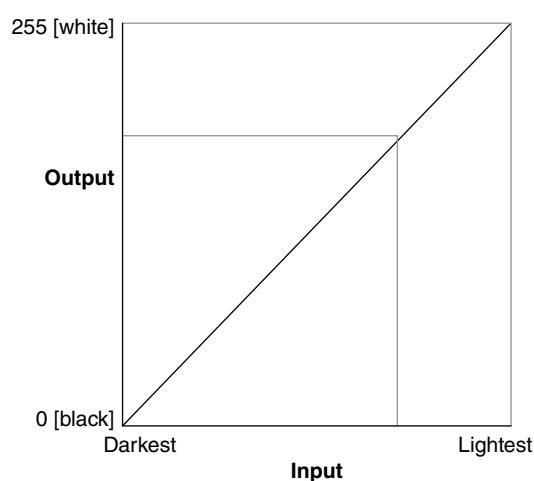


Figure 15.2 Computer gray levels.

15.5.2 Gray scale

Understanding how these values are processed by the computer can be clarified with the assistance of a photographic gray scale or zone system (Figure 15.3) developed by the famous American photographer, Ansel Adams [3]. This system is a graphic aid used in photographic developing to represent all the possible tones or strengths of grays from black to white. Zone 0 is maximum black; V is the tonal value that prints as middle gray; and IX is the pure white of the paper base of the print [4]. The black and white photograph (Figure 15.4) illustrates the gray tones of zones 3, 5, and 7. A computer-enhanced metaphase image (Figure 15.5) also displays the varying tones of gray as seen in the zone system. In this metaphase, the staining time is adjusted so that when digitized, the maximum range of gray values is seen. The computer's enhancement features are best utilized when metaphases are banded in this manner. Metaphases that are lightly stained allow the contrast enhancement features of the computer to encompass the entire range of values from black to white to heighten image quality.

A metaphase that is overstained (Figure 15.6) limits the range of gray values causing the range to shift towards the gray-black and black tones of the photographic gray scale. Consequently, the white tones of the chromosomes now appear

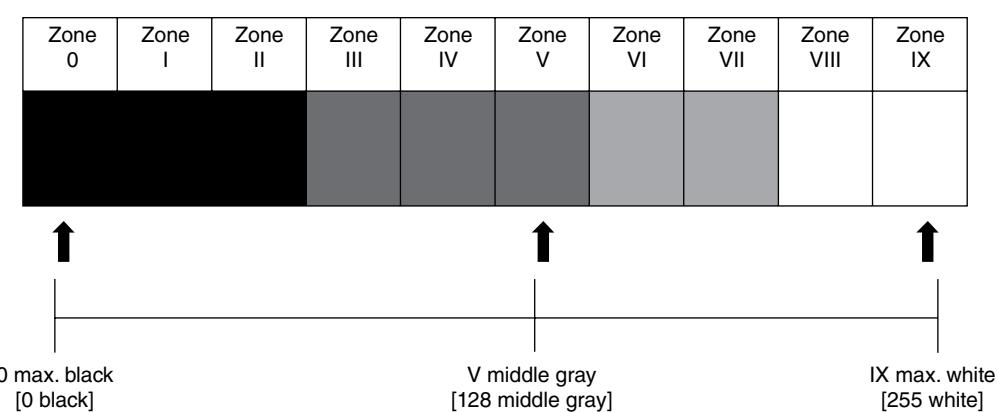


Figure 15.3 The photographic zone system or gray scale illustrates the tones of gray ranging from black to white.

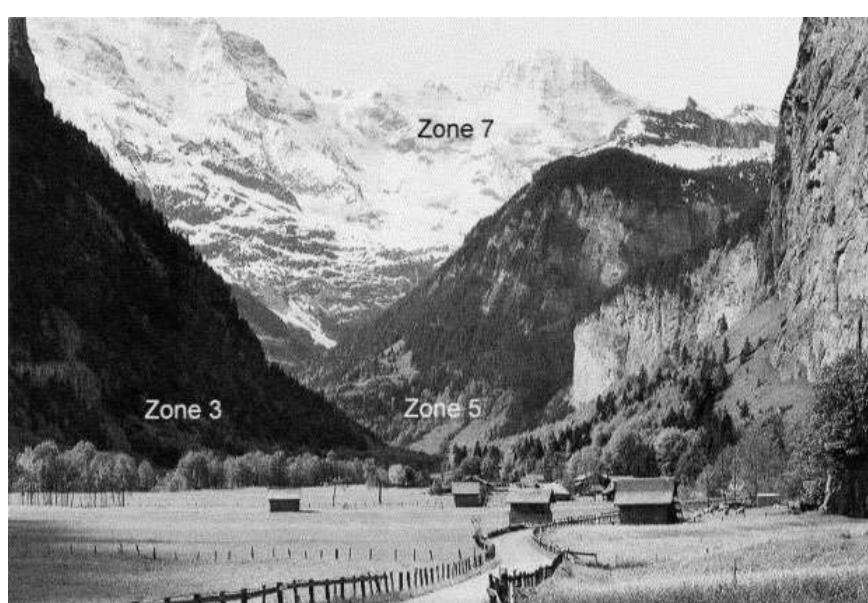


Figure 15.4 Gray tones of zones 3, 5, and 7 are depicted in this black and white photograph. Reprinted by permission of Norman Koren.

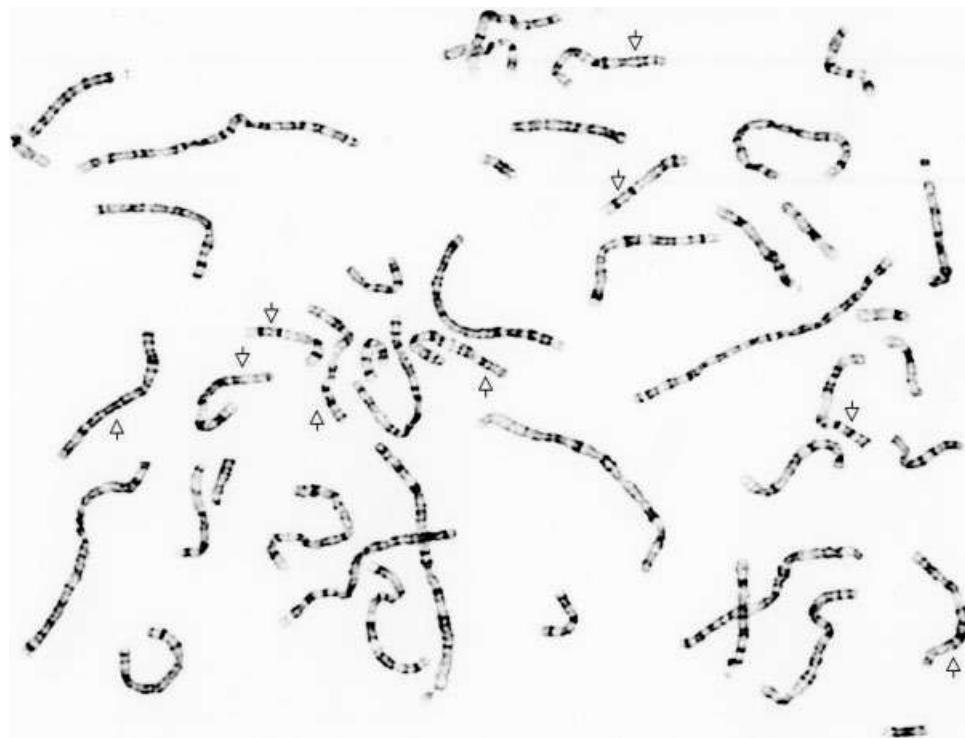


Figure 15.5 A computer-enhanced image of a fused metaphase that is optimally stained for enhancement. Finer gray bands appear distinct and separate. Arrows designate quality indicators. Courtesy of Christine Haessig.

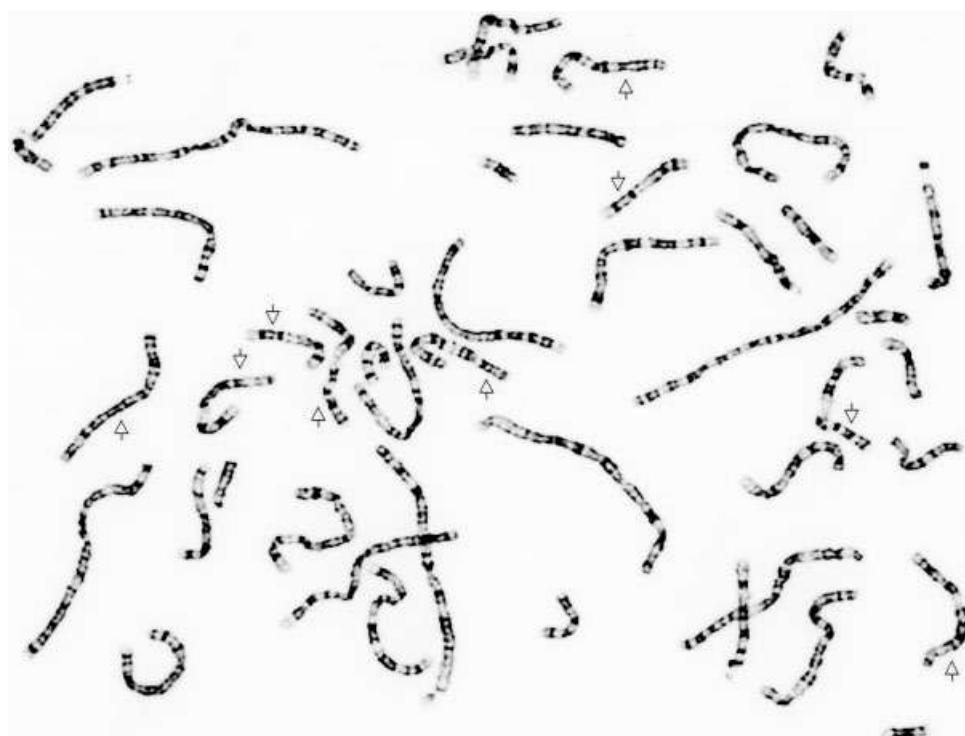


Figure 15.6 An overstained or overly contrasted image of the same metaphase as in Figure 15.5. Arrows indicate the chromosome regions where finer bands tend to merge and result in a loss of resolution. Courtesy of Christine Haessig.

light-gray, gray tones appear dark-gray, and dark-gray tones appear gray-black or black, producing a very dark and “muddy looking” image. Using the computer’s enhancement features in an attempt to reduce the intensity of the darker gray and gray-black areas of overstained chromosomes can cause the lighter gray and white-gray areas of the chromosomes to shift towards the white tones of zone IX. This leads to loss of the satellites and telomeric ends of the chromosomes on the resulting print. The enhancement capabilities of the system cannot improve resolution of a metaphase that is stained too darkly. It is also important to note that any dark-gray or black artifacts, such as debris, cytoplasm and nuclei in the field of view, can also cause a shift in the range of gray levels and will thus interfere with the computer’s enhancement capabilities. Therefore, it is best to delete debris from the original image before proceeding with sharpness or contrast adjustments of the captured image.

15.5.3 Indicator chromosomes

The dark bands of the chromosome regions such as X, 10, 11p, 12q and 17q can be used as quality indicators (Figure 15.5). If these areas are adequately banded, the finer bands display a wide range of gray values and appear distinct and separate. In a metaphase that is overstained or over contrasted (Figure 15.6), the finer bands appear to merge, subsequently giving fewer, darker, and thicker bands.

In summary, overstained metaphases limit the enhancement capacity of the computer, resulting in images with a reduced range of gray values that appear too dark or are poorly contrasted with decreased resolution. Optimally stained metaphases allow the enhancement features of the computer to encompass the entire range of gray values from black to white to produce prints of good quality and resolution.

15.6 Enhancement

15.6.1 Sharpening

Before adjusting the overall contrast, it is advisable to sharpen the metaphase, as the process of sharpening can also make the entire image slightly darker. Sharpening increases the contrast between pixels (or increases the difference between gray levels) so that details are more visible. Smaller increments of sharpening are recommended over large ones. This allows for greater precision when choosing the correct degree of sharpness for the metaphase. Using a sharpening increment that is too large can cause the image to appear “digital, pixelated, or jagged,” similar to chromosomes that are overtrypsinized.

As an example, if a computer imaging system has a sharpening factor from 0 to 9, and 6 is the recommended increment for sharpening a metaphase, it may be preferable to set the sharpening factor to a smaller increment number, such as 2 or 3. The metaphase can then be sharpened more than once to achieve the desired sharpness. As another example, a metaphase could have a required sharpening factor of 4, but if sharpened at a factor of 6, the resulting image would look oversharpened. By sharpening twice, using a factor of 2, the ideal sharpness is obtained.

15.6.2 Contrast

Contrast is the difference in subject tones from black-to-gray-to-white or from the darkest tone to the lightest tone. Once the image has been sharpened, contrast can be improved by adjusting the gray levels of the image. At this point, the dark levels can be intensified to increase the visibility of the fine gray bands. Avoid adding too much contrast to the image as this can cause dark bands to merge into each other, causing loss of resolution (Figure 15.6). If chromosomes are overstained, it may be difficult to contrast the image optimally. In metaphases that are undercontrasted, the dark chromosome bands do not appear as distinct. To increase the resolution, simply adjust the contrast slightly to increase the dark values so that the finer gray bands become more distinct.

15.7 Advanced contrast

In the **Advanced Contrast** feature (Figure 15.7) of the Leica Microsystems – CytoVision® computer imaging system, contrast can be adjusted in greater detail with the use of the following features [2]:

- Pink/Blue Sliders
- Brighten/Darken Contrast Sliders
- Cutoff % Sliders.

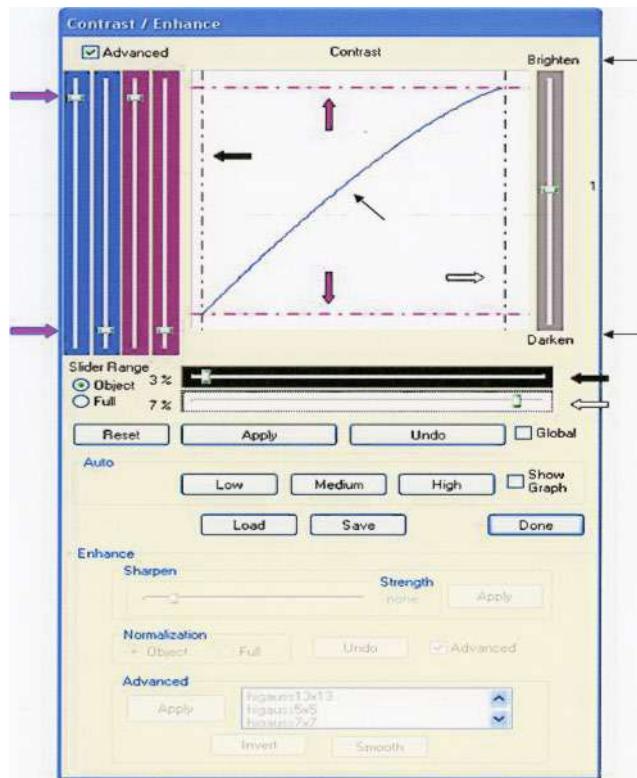


Figure 15.7 Advanced contrast feature of the CytoVision® computer imaging. See insert for color representation of this figure.

15.7.1 Pink/blue sliders

To prevent areas in the image from becoming too black or too white, adjust the **Pink/Blue Slider** commands in **Advanced Contrast** (pink or shaded arrows in Figure 15.7) slightly down from the top and up from the bottom to limit the upper and lower limits of gray. This helps to avoid the loss of chromosome satellites or telomeres and should always be used in conjunction with the **Brighten/Darken Contrast** and the **Black and White Cutoff % Slider** commands.

15.7.2 Brighten/darken contrast slider

The **brighten/darken slider** pushes the contrast stretch (curve) up or down (thin black arrows in Figure 15.7). This command allows the relative intensity of black, gray and white levels to be changed within the screen image.

Brighten Slider bends *up* the contrast stretch to lighten the overall contrast of the image; for example, when the slider is moved up to **+1**, this changes the relative intensity of the tones so that the gray and light-gray bands shift towards white and become brightened.

Darken Slider bends *down* the contrast stretch to darken the overall contrast of the image when, for example, the slider is moved down to **-1**. If an image is too light after enhancing, moving the slider to **-1** helps increase the image's overall contrast so that all mid-range grays will be darkened without making the image look too dark or muddy.

15.7.3 Cutoff % sliders

The **cutoff % sliders** use the value selected as the darkest or lightest band and calculates the contrast stretch based on that value. Any grays outside the slider will be changed to white or black [2].

Black Cutoff % Slider shifts the gray levels towards black so that the grays become darker and whites become grayer – for example, when the black cutoff is set at 3% (thick black arrows in Figure 15.7), the darkest 3% of gray levels will shift towards black.

White Cutoff % Slider takes the selected section of light grays and turns them to white (white arrows in Figure 15.7). For example, when the white cutoff is set at 7%, the lightest 7% of gray levels will shift towards white.

15.8 Macro programming

A macro automates a series of commands by grouping them together into a single command. A single keystroke can be used to replace a series of repetitive actions [2]. Macros can be programmed for enhancement applications, such as standard sharpening and contrast settings, for quick repeat applications or to quickly add text or delete material. Macro programming can also be used for MetaSystems imaging software.

To program a **Macro** (Figure 15.8):

1. Click on the circle for a macro **F key** (e.g., F7).
2. Enter the name to label the macro program.
3. Click on **Record** and program each step of the program.
4. Click on **F11** key on keyboard to stop recording.

For example, a macro program can be used to clear any faint outlines that appear around each chromosome in a karyogram. This macro should be used as the very last enhancement application for a karyogram.

To program the **Clear Fuzzy Edges** macro:

1. Click on the circle for a macro **F key** (e.g., F7 in Figure 15.8).
2. Enter *Clear Fuzzy Edges* to label the macro program.
3. Click on **Record**.
4. Click on the **Contrast** icon for enhancement.
5. Set the **White Cutoff % Slider** to 1% (e.g., Figure 15.9).
6. Click **Apply** and **Done**.
7. Press **F11** key to stop recording.

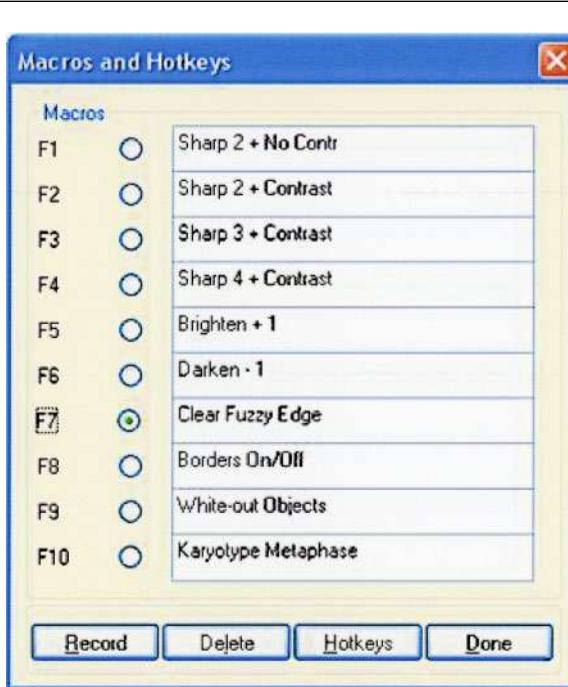


Figure 15.8 Macro programming.

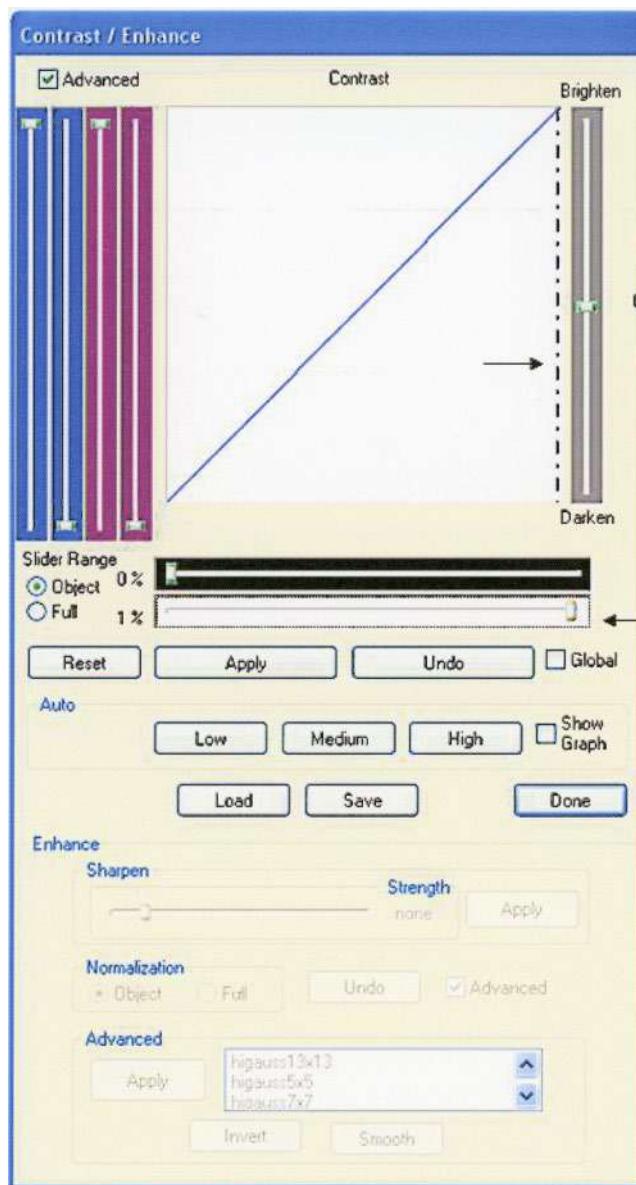


Figure 15.9 An example of a macro program, to clear the fuzzy edges from the chromosomes of a karyogram. See insert for color representation of this figure.

15.9 FISH imaging

15.9.1 Microscope setup

FISH imaging requires a 100-watt fluorescent bulb that is centered and focused. Uneven illumination from an uncentered bulb can make thresholding an image difficult. The correct filters should be used for the type of fluorescent dye needed (e.g., select the right filter for the dye 82000 vs. 83000). Filters should be less than 3 years old; otherwise, the colors may not be as intense and amplification may be necessary [5].

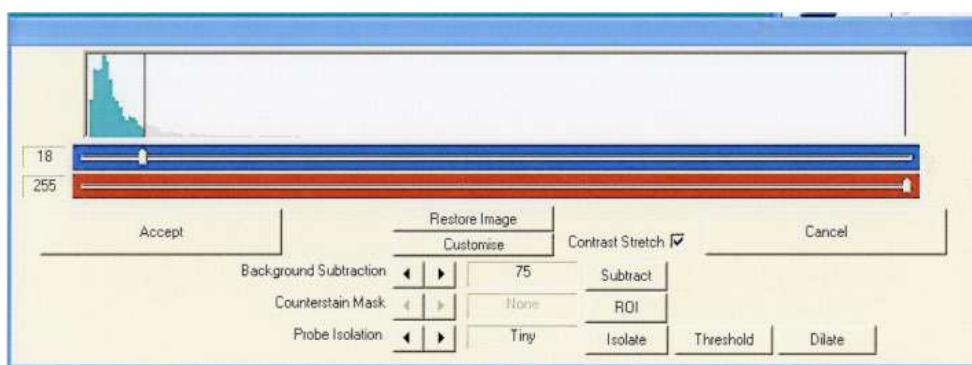


Figure 15.10 Thresholding tools. See insert for color representation of this figure.

15.9.2 Thresholding

Thresholding probe images is similar to thresholding for brightfield. The difference is that when thresholding probes, any area not covered by the blue mask will have the color chosen for that fluorochrome applied to it. Background noise and debris can make thresholding difficult. The following tools (Figure 15.10) are useful when thresholding probe images [2]:

- **Counterstain mask** – eliminates all parts of the image that do not contain counterstain. This is a good tool to use if there is a lot of background noise.
- **Region of interest (ROI)** – defines one or more areas for thresholding. This tool aids in isolating small signals from artifacts or debris.
- **Probe isolation** – looks for signals that match the size chosen and isolates them from the rest of the image [2].
- **Dual-gated sliders** – the blue slider is used to set/reduce the background level. Any grays to the left of the slider will be turned black. The red slider sets the cut-off for white. Moving it left will brighten the image and any gray level to the right of the slider will be changed to white.
- **Background subtraction** – is used to remove background noise and objects based on the size of their banding profiles. For metaphases, 15–25 is recommended. Often nuclei are broken up by background subtraction levels set for chromosomes or only the edges remain. This will happen if there is uneven staining within the nucleus that is approximately the same intensity and size as chromosomes or signals. Err on the large side, and if unsure, choose a larger number. To capture nuclei, use background subtraction values of 50–100. Automatically apply Background Subtraction during capture by turning on Auto Background Subtract in Customize [5].

15.9.3 Probe enhancement tips

- Use **Preview** enhancements to view enhancements before printing.
- The **Sharpen** command increases the contrast between pixels, so that details are more visible.
- The **Smooth** command reduces the contrast between pixels, so that details are less visible.
- The tools in **Contrast** allow you to change the range of gray levels to lighten or darken the screen image. Contrast stretches can be applied to whole images or to selected objects only.
- If signals need to be consistently brightened, try lowering the gamma in the fluorochrome's color settings. This will brighten the signals as part of the capture process.
- Keep the relative brightness of signals by using the contrast and not sharpening the signals.
- Usually counterstain should be enhanced or contrasted separately from the signals.
- When karyotyping a metaphase, perform enhancements on the fluorochromes before classification. Their appearance cannot be changed once a karyogram is created [5].
- **Higauss filters** (7×7 , 9×9 , 13×13) in the **Advance Contrast** command are particularly useful when working with inverted DAPI images. These filters attenuate low spatial variations and accentuate high spatial variations to achieve the overall effect of sharper edges [2].

15.10 Printing

When printing black and white karyograms and metaphases or color FISH images, it is advisable to use high-quality, color copy paper (28 lb. with 98 Brightness) for color laser printers. The higher the number on brightness, the more light is reflected off the page for better image contrast and clarity. Printing with regular photocopy paper of lesser quality affects the resolution and can lower image quality. Laser printers seem to be the printer of choice in a cytogenetic laboratory setting, since they produce prints with very good quality at a reasonable expense (e.g., Ricoh Aficio or Lexmark printers).

15.11 Quality control

It is important to establish regular quality meetings amongst staff to discuss image quality using the quality indicators and band resolution system. Protocols may need to be adjusted in the staining process or during enhancement of the image to ensure that optimal resolution and high standards are maintained. As an additional quality measure, it is also important to perform monthly maintenance on your imaging system. This includes simple measures to ensure the CPU unit is free of any dust accumulation; cords belonging to different components of the unit are free of any damage or do not appear frayed; and monitor, mouse, and keyboard are cleaned with an appropriate solution. For example, a screen cleaning kit solution free of alcohol, ammonia or any surface protection coating is generally best for the monitors. It is also advisable to not block the fan area of the CPU with any object as it can cause poor ventilation and unnecessarily elevate the temperature of the computer.

15.12 Archiving

Completed cases should be transferred from the hard drive of the computer to a separate medium for long term storage on a monthly basis. Storage of electronic cytogenetic images can vary from few to several years or indefinitely, depending on state or provincial guidelines for individual laboratories. Archiving becomes necessary, as there is limited space on an imaging system's hard drive and also to guard against data loss during disasters. Completed cases from a given month can be transferred from the root directory on the imaging system to a DVD, an external hard drive, or an in-house computer network. Additionally, once cases have been archived, they should be checked to see if they are indeed retrievable from their archived destination before deleting them from the hard drive, so that any files that become corrupt can be re-archived immediately. When archiving on DVDs, it is advisable for laboratory accreditation purposes to archive each case on two separate DVDs so that one set can be stored at a different location to be used for back-up purposes.

Acknowledgments

I wish to thank our director, Dr. Helene Bruyere, and cytogeneticist, Dr. Tanya Gillan, at the Vancouver General Hospital, Cytogenetics Laboratory, for their helpful review and editing of this chapter.

References

1. Josifek K, Haessig C, Pantzar T. Evaluation of chromosome banding resolution: A simple guide for laboratory quality assurance. *Applied Cytogenetics* 1991; 17:4.
2. Leica Microsystems, *CytoVision®* 3.7, 2005.
3. Koren N. A simplified zone system for making good exposures. Images and text copyright © 2000-2005 by Norman Koren, www.normankoren.com.
4. Balsys A, DeCock-Morgan L. *The Morgan & Morgan Darkroom Book*. Dobbs Ferry, NY: Morgan & Morgan Inc, 1980.
5. Haessig C, Merrick S. Techniques to improve your CytoVision® karyotypes. AGT Workshop, Jacksonville, FL, 2009.

CHAPTER 16

Fluorescence in situ hybridization (FISH)

Helen J. Lawce¹ and Jeffrey S. Sanford²

¹Oregon Health & Science University Knight Diagnostics Laboratory, Portland, OR, USA

²MetaSystems Group, Inc., Newton, MA, USA

16.1 Introduction

16.1.1 Molecular mechanisms of FISH

DNA has a special property which allows molecular investigation using various probes, and that is the complementary nature of its nucleotide bases: G (the purine, guanine) preferentially pairs with C (the pyrimidine, cytosine) and A (the purine, adenine) preferentially pairs with T (the pyrimidine, thymine) (see Chapter 1 and Figure 16.1). Base sequences in the strand will be different in various regions of the genome. The complementary strands are weakly held together by hydrogen bonds, which can be broken with heat or alkaline pH into two separate strands. This separation is termed DNA denaturation or dissociation. When the strands are cooled, if the salt conditions are correct, the base-specific hydrogen bonds will form again, and the DNA will return to double-stranded, with the G–C and A–T bonds restored. This is termed DNA renaturation, reassociation, or reannealing. If there is DNA present that matches a denatured DNA sequence, base for base, it can reanneal with the strand, even if it is not from the complementary original strand. Thus, the addition of a denatured DNA “probe” to a denatured chromosome can give rise to a hybrid DNA. The probe will usually hybridize to the chromosome in spite of small mismatches or a point mutation in the chromosomal DNA.

Within the probe, a detectable reporter molecule (usually fluorescent in today’s methods) allows visualization of the probe’s complementary sequence location. Using a fluorescence microscope, there will be a bright spot or spots (signals) on the nucleus or chromosomes (the “target”), where the fluorescent-tagged probe has hybridized to a cell on the slide (“*in situ*”). The rate at which the strands reanneal is dependent upon the DNA concentration, the size of the probe, and the degree of similarity between the probe and the target DNA, as well as other less defined variables, such as how much protein the DNA probe must penetrate to get to the target.

The term fluorescence *in situ* hybridization (FISH) represents the methodology to detect specific nucleotide sequences by using DNA probes labeled with a fluorochrome that will hybridize with denatured chromatin (DNA) on a microscope slide (Figure 16.2). The natural evolution of the cytogenetics laboratory into the realm of FISH has lead to the term “molecular cytogenetics” as a synonym for FISH. Other terms used include molecular pathology or molecular cytology.

16.1.2 Historical development of FISH

The development of FISH began with nonfluorescence *in situ* hybridization (reviewed in [1]). The first *in situ* hybridization (ISH) was performed in 1969, utilizing tritiated thymidine-tagged RNA probe and a silver photographic emulsion over slides containing mouse and *Drosophila* chromosomes, to track the radioactive emissions as they exposed the emulsion [2]. When the hybridized slide with emulsion was exposed for several days and the emulsion was photographically developed, the location of the tritiated-probe was visualized as silver dots from the exposed emulsion. The method evolved to include DNA probes and single genes. For example, Harper et al. [3] mapped the insulin gene to chromosome 11p15 using radioactive methods.

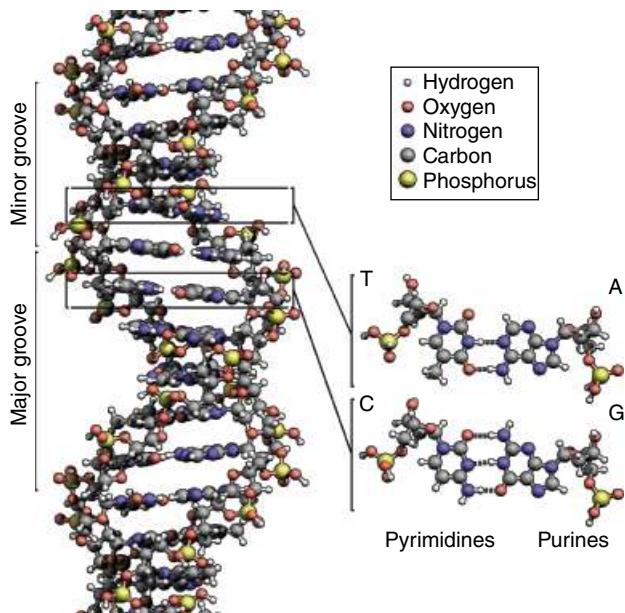


Figure 16.1 An element replica of DNA. Figure shows the detailed structure of the four bases -adenine, cytosine, guanine and thymine, and the location of the major and minor groove. By Richard Wheeler, at the Sir William Dunn School of Pathology, University of Oxford, used with permission. [http://commons.wikimedia.org/wiki/File:DNA_Structure%2BKey%2BLlabelled.pn_NoBB.png]. See insert for color representation of this figure.

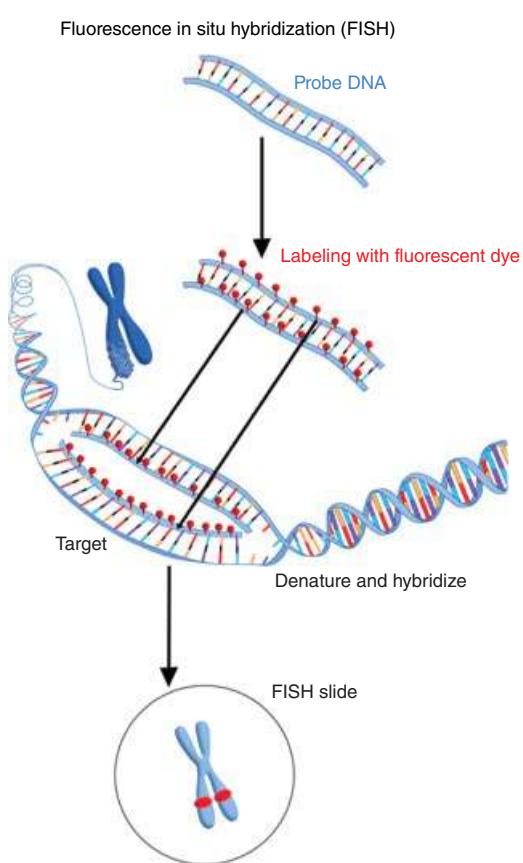


Figure 16.2 FISH process. The probe DNA (above) with a specific sequence is labeled with a fluorophore (in this case, one that emits red wavelengths when exposed to UV light). The probe (light blue with red label) and target (in this case a metaphase chromosome, but interphase cells are also appropriate) are denatured, usually using heat. The temperature is lowered to allow the probe to reanneal, or hybridize, to the target wherever they share complementary sequences (arrows). Nonspecific probe and excess probe are rinsed off during the post-hybridization stringency wash (not shown). Cells are counterstained to show the chromatin location, and observed on a fluorescence microscope to determine the location of the probe signals (lower right). See insert for color representation of this figure.

However, the resolution was low because the electrons produced by the decay of tritium scattered widely; therefore, statistical methods were needed to discern the significance of the signals and exclude the background noise [3, 4]. The background was partly due to the presence in the probes of highly repetitive DNA sequences, which are also present throughout the genome. Workers in the 1980s [5–7] learned to use unlabeled repetitive DNA from various sources, such as salmon sperm DNA, to block these repeats by pre-hybridizing (preannealing) them to the test DNA, preventing the repeats from being available to hybridize to the test DNA, thus obtaining a cleaner signal.

There were, however, significant drawbacks to this process: radioactive signals were imprecise due to scatter, tritium required long exposure times, and special safety techniques were needed due to the radioactivity. These drawbacks were eliminated in 1980 with the first use of a fluorescent dye (fluor) to detect RNA/DNA hybridized sequences [4]. This new method also allowed for multiple probes to be simultaneously investigated with different colors [8].

The first fluorescence detection methods to be used were performed with chemical labels attached to the DNA probe called haptens – historically, biotin (vitamin B₁₂), and digoxigenin (DIG). Estradiol and dinitrophenyl (DNP) are also haptens that are sometimes used in research for probe detection in three-dimensional nuclei. The hapten-labeled probes were detected by exposure to fluor-conjugated antibodies specific for the hapten label after the hybridization steps. For example, a hybridized probe labeled with DIG can be detected using an anti-DIG antibody conjugated with a fluorescein (FITC) molecule. Similarly, a probe labeled with biotin can be detected using avidin, a tetramer with a very strong affinity for biotin, or streptavidin, a bacteria-produced variation of avidin conjugated with a Texas Red dye. If the probe signal was not bright enough, it could be amplified with fluorescent antibodies specific for the initial hapten-targeted antibody [9]. Probes requiring detection steps are known as “indirectly labeled” probes. Later on, methods to attach fluorescent dyes directly to the DNA molecule were developed which do not require any detection steps to visualize the fluorescent dyes (“directly labeled” probes).

Directly labeled probes are cleaner and easier to use, and have become the label of choice for most purposes. There are a number of different fluorescent dyes that can be used, with up to five or six different colors in one assay, allowing detection of multiple chromosomal targets. Further refining of FISH signals led to development of binary color combinations and color ratios (called multiplexing) to identify multiple targets by varying the relative contributions of each color combination for each target. This development made SKY (Spectral Karyotyping) and M-FISH (Multicolor) possible (reviewed in [10]) (see Chapter 17, Multicolor FISH and CGH). Using both combinations and ratios, one form of multiplex FISH is often called COBRA (combined binary ratio) [11]. A 12 color M-FISH subtelomere panel has been used to discover small rearrangements invisible by G-banding studies [12].

16.1.3 FISH of today

FISH applications have grown to include detection of submicroscopic cytogenetic deletions), simple and complex translocations, inversions, gene amplifications (e.g., *HER2/neu*, *MYCN*), and numerical abnormalities on both metaphase and interphase cells (see Table 16.1 for examples of cancer applications). It has led to the development of comparative genomic hybridization (CGH) (reviewed by Forozan et al., in [13]), a method which allows the study of genomic changes in cells by co-hybridizing their fluorescent-labeled DNA competitively with differently colored, normal DNA against unlabeled, normal metaphases, and detecting over- or under-representation by which color predominates on the chromosomes of the normal metaphase cell.

Comparative genomic hybridization

Refinement of this method then led to the development of other chromosome microarray technology, such as array CGH (aCGH), which carried the idea of CGH further by using specific target sequences (instead of genomic DNA) that are spotted on a slide or special glass chip. As with CGH, aCGH involves competitively hybridizing differently colored patient genomic DNA and normal control DNA against the known target sequences on the slide to assess loss or gain of the sequences in the test sample in a high throughput assay, looking at hundreds or thousands of sequences in a single assay. Regions of genomic gain will appear chromatically skewed toward the patient label (e.g., green), while regions of genomic loss will be skewed toward the normal control DNA (e.g., red). Normally represented regions will be an equal combination of the test and normal DNA (e.g., neutral yellow) (see Chapter 18, Genomic microarray technologies for the cytogenetics laboratory). The applications of microarray technology are expanding every year, and are blurring the boundaries between conventional cytogenetics and molecular genetics. It does have some limitations, such as an inability to detect intra-chromosomal changes and balanced rearrangements, and it is not currently sensitive to low-levels of mosaicism. In such cases, confirmation is usually performed by standard FISH on metaphase chromosomes (see Chapter 18, section 18.3.7, Confirmation of abnormal CMA results). Other microarray methods are also currently used that are not based on comparative genomic methods, but rather work more like a straight FISH assay with a high throughput, such as single nucleotide polymorphism (SNP) and expression microarrays.

Table 16.1 Commonly used probes for hematological malignancies and solid tumors

Probe	Location	Disease(s)	Abnormality
1p36/and control probe, plus 19q13/and control probe	1p36/19q13	Glioma	Deletions of 1p36 and 19q13
ALK BAP	2p23	Anaplastic lymphoma; carcinoma of lung; colorectal carcinoma	Translocation in lymphoma; inversion or translocation in lung
Alpha satellite 12	12 centromere	Chronic lymphocytic leukemia (CLL)	Trisomy 12
Alpha satellite 3, 7, and 17, plus a unique sequence probe for p16 (9p21)	Centromeres of 3, 7, and 17 for aneuploidy; 9p21 for deletions	Bladder cancer	Ploidy of 3, 7, and 17; deletion of p16 gene
Alpha satellite 8	8 centromere	Acute myelocytic leukemia (AML); myelodysplastic syndrome (MDS); myeloproliferative disease (MPD); chronic myelocytic leukemia (CML)	Trisomy 8
Alpha satellite X and Y	Centromere X and centromere Y or Yq	Monitor opposite sex donor transplants	Male/female chimerism/engraftment post transplantation
API2/MALT1 fusion	11q21/18q21	MALT lymphoma	t(11;18)(q21;q21)
ATM with control probe	11q22.3	CLL	Deletion
BCL6 BAP	3q27	Non-Hodgkin lymphoma (NHL)	t(3q; multiple partners including 2, 4, 6, and 14)
BCR/ABL1 fusion	22q11.2/9q34	Acute lymphocytic leukemia B-cell (ALL)/CML in blast crisis (CML) may present as AML	t(9;22)(q34;q11.2)
BIRC2 (API2)/MALT1	11q21/18q21	Lymphoma	Translocation
CBFB BAP	16q22	Acute myelomonocytic leukemia (AMMoL) (FAB M4)	inv(16)(p13q22), t(16;16) (p13;q22)
CCND1	11q13	Distinguish CLL from Mantle Cell Lymphoma (MCL); Multiple myeloma	t(11;14)(q13;q32)
CDKN2A (p16)	9p21	Leukemias, solid tumors	Deletions
CHIC2/FIP1L1/PDGFR α Fusion/deletion	4q12	Hypereosinophilia (HES)/chronic eosinophilic leukemia (CEL)	Deletion of CHIC2 and fusion of FIP1L1 (4q11-q12) and PDGFR α (4q12)
CSF1R/control probe	5q33-34	AML	Deletions
D13S319 or D13S25 with control probe	13q14.3	CLL MM	Deletion/nullisomy Deletion
D7S522 or D7S486 with control probe	7q31	AML, MDS	del(7)(q31)
DDIT3 (CHOP) BAP	12q13	Mixoid liposarcoma	t(12;16)(q13;p11)
EGFR and control probe	7p12	Glioblastoma	Amplification
EGR-1 with control probe	5q31	AML, MDS	del(5)(q31)
EMSY	11q13.5	Breast tumors	Amplification
ETV6 (TEL)/RUNX1 (AML1) fusion	12p13/21q22	ALL	t(12;21)(p13;q22)
EVI1 break-apart	3q26.2	AML/MDS/CML	inv(3)(q21q26) or t(3;3) (q21;q26)
EWS/ERG fusion	22q12/21q22.2	Ewing sarcoma/PNET/etc	t(21;22)
EWSR1 BAP	22q11	Ewing sarcoma/ PNET, desmoplastic small round cell tumor, clear cell sarcoma, others	Rearrangement usually with 11q but other partners are possible
FGFR1	8p12	Hypereosinophilia, MPD	Rearrangement
FL1/EWS fusion	11q24/22q12	Ewing sarcoma/PNET/ etc.	t(11;22)(q24;q12)

Table 16.1 (Continued)

Probe	Location	Disease(s)	Abnormality
FOX01 (FKHR) BAP	13q34	Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) or variant t(1;13)(p36;q14)
FUS BAP	16p11	Malignant liposarcoma, mixoid liposarcoma, AML, fibrous histiocytoma	mixoid liposarcoma: t(12;16)(q13;p11); fibromyxoid sarcoma: t(7;16)(q33;p11); AML: t(16;21)(p11;q22)
HER2/neu, also called c-erb-b2, usually with a control probe	17q11.2-12	Breast cancer, ovarian cancer	Amplification
hTERC	3q26	Various solid tumors	Low level amplification
IGH BAP	14q32	Lymphoma, MM, CLL	t(14q; multiple partners including 4, 8, 11, and 18)
IGH/BCL2 fusion	14q32/18q21	Follicular lymphoma	t(14;18)(q32;q21)
IGH/CCND1 fusion	14q32/11q13	Mantle cell lymphoma; MM; (CLL)	t(11;14)(q13;q32)
IGH/FGFR3 fusion	14q32/4p16	MM	t(4;14)(p16;q32)
IGH/MAF fusion	14q32/16q23	MM	t(14;16)(q32;q23)
IGH/MALT1fusion	14q32/18q21	MALT lymphoma	t(14;18)(q32;q21)
IGH/MYC	14q32/8q24	Burkitt lymphoma; Diffuse large B cell lymphoma (DLBCL)	t(8;14)(q24;q32)
IGK BAP	2p12	Leukemia, ALL, DLBCL	t(2p; multiple partners)
IGL BAP	22q11.2	Lymphoma	t(22q; multiple partners)
MDM2	12q13-14	Soft tissue tumors	Amplification
MET (c-MET)	7q31.2	Lung cancer	Amplification
MLL BAP	11q23	Mixed lineage leukemias, both myeloid and lymphoid; some MDS	t(11q23); multiple partners including chromosomes 4, 6, 9, and 19)
MYB	6q23	CLL and other lymphoid malignancies	Deletion
MYC (c-MYC)	8q24.1	Lymphomas; other lymphoid malignancies Brain tumors	Translocation, often with IGH (14q32) Amplification
MYCN/CEP 2 control probe	2p24	Neuroblastoma	Amplification
PBX1/TCF3	19p13.3/1q23	ALL	t(1;19)(q23;p13.3)
PDGFRB	5q33.1	MDS/MPD	Translocations, especially with ETV6 on 12p
PML/RARA fusion	15q22/17q21.1	APL AML (FAB M3)	t(15;17)(q22;q21.1)
PTEN/CEN 10 control probe	10q23.3/cen	Glioblastoma	Deletion, monosomy
RB1	13q14	MM; CLL	Deletion
RET BAP	10q11.2	Carcinoma of lung, colorectal carcinoma	Translocation
ROS1 BAP	6q22	Carcinoma of lung	Translocation
RUNX1 (AML)/RUNX1T1 (ETO) fusion	8q22/21q22	AML with maturation (FAB M2)	t(8;21)(q22;q22)
RUNX1 (ETO)/RUNX1T1 (AML1)	8q22/21q22	AML	Translocations
SS18 (SYT) BAP	18q11.2	Synovial sarcoma	t(X;18)(p11.2;q11.2)
TP53 with control probe	17p13.1	Many different oncology disorders, including CLL, MDS, MM	Deletion
TRA/D	14q11	T cell ALL	Translocation

BAP, break-apart probe strategy.

The availability of robust commercial probes and automation platforms has allowed the development of specific clinical tests on commonly performed methods, such as with BCR/ABL1 and HER2/neu (ERBB2), to be performed by spot counting image analysis systems [14]. These platforms allow a higher throughput than manual methods. However, manual analysis is still the gold standard, and is often used to confirm automated results; this chapter will cover basic theories and practices that can be utilized with or without automation.

16.2 Clinical applications of FISH probes

16.2.1 In vitro diagnostics versus analyte-specific reagents

The clinical applications of FISH are expanding as new commercial probes are developed. Probes are described as either in vitro diagnostics (IVD) or analyte specific reagents (ASRs) by the manufacturers and by regulating organizations. ASRs are probes for which the manufacturer has not established performance characteristics, and it is up to the purchaser to determine the analytic sensitivity, specificity, and utility of the probe. IVDs have been characterized by the manufacturer as useful for a particular application when used as directed and the analytical performance has been worked out and published. The FDA will only approve such IVD probes for specific uses, and the probes must be used exactly as the manufacturer specifies; therefore, if not used exactly as specified, the probe becomes classified an ASR probe, and the characteristics must be worked out by the purchaser/user. Home-brew probes (e.g., probes produced by the user) are treated similarly to ASRs. In addition, because the European definition of an IVD differs from the U.S. FDA definition, probes termed CE/IVD in Europe, may still be registered as ASR in the USA.

16.2.2 Probe designs

Several types of probe designs have been developed in the clinical setting for use in interpreting abnormal karyotypes, diagnosing various diseases, assessing prognoses, monitoring response to cancer therapies, and determining what therapy would be most effective in different situations. These probes include locus-specific probes, chromosome paints, alpha satellite DNA probes, fusion probes and break-apart or fission probes for translocations, plus several other designs. These probe strategies are all valuable for specific purposes and are discussed below.

Besides clinical uses of these technologies, research laboratories use FISH for many different investigations. Using paints and various conserved cosmids, researchers have traced the relationships of selected animals [10, 15–17]. FISH can also be used as a measure of chromosome instability, such as in evaluating the effect of certain environmental factors on aneuploidy. For example, interphase FISH studies using probes for certain aneuploidies have been resourceful in assessing the effects of smoking, caffeine and alcohol use in the sperm of healthy men [18]. FISH thus provides a means for detecting ploidy, amplification status, and chromosomal susceptibility to rearrangement through the presence or absence of certain chromosomes, sequences, and genes. This chapter will focus on the clinical uses of FISH.

Locus-specific probes

Locus-specific probes, also known as single copy or unique sequence probes, are used to detect small regions, including gene, telomeric and subtelomeric sequences (Figure 16.3). Their uses include detection of deletions, duplications, amplifications, translocations, trisomies, monosomies, inversions, and other abnormalities. They can be fusion-type probes, as well as dual-color break-apart probes, which are used in oncology/hematology studies. They may also be included in a probe mixture in order to act as an internal control for the number of copies of a specific chromosome. Locus-specific probes are usually of moderate fluorescence intensity.

Paint probes

Paints are libraries of multiple overlapping chromosome or chromosome-region specific probes. They are used to detect whole chromosomes, whole chromosome arms, or band-sized chromosomal regions. They are produced by flow sorting chromosomes or by needle microdissection of the chromosome bands (see Figure 16.4). Paints are useful for identifying unknown chromosomes, regions of chromosomes, and markers. Paints are also used in some types of multiplex (multicolor) FISH testing, such as M-FISH (Figure 16.5) and SKY (see Chapter 17, section 17.2, Multicolor FISH). These probes are moderate to strongly fluorescent, and are generally used only for metaphase analysis because the interphase chromosome signals are usually diffuse and difficult to interpret.

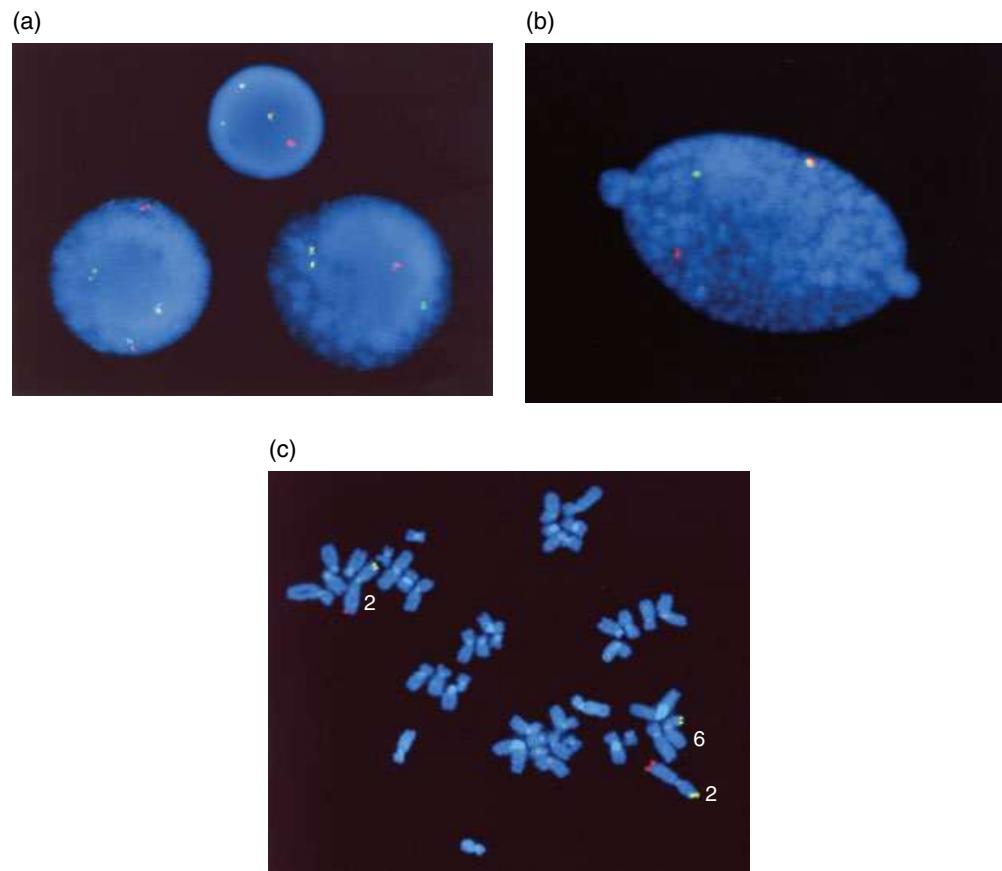


Figure 16.3 Single copy locus probes of several types. (a) Fusion probe. Translocations are indicated by the juxtaposed red and green signals, in this case for each derivative (a “D-FISH” strategy, for “dual fusion” probe type). The single red and green signals usually indicate the normal homologues that have not translocated. (b) Break-apart probe. Translocations are indicated by red and green signals that have become separated from each other. The red and green signals that are close together are on the normal homologue. (c) Subtelomeric specific probes for 2p (green) and 2q (red). Note that a chromosome 6 is exhibiting a signal for 2p, and represents a derivative 6 from an unbalanced t(2p;6p). See insert for color representation of this figure.

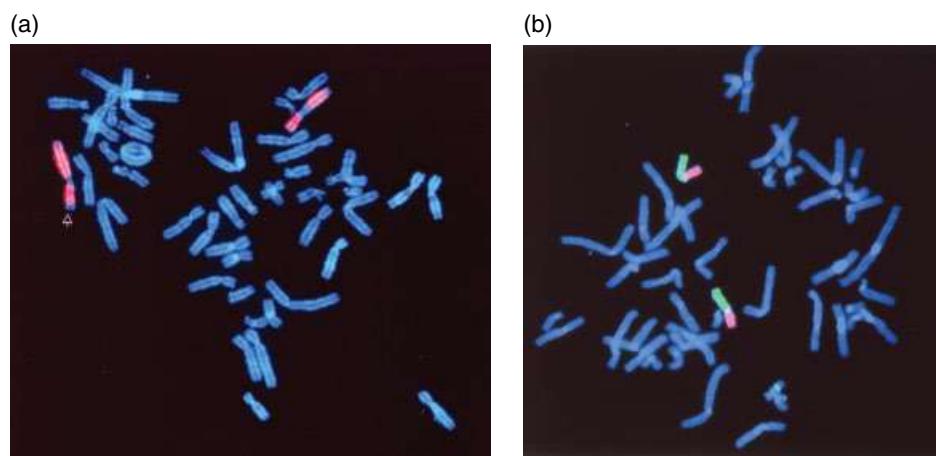


Figure 16.4 Chromosome paints. (a) A whole chromosome paint for chromosome 9. Note that the heterochromatic region in the pericentric region does not paint. This can either be caused by the repetitive sequences not being included in the paint mixture or the repeats are blocked with unlabeled DNA so that the paint will be more specific to the euchromatin of the 9 and will not cross hybridize with other chromosomes. The arrowed 9 has material from another chromosome at the end of the short arm and does not paint with this probe. (b) A whole arm paint for 11p (red) and 11q (green). Note that a small metacentric chromosome (arrow) is exhibiting green signal, and represents an insertion of 11q material into a 20q. See insert for color representation of this figure.

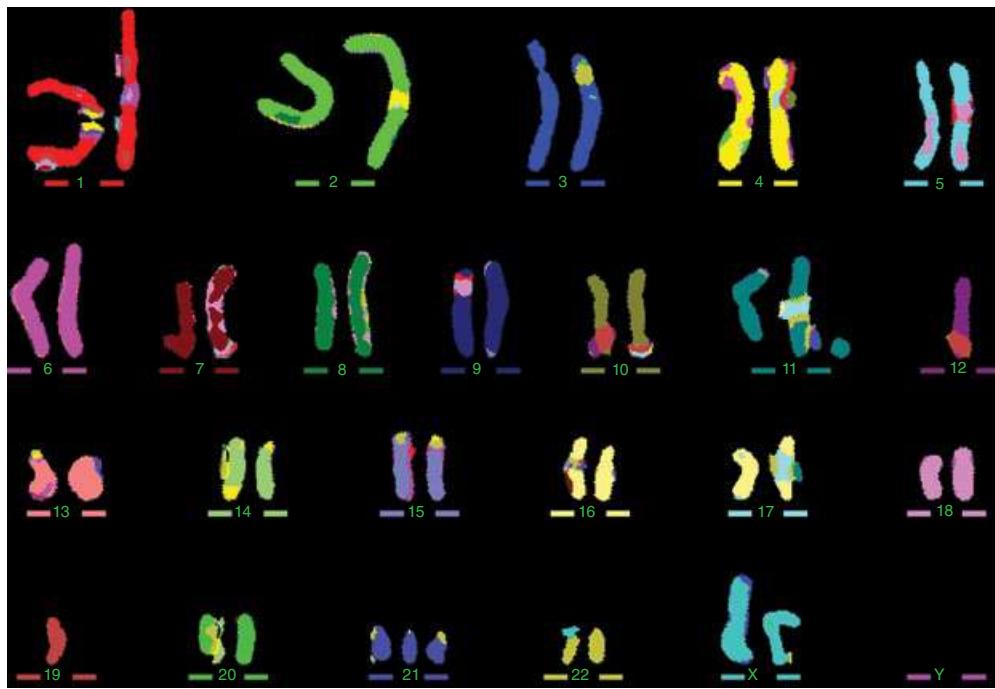


Figure 16.5 M-FISH (Multicolor FISH). M-FISH (multicolor FISH) using combinatorial labeling to obtain specific signals from each chromosome pair. The final color is assigned by the imaging system software to reflect the combinations of fluorophores exhibited during capture. There is an extra chromosome 21 and a small ring 11 (both constitutional) present, and technical loss of chromosomes 12 and 19. See insert for color representation of this figure.

Multiplex FISH uses paints labeled with combinations of fluorochromes to paint each chromosome a different color (or different colors) and create a karyogram (see Chapter 17, section 17.2, Multicolor FISH). It is very useful in identifying rearrangements between different chromosomes or in identifying marker chromosomes (Figure 16.5), and can be accomplished by several methods.

Satellite DNA probes

Satellite DNA is made up of short sequences of highly repetitive DNA that reside in the heterochromatic regions of the chromosomes. It has a different density than the majority of DNA and thus can be separated on a density gradient, making it visible as “satellite” bands found apart from the major concentration of other types of DNA – hence, the name. It also has the ability to fold in on itself, contributing to the compaction of heterochromatin. Along with minisatellite (VNTR) and microsatellite DNA, the pericentromeric satellite DNAs constitute most of the tandem repeats in genomic DNA. Satellite DNA is not to be confused with chromosome satellites, which are structures found on some acrocentric chromosomes. Both were named “satellite” because they appear as outliers (see Chapter 1, Table 1.2, for a list of satellite DNA).

Generally, satellite probes are very bright and easy to use, with the greater number of copies in the target chromatin yielding a brighter signal; however, even though target satellite regions are repetitive, the number of repeats varies from chromosome to chromosome and to a lesser degree from individual to individual. They may therefore occur as heteromorphic variants with either a very small size or a very large, often segmented appearance [19]. If the repeats are segmented, the probe may appear multipartite, making enumeration more difficult.

Centromeric alpha satellite DNA (see Chapter 1, section 1.1.6, Satellite DNA) probes are commonly used for interphase enumeration of specific chromosomes (Figure 16.6) or to help identify marker chromosome origin. Because the probe spans the centromere, they have also proven of value identifying whole arm translocations or a small inversion in metaphase FISH; however, because an inversion breaks the signal, interphase nuclei should be used with caution, as the probe may show two separate signals, creating a misleading signal pattern [20]. In addition to the pericentromeric regions, satellite DNA is also specific to the qh regions of 1, 9, and 16, the acrocentric chromosome satellites and short arms, and the distal Yq in the brightly fluorescing Q-band region.

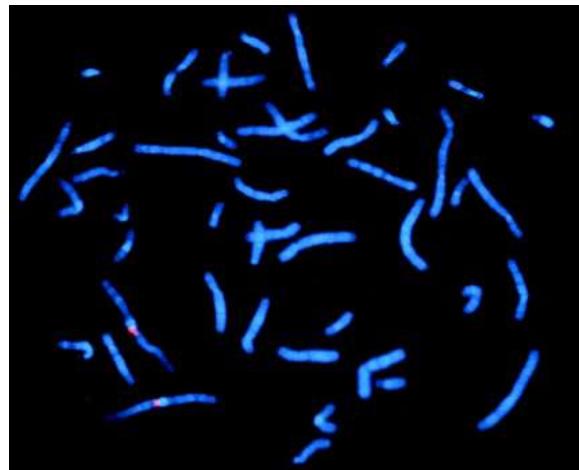


Figure 16.6 Alpha satellite DNA probes. Alpha satellite DNA probes can be used to enumerate all of the centromeric regions, except for chromosomes 5, 13, 14, 19, 21, and 22, which do not have specific sequences. This photograph shows a centromere enumeration probe (“CEP”) for chromosome 1. These regions are variable from person to person in size and position around the centromere, like C-bands, to which they roughly correspond. See insert for color representation of this figure.

Table 16.2 Some common cross-hybridizations between human alpha satellite DNA Sequences. Experienced cytogenetic technologists should keep in mind that small, extra signals may be due to cross hybridization from similar pericentromeric sequences between chromosomes

Alpha satellite probe	Cross-hybridizes with
1	5, 19
4	9
5	1, 19
9	4
13	21
14	22
15	14 [19]
19	1, 5
21	13
22	14

Classical satellite DNA is sometimes used for special probes. They are heavily methylated repeat sequences, and are mostly located in the qh regions of chromosomes 1, 9 and 16, and on the chromosome 15 short arm. For example, there is a classical satellite probe for 9qh and a centromeric alpha satellite DNA probe, either of which may be used for enumeration of chromosome 9 in interphase.

Alpha satellite probes are only useful for those chromosomes having unique repetitive DNA. But because these highly repetitive sequences can sometimes be found on multiple chromosomes, the probe cross-hybridizes more frequently than locus-specific probes, and is thus used with caution (see Table 16.2 for the common cross-hybridization patterns of alpha satellite DNA probes). Alpha satellite DNA can also be located at loci other than the usual, without affecting the patient; for example, the alpha satellite DNA on the Y long arm has been found on acrocentric short arms as a variation, usually with no phenotypic effect. Another example is chromosome 15, which has both alpha and classical satellite DNA

sequences, but the classical satellite DNA that corresponds to the DAPI-bright region of chromosome 15 short arm is also found on chromosome 14 in about 10% of normal individuals [19]. Using the pericentromeric D15Z4 alpha satellite DNA probe may help confirm chromosome 15 identification, since it may be present even if the classical satellite DNA is missing [19]; however, if the alpha satellite is missing, results can become inconclusive or misleading. Likewise, chromosomes 1, 5 and 19, 13 and 21, and 14 and 22 share alpha satellite sequences with each other, and are thus not currently available as unique probes.

Alpha satellite DNA probes may also be included in a probe mixture as an internal control for the number of copies of the chromosome they reside on. For example, a probe used to detect interphase deletions of *TP53* (*tumor protein p53*) on chromosome 17 short arm at 17p13.1 would include an alpha satellite DNA probe in a different color for the centromere region of chromosome 17 in order to differentiate monosomy 17 from a deletion and also to document ploidy. However, centromeric probes are not useful for certain chromosome abnormalities where the alpha satellite DNA is missing.

Centromeres also contain common sequences that can be hybridized with a human All- or Pan-human centromere probes to detect all centromeres simultaneously in a single color. These are used in xenograft studies to identify human cells, and may also be used in aneuploidy- and opposite-sex engraftment studies. However, the signal strength varies greatly between chromosomes. See 16.10.2 Scoring cells later on FISH scoring for difficulties in scoring some alpha satellite DNA probes.

Alpha satellite DNA is only found in primates, but is not conserved between members of different species; therefore, alpha satellite probes designed for humans will not bind efficiently to the centromeres of other apes, or to monkey chromosomes. Beta-satellite DNA, on the other hand, has a structure similar to mouse satellite DNA, and is not commonly used for commercial probes for humans.

NOR probes

Using a FISH probe for the 18S rDNA found on the satellites of the acrocentrics may be useful for cases with stalks present in unusual positions, for rearrangements involving the stalk regions [21], and for research purposes. The advantage of using FISH over NOR-staining is that FISH stains DNA whether or not it is active in the cell.

Fusion probes

To detect certain, specific translocations in interphase cells, fusion probes have been designed to label the breakpoints which flank or span DNA segments with different colors (Figures 16.3, 16.7, and 16.31). When the translocation is absent, the two probes each show two distinct signals from the two normal homologues of each partner. When present, the translocation causes the signals to “fuse” and appear so close that sometimes a third color is produced by wavelength interference between the emitted probe colors. For example, with red- and green-labeled probes, a yellow ‘fusion’ signal may be formed from the adjacent red and green signals.

There are several types of fusion probes:

- Dual-color single-fusion probes flank the breakpoints so that only one fusion signal, which is emitted by the derivative chromosome, is produced (see Figure 16.7a).
- Dual-color dual-fusion (DCDF) probes span the breakpoints so that both derivative chromosomes can be detected, producing two fusion signals. These probes are more sensitive in detecting residual disease, because the abnormal pattern is rarely seen as an artifact of chromosome overlap in interphase (see Figures 16.3, 16.7c, 16.8). This is sometimes termed “D-FISH.”
- Extra-signal (ES) probes, in which one probe flanks and the other spans their respective breakpoints, are designed to produce a fusion on one derivative, but instead of a second fusion, the proximal region of one breakpoint remains behind as an extra signal (see Figures 16.7b, 16.9).
- Centromere alpha satellite probes can also be used as fusion probes when derivatives involve close centromere regions. For example, centromeric probes for chromosomes 9 and 20 may be used with interphase cells to detect the dicentric derivative that is formed from a translocation between chromosomes 9 and 20 and is associated with acute lymphoblastic leukemia (ALL).

Keep in mind that each probe manufacturer has a different probe design, with some covering more or less of the breakpoint regions. It is important to know that validation of a certain translocation probe type from a given manufacturer does not translate to a similar translocation probe from another company, and the abnormal pattern of a patient can appear different from one probe to the other. For example, if there is a microdeletion concurrent with the translocation near one or both of the breakpoints that removes part or all of the residual signal of one or both chromosomes (e.g., the part of the signal that stays on the native chromosomes), the extent of coverage (number of kilobases) that the manufacturer has designed to hybridize in that region will

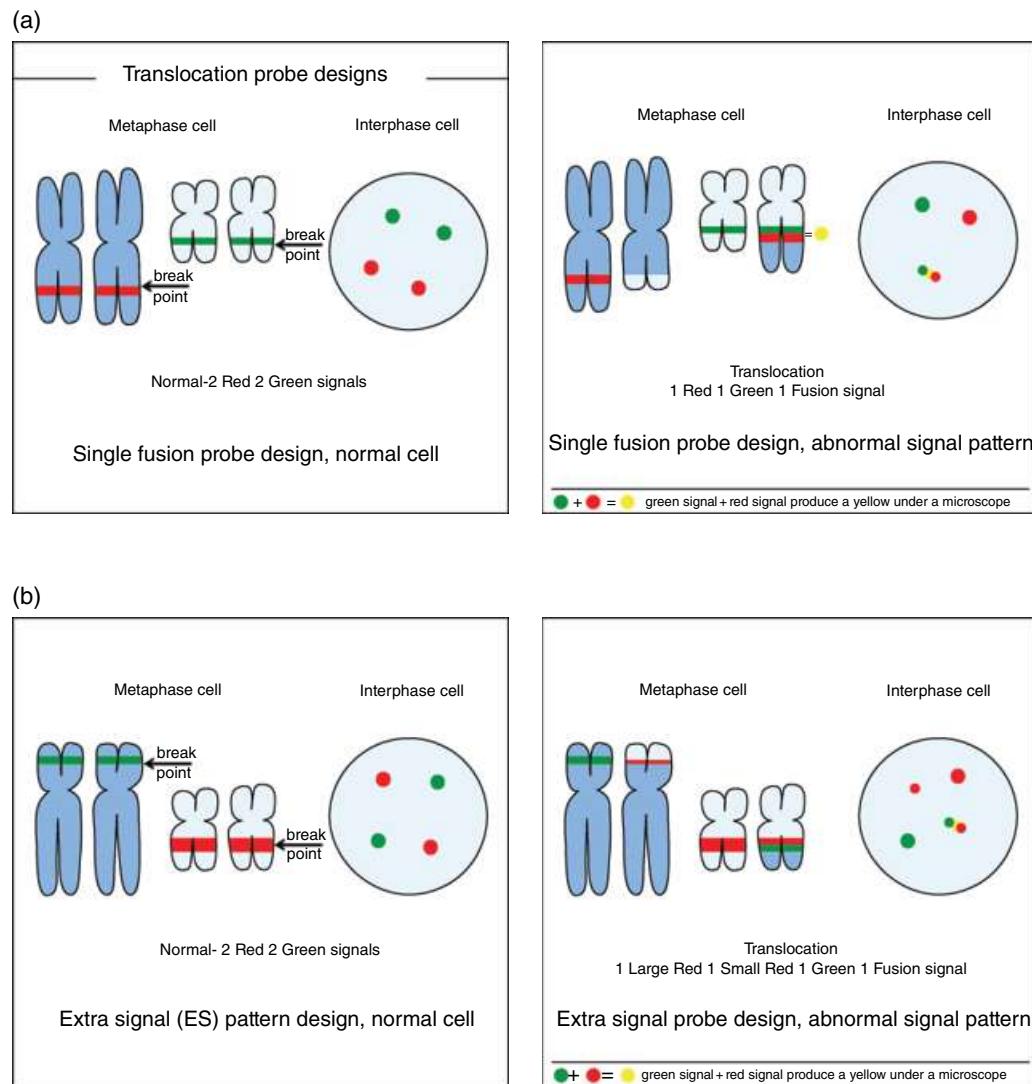


Figure 16.7 Translocation probe strategies. Various probe design strategies have been used to detect certain oncogenic translocations in order to facilitate diagnosis and monitoring of residual disease in interphase cells. Cells that are not mitotic can be assessed for presence of disease. The main types of probes used for this purpose are single fusion, extra-signal, dual fusion, and break-apart probes. (a) Single fusion (SF) translocation probe design: Probes are designed with the breakpoint on one chromosome (red, above) proximal to the signal and the other chromosome breakpoint distal to the signal (green). When the translocation occurs, one of the signals (red) is completely moved over to the other chromosome, forming a fusion signal (below) that can appear as a closely positioned red and green signal, as a single yellow signal, or as a red/yellow/green signal, depending upon how the chromatin condenses or opens up. There are also normal signals (one red one green) present from the normal homologues that did not participate in the translocation. The drawback to this design is that the red and green signals can be positioned by chance very close together in the normal interphase nucleus, creating an artifactual fusion signal pattern in normal cells. There may be as many as 10% or 12% of nuclei with artifactual fusion signals in normal cells. (b) Extra-signal (ES) translocation probe design: This strategy was designed to help overcome the problem of artifactual fusion signal patterns encountered with single fusion probe designs. The signal on the submetacentric chromosome (green, above) is distal to the breakpoints, and the signal on the metacentric chromosome (red) spans the breakpoint. When the translocation occurs, the entire green signal moves to the translocation partner to form the fusion signal. The distal portion of the red signal on the metacentric chromosome is moved to the submetacentric chromosome, while the proximal red signal is retained by the metacentric chromosome to participate in the fusion. The result is the fusion signal on the metacentric partner and a small red signal on the submetacentric. There are also one red and one green signal from the normal homologues present. This pattern is much less likely to be obtained from artifactual signal overlap in the nucleus than is the single fusion pattern. (*continued on next page*)

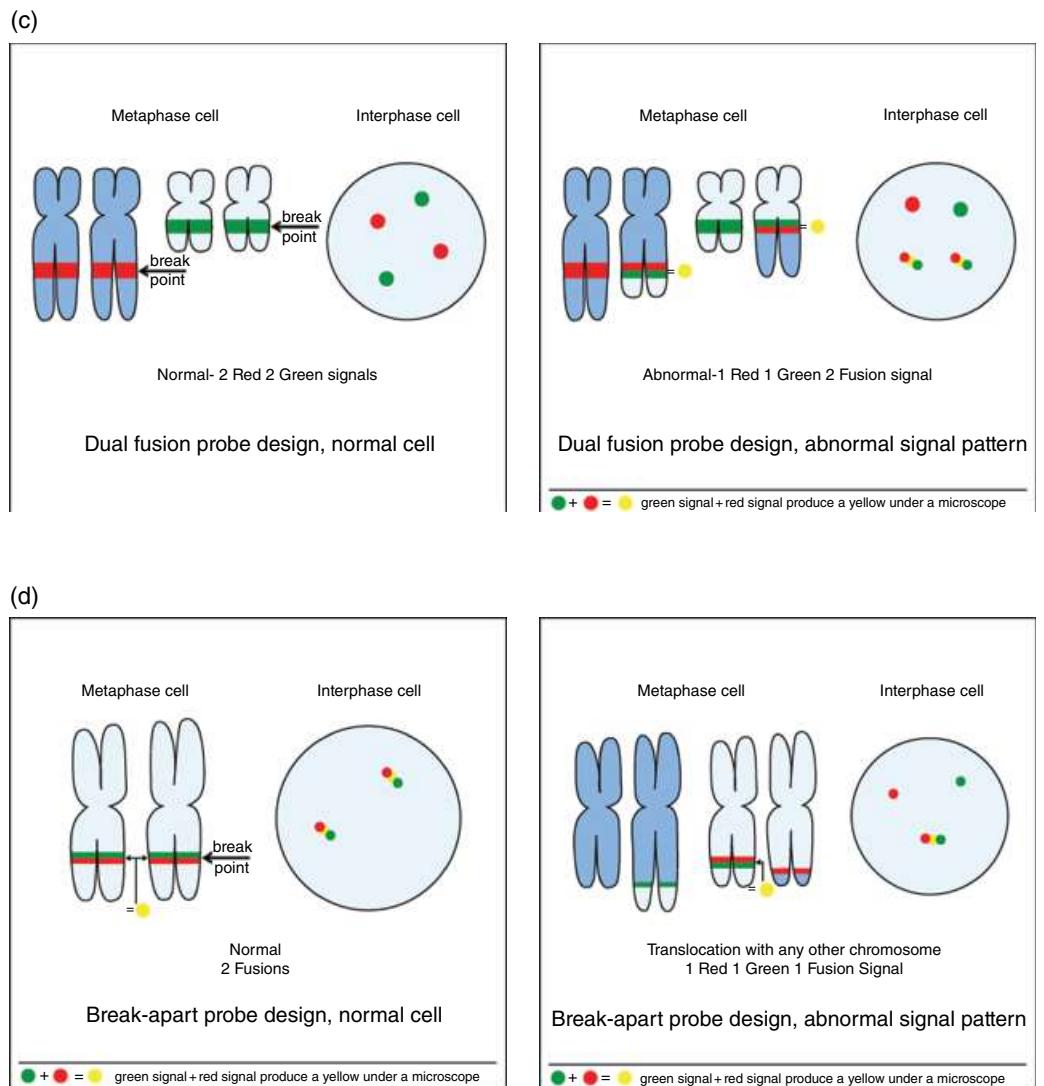


Figure 16.7 (Continued) The drawback is that the small red signal is sometimes difficult to visualize, and in some translocations it can even be deleted. (c) Dual fusion (DF or D) translocation probe design: This probe type was designed to help distinguish between artifactual and true fusion signals. Both signals span the breakpoints on each partner in the translocation, so that residual signal is present on both chromosomes to form two fusion signals per nucleus, plus the normal red and green signal from the normal homologues. This pattern is the least likely to be formed in a normal cell by overlapping signals, because if there are two fusion signals present in a normal nucleus, it would require both normal homologues to overlap both of the other chromosomes, and the resulting pattern would be 0 red, 0 green, 2 yellow. This pattern would not be considered scorable. This design is considered the most reliable for translocations that take place between two specific chromosomes. The drawback is that if there is a complex translocation, or if one of the partners is different than the usual one (variant partner), the pattern will either be different from the expected red/green/yellow/yellow, or the design could miss the translocation altogether. Another drawback to dual fusion probes is that they are sometimes difficult to interpret when used in FFPE tissue sections due to truncation of signals during sectioning. (d) Break-apart (BAP) translocation probe design: The break-apart probe type was designed to help with the problem of multiple partner involvement with certain “promiscuous” oncogenes such as MLL, which has many partners. It would be impractical to use dual fusion probes for each possible partner. Break-apart probes are designed to have a different color signal on each side of the breakpoint. When the translocation has not taken place, both homologues will show a fusion type signal pattern. When there is an exchange with any other chromosome, the proximal and distal portions of the probe are separated in the nucleus, producing a red and green signal. Also present will be the normal fusion from the other homologue. This design is also more easily interpreted than dual fusion probes in FFPE tissue sections. See insert for color representation of this figure.

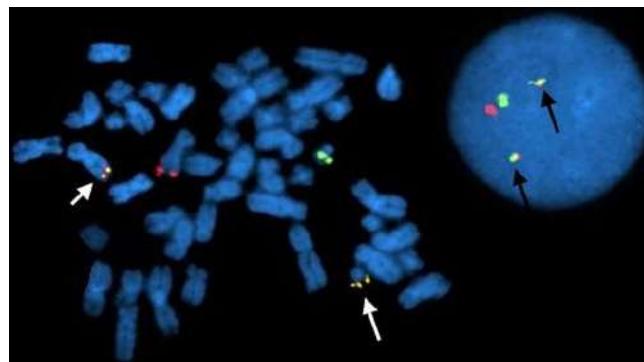


Figure 16.8 Fusion probe for BCR/ABL1 t(9;22) FISH. Metaphase and interphase cell with dual fusion probe showing a red (normal 9), green (normal 22), fusion on der(9), and fusion on der(22), both appearing yellow. This is the most common signal pattern for a *BCR/ABL1* positive sample; however, other patterns can be exhibited, depending upon the cytogenetics of the patient (see Figure 16.31). See insert for color representation of this figure.

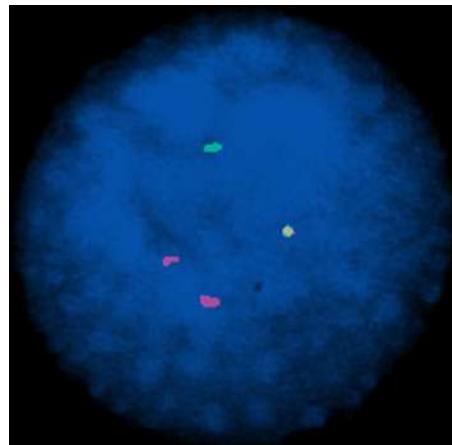


Figure 16.9 Extra-signal design probe for TEL (*ETV6*)/AML1 (*RUNX1T1*) fusion t(12;21). The extra-signal probe design has a residual proximal signal from one of the breakpoints instead of a second fusion. This is an Abbott Molecular TEL/AML1 probe, where green is the normal 12p, red is the 21, and the fusion is the der(21) with 12p on it. The smaller red signal is the residual probe signal from the rearranged 21. Thus, a juxtaposition artifact is distinguishable from a true fusion by the presence of the second red signal in the abnormal cells. See insert for color representation of this figure.

determine whether there is a signal present or how bright it appears. Larger probe coverage may make the deletion less visible. The abnormal pattern will also depend on whether the manufacturers have chosen to label probes with the same fluorochromes. For example, using two different dual fusion probes on a sample in which there is a microdeletion of part of one BCR (*breakpoint cluster region* at 22q11) signal, one BCR/ABL1 probe, where BCR is red and ABL1 (*c-abl* oncogene 1, nonreceptor tyrosine kinase at 9q34.1) is green, might show a red-green-green-yellow pattern, while another BCR/ABL1 probe with opposite color assignments (with BCR in green and ABL1 in red) could show a red-red-green-yellow pattern. If the laboratory changes from one probe manufacturer to another, this may cause some confusion in monitoring such a patient over time.

Break-apart probes

Along with fusion probes, break-apart probes are used for the detection of specific translocations in interphase (see Figure 16.10), and to detect inversions (see Figure 16.7d). They are also used for translocations of promiscuous genes in which a constant partner can rearrange with several different chromosomes or genes. This constant breakpoint is labeled in two colors, one on

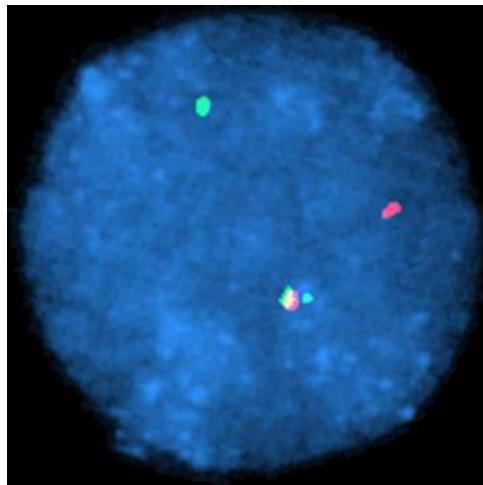


Figure 16.10 Break-apart probe for *KMT2A* (*MLL*). This 11q23 region is rearranged with many partners, so a dual fusion probe would not be practical. The break-apart probe is used to delineate any translocation involving this 11q23 locus, regardless of the partner chromosome in the translocation. The juxtaposed red/green (appearing yellow) is the normal 11q23, and the separated red and green signals represent the rearranged 11q23. It would require a metaphase to determine which other chromosome is involved. See insert for color representation of this figure.

each side of the breakpoint. Normal cells have two “fusion” signals (e.g., yellow), and rearranged cells show one fusion signal and one signal for each part of the “broken apart” breakpoint (e.g., one red and one green). The advantages of this system are that metaphases are not required to detect small numbers of abnormal cells and that gene breakpoints which rearrange with multiple partners can be assessed without knowing the partner, such as *IGH@* (immunoglobulin heavy locus, 14q32.32-q32.33), *MLL* *KMT2A*, 11q23 (more commonly referred by its former name *MLL*, i.e., myeloid/lymphoid or mixed lineage leukemia), and *EWSR1* (Ewing sarcoma breakpoint region 1, 22q12.2) (see Chapter 11, Cytogenetic analysis of hematologic malignant diseases). If necessary, additional FISH probes can be performed to find out what the exact rearrangement is. Some break-apart probes have a tendency for the red and green elements to separate in normal cells when the chromatin is extended in interphase, so it is important to determine cutoff levels for a normal result.

A caveat for interpreting the results of a break-apart signal pattern is that the partner is unknown. For example, if *SS18* (synovial sarcoma translocation on chromosome 18, synonym *SYT* at 18q11.2) is rearranged, and the differential diagnosis includes synovial sarcoma, one can only infer that the partner is most likely the X. Another problem is that if the differential diagnosis includes two different tumors, such as Ewing sarcoma versus desmoplastic small round cell tumor, the break-apart probe for *EWSR1* cannot distinguish between them. Ewing sarcoma usually has a t(11;22)(q24;q12), while desmoplastic small round cell tumors have a t(11;22)(p13;q12).

Sometimes a break-apart probe is more reliable for detection of specific rearrangements than a dual fusion probe, as when variant partners are involved. For example, because of the design of the dual fusion probes and the nature of the rearrangement, some versions of the *IGH/MYC* probe for detecting t(8;14) (see Chapter 11, section 11.4.1, B-cell lymphomas) can appear normal when the *MYC* gene is rearranged with chromosome 2 (*IGK*) or 22 (*IGL*) instead of chromosome 14. Use of the *MYC* break-apart probe will demonstrate the rearrangement. Break-apart probes can also be simpler to interpret than fusion type translocation probes for formalin-fixed paraffin-embedded (FFPE) tissues because of fewer issues with signal artifact that result from nuclear truncation during sectioning.

16.3 Deletion/duplication probes for constitutional abnormalities

Deletion/duplication probes are available for the detection of several microdeletion syndromes, such as Prader–Willi (PWS)/Angelman (AS), Williams (WS), Smith–Magenis (SMS), and velocardiofacial (VCFS)/DiGeorge (DGS) syndromes (Table 16.3, Figures 16.11, 16.12). Since some microdeletions are very difficult to visualize on G-banded metaphase material, these probes have become the standard of care in cytogenetic studies when the reason for referral includes microdeletion phenotype findings (see Chapter 9, Table 9.1). It is important to remember that each microdeletion locus is also prone to

Table 16.3 Constitutional deletions. Some deletions are common enough to have commercial probes available for their detection

Clinical condition	Probes available	Locus	Percent detected with FISH	Comment
Prader–Willi	SNRPN, D15S10, GABR β 3, D15S11, UBE3A	15q12	70% ¹	Some are due to UPD/methylation defects and do not have deletion
Angelman	SNRPN, D15S10, GABR β 3, D15S11	15q12	70% ²	1/25,000 incidence
Smith–Magenis	SMS, RAI	17p11.2	>90% ^{3,4,5}	1/25,000 births
Williams	ELN, LIMK, D7S613	7q11.23	>90% ⁶	Usually submicroscopic
Sotos	NSD1 (deletions/mutations)	5q35	4/59 patients ⁷	Usually a mutation
Wolf–Hirshhorn	WHSC1	4p16.3	>95%	1/50,000 live births
Cri-du-chat	D5S721/D5S23	5p15.2	98%	1/20,000–50,000 livebirths
Miller–Dieker/isolated lissencephaly	LIS1	17p13.3	Almost 100% have deletions	~11/million livebirths
DiGeorge/ Velocardiofacial	22q11 from proximal to distal: DGCR2, D22S75 (N25), TUPLE1 (HIRA), TBX1	22q11.2	85% for 3 megabase deletion	Occurrence: 1/3000–4000 live births
DiGeorge	DGS2 or DGCR2II	10p14	Rare ⁸	
Kallman	KAL	Xp22.3		
Steroid sulfatase deletion – X-linked ichthyosis syndrome	STS	Xp22.3	Deletion in Type 1 only	
Sex reversal	SRY	Yp11.3		
1p36 Microdeletion syndrome	p58	1p36	Breakpoints vary from 1p36.13 to 1p36.33	Occurrence: 1/5000 births ⁹
Idiopathic short stature; Leri–Weill dyschondrosteosis (LWD)	SHOX	Xp22.33	2%	2% of idiopathic short stature patients
Alagille	JAG1	20p12	6–7%	1/100,000 live births; 6–7% by deletion, others are mutations
Neurofibromatosis type 1	NF1	17q11.2		1/3000–4000 people – usually a mutation
X inactivation center	XIST	Xq13.2	May be deleted in Turner patients with structurally abnormal Xs	

¹ Bittel DC, Butler MG. Prader–Willi syndrome: clinical genetics, cytogenetics and molecular biology. *Expert Rev Mol Med* 2005;7(14):1–20.

² Watson P, Black G, Ramsden S, Barrow M, Super M, Kerr B, Clayton-Smith J. Angelman syndrome phenotype associated with mutations in MECP2, a gene encoding a methyl CpG binding protein. *J Med Genet* 2001;38:224–228.

³ Greenberg F, Guzzetta V, Montes de Oca-Luna R, Magenis RE, Smith AC, Richter SF, Kondo I, Dobyns WB, Patel PI, Lupski JR. Molecular analysis of the Smith–Magenis syndrome: a possible contiguous-gene syndrome associated with del(17)(p11.2). *Am J Hum Genet* 1991;49(6):1207–1218.

⁴ Juyal RC, Figueira LE, Hauge X, Elsea SH, Lupski JR, Greenberg F, Baldini A, Patel, PI. Molecular analyses of 17p11.2 deletions in 62 Smith–Magenis syndrome patients. *Am J Hum Genet* 1996;58: 998–1007.

⁵ Chen K-S, Manian P, Koeuth T, Potocki L, Zhao Q, Chinault AC, Lee CC, Lupski JR. Homologous recombination of a flanking repeat gene cluster is a mechanism for a common contiguous gene deletion syndrome. *Nat Genet* 1997;17:154–163.

⁶ Lowery MC, Morris CA, Ewart A, Brothman LJ, Zhu XL, Leonard CO, Carey JC, Keating M, Brothman AR. Strong correlation of elastin deletions, detected by FISH, with Williams syndrome: evaluation of 235 patients. *Am J Hum Genet* 1995;57(1):49–53.

⁷ de Boer L, Kant SG, Karperien M, van Beers L, Tjon J, Vink GR, van Tol D, Dauwerae H, le Cessie S, Beemer FA, van der Burgt I, Hamel BC, Hennekam RC, Kuhnle U, Mathijssen IB, Veenstra-Knol HE, Stumpel T, Breuning MH, Wit JM. Genotype–phenotype correlation in patients suspected of having Sotos syndrome. *Horm Res* 2004;62(4):197–207.

⁸ Schuffenhauer S, Lichtner P, Peykar-Derakhshan P, Murken J, Haas O, Back E, Wolff G, Zabel B, Barisic I, Rauch A, Borochowitz Z, Dallapiccola B, Ross M, Meitinger T. Deletion mapping on chromosome 10p and definition of a critical region for the second DiGeorge syndrome locus (DGS2). *Europ J Hum Genet* 1998;6(3):213–225.

⁹ Shaffer LG, Lupski JR. Molecular mechanisms for constitutional chromosomal rearrangements in humans. *Annu Rev Genet* 2000;34:297–329.



Figure 16.11 Constitutional deletion of chromosome 15q13. SNRPN (red)/PML (red)/CEP 15 (green) probe for Prader–Willi/Angelman syndromes: This patient has a microdeletion in one chromosome 15. The normal chromosome (15) exhibits a green CEP 15 signal and a red PML (control probes to verify the pericentromeric region of the 15 and the distal 15q), and a red SNRPN signal proximal to the centromere on the long arm. The deleted chromosome 22 below it exhibits only the PML and CEP 15 control signals and is missing the SNRPN (arrow) signal. See insert for color representation of this figure.

microduplications, as well (Figure 16.12). For example, deletions and duplications of chromosome 22q11.2 can both cause heart anomalies; therefore, it is good practice to look at interphase cells, as well as metaphase cells, since duplications are more easily visualized in interphase. Most microduplications, however, have different phenotypes from their deletion counterparts.

16.3.1 Subtelomeric-specific probes

Subtelomeric probes are specific to sequences 70–300 kb immediately proximal to the telomeres (except for the short arms of the acrocentrics, which lack these sequences). These probes are distinct from the telomere sequence (TTAGGG)_n repeats that are found at the end of all chromosome arms, and are thus used to identify subtle structural abnormalities that are not visible with G-bands, such as cryptic translocations and duplications/deletions (Figures 16.3 and 16.13). Probes that target the actual telomeric regions of the chromosomes will hybridize to all normal telomeres in the cells of many species, and are not used in clinical practice, but can be of use in research projects, such as in studying the loss of telomere sequences due to aging or malignancy.

There are several different DNA clones available for the subtelomere regions, and each probe company may use a different one, so if the probe from one company is unsatisfactory for the purpose at hand, another brand using a slightly different region in the subtelomeric repeats may be satisfactory. Panels are available that will test the subtelomeric regions for every chromosome (except the acrocentric short arms); these panels are used for detecting cryptic rearrangements of these regions, including deletions, duplications, and translocations. Chromosome microarray has lessened demand for such probes.

Note: Some manufacturers use control probes in the panels that can also detect abnormalities in nontelomeric regions. For example, the manufacturer may have used the *BCR* gene locus as the control probe (identifier) in case of a deletion or rearrangement to confirm the presence and identity of the deleted/rearranged chromosome 22. Because the *BCR* region can be deleted as a constitutional finding, the subtelomere panel could also detect a rearrangement or deletion within the *BCR* locus. This holds true for any of the other control probes used in the panel.

Subtelomere-specific probes are used to look for cryptic translocations and also for submicroscopic deletions and duplications within those regions. However, care in the interpretation of any abnormal findings is important, because studies on apparently normal parents of deleted probands may also show a similar abnormality [22]. One important question, if both the patient and a parent contain a subtelomeric deletion, is: did the deletion expand in the patient? Currently, the best method for investigation of subtelomere deletions and duplications is with chromosome microarray analysis, so that inherited aberrations can be compared at the DNA level to parental deletions for size and content. Note, however, that microarray analysis cannot detect a balanced rearrangement in a parent that is responsible for the duplication and/or deletion in an offspring. For detection of balanced

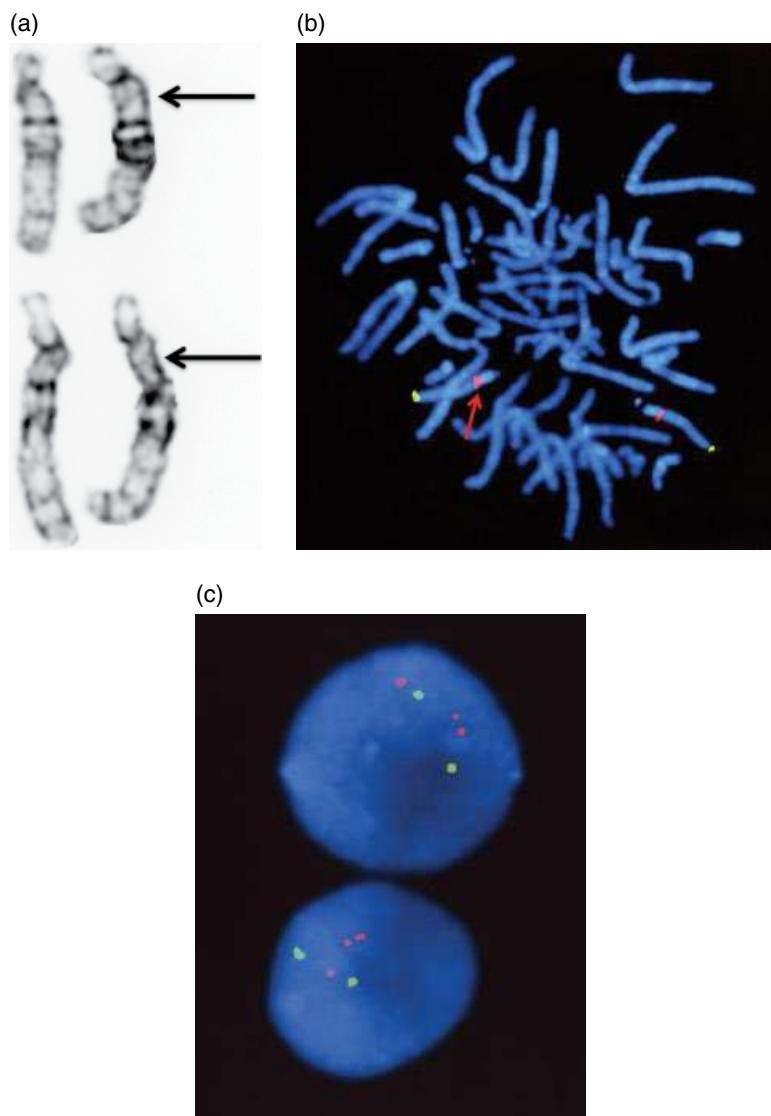


Figure 16.12 Locus-specific probes for duplication. For most microdeletion syndromes, there is a corresponding microduplication syndrome, usually with a completely different phenotype. For example, the region that is deleted in Prader-Willi/Angelman syndromes, at 15q13, can cause symptoms of autism in patients in whom the region is duplicated. The G-banded chromosome on the right in each pair is too long between the centromere and the first major dark band, and SNRPN probes with a 15 subtelomere-specific control probe were hybridized to metaphase chromosomes (left) and interphase cells (right). Note the right-hand 15 in the metaphase has a large red SNRPN signal. In interphase, the double nature of this signal becomes more apparent due to opening up and elongation of chromatin, and three red signals are evident, one from the normal homologue and two from the duplicated homologue. See insert for color representation of this figure.

parental rearrangements, FISH is preferred. Deletions and duplications that are detected with array technologies can also be confirmed using standard FISH, sometimes using subtelomeric sequences if these regions are involved.

16.3.2 All-human telomere probes

All-human telomere probes are used to detect the TTAGGG repeat sequences that are used for prevention of chromosome deterioration and for attachment of replication enzymes at the ends of all chromosome arms. These probes are not generally used for clinical cytogenetics, but are used in cancer research to quantitate telomere shortening in some tumors that can lead to breakage–bridge–fusion type abnormalities [23].

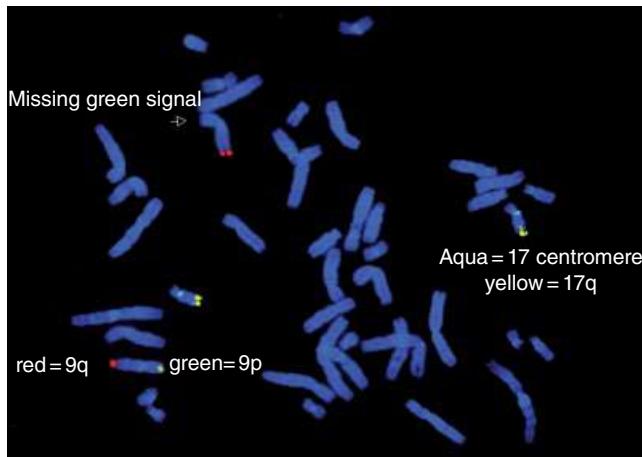


Figure 16.13 Subtelomere-specific panels. These FISH panels have several probe mixtures in each assay, and when the entire probe set has been utilized, all of the telomeres except for the acrocentric short arms are scrutinized for rearrangements, duplications, and deletions. This probe set in this assay includes the 9p (green), 9q (red), 17q (yellow), and 17 alpha satellite (aqua). The two chromosomes 17 are normal, but one 9p is missing a signal. This 9p on another hybridization exhibited the subtelomere-specific probe signal from 7p, while both 7 short arms had normal green signals (not shown). All of the rest of the subtelomere-specific probe panel was normal. Thus, this patient has a derivative 9 with chromosome 7 short arm material replacing part of the short arm of the 9. See insert for color representation of this figure.

16.4 Hematology/oncology and solid tumor probes

FISH has revolutionized the study of oncological specimens. It is faster than conventional cytogenetics, which can take several days for the culture and harvest of metaphase cells and more sensitive. Probe sets that are designed to detect acquired abnormalities in malignancies can be used in interphase nuclei so that large numbers of cells (compared with metaphase studies) can be assessed and so that FFPE tissues and other nondividing cells may be utilized. Results on hematologic specimens for which a quick diagnosis can be a matter of life or death, such as acute promyelocytic leukemia (APL-M3) (see Chapter 11), can be obtained in 4 or 5 hours on non-FFPE samples using a short hybridization time. Note: Be sure the abbreviated procedure has been properly validated. Interphase FISH is often used to monitor disease remission/relapse by first setting a baseline at diagnosis, and then performing periodic tests at specific intervals to detect any changes.

Since interphase FISH probes are available for many of the chromosome rearrangements seen in cancer, metaphase cells may not always be necessary for diagnosis. Interphase studies are useful for indolent cancers that proliferate slowly, such as chronic lymphocytic leukemia (CLL) and multiple myeloma (MM); for cell types that are difficult to culture or analyze, such as ALL and neuroblastoma; or for cancers that have been treated and are in remission but may have some low-level residual disease. Paraffin-embedded formalin-fixed tissue is also a potential resource for interphase FISH; it does not contain analyzable metaphase cells, but in some cases, it may be the only available specimen that can be studied. Another advantage of interphase FISH is for detecting “masked” rearrangements, such as complex or cryptic translocations. An example of this is the use of the fusion probes that target the gene loci *ETV6* (at 12p13, also known by its synonym *TEL*) and *RUNX1* (at 21q22.3, previously known as the *AML1* gene locus) to detect the cryptic t(12;21) associated with pediatric B-cell acute leukemia, even when cytogenetic studies appear normal.

All these probes are also useful in metaphase FISH to confirm the nature of rearrangements and extra or missing probe signals. The number of probes available increases yearly, and the reader is encouraged to visit the websites of probe companies to determine what is currently offered. See Chapters 11 and 12 for discussions on neoplastic tissue FISH.

16.4.1 Cancer-related deletion probes

Common cancer-related deletions for which FISH is useful include chromosomes 11q, 13q and 17p in CLL, 1p36 and 19q13 in gliomas, and 5q, 7q, 20q in AML/MDS, but there are many others (see Table 16.1). Acquired deletion probes (see Figure 16.14) are often two-color, with the target probe in one color, and the control probe in a second color. For example,

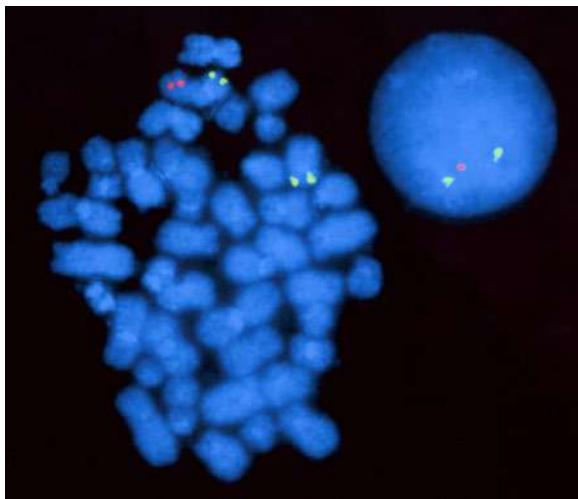


Figure 16.14 Acquired deletion of *EGR1* (5q). A metaphase and interphase cell with an interstitial deletion of 5q in bone marrow from a patient with myelodysplastic syndrome (MDS). The red signal is in the region that has been deleted. The green signal is a control probe on the short arm of the 5. See insert for color representation of this figure.

if the EGR1 probe for 5q has a red EGR1 signal, then its control probe on 5p would be labeled with a green signal so that the total number of chromosomes 5 can be assessed in interphase or metaphase, and euploid interphase nuclei can be differentiated from an aneuploid situation - in other words, whether the missing red EGR1 signal is due to a deletion of the gene or the loss of the entire chromosome.

16.4.2 Cancer-related enumeration probes

Centromere-specific probes are used in cancer to enumerate certain chromosomes or regions, such as detection of the interphase status of trisomy 8 in various leukemias or *EGFR* (epidermal growth factor receptor at 7p12) amplification in soft tissue tumors. They are also used as an internal control for ploidy and polysomy/monosomy determinations. Another widely used alpha satellite probe set for hematology disorders is the centromeric X and Y probes for determination of engraftment in opposite-sex bone marrow or stem cell transplants (see Table 16.1). Note: for interphase detection of the Y chromosome it is important to use the centromeric probe, DYZ3, rather than DYZ1 on the long arm since it is possible for the DYZ1 region to be present in some females. Locus-specific probes (see 16.4.3 Cancer-related locus-specific probes) are also used for chromosome enumeration in some instances; for example, hypodiploidy in ALL can be detected in interphase using PML/RARA probes, since the 15 and 17 are often monosomic in hypodiploid ALL.

16.4.3 Cancer-related locus-specific probes

Locus-specific probes are used to detect structural changes, such as gains/losses corresponding to deletions (e.g., 1p36 and 19q13 for gliomas) and amplifications (e.g., HER2/neu for invasive breast carcinoma and MYCN for neuroblastoma). In addition, many probes have been designed to detect reciprocal translocations (e.g., BCR/ABL1 in chronic myelogenous leukemia) and inversions (e.g., inv(16) in AML-M4 with eosinophilia) spanning specific breakpoints. Fusion and break-apart probes are made from locus-specific probes, and may be used in metaphase and/or interphase (see Table 16.1).

16.4.4 Cancer-related paint probes

Chromosome paints are used only in metaphase, and can be used to clarify chromosome exchanges, either as one- or two-paint assays or as a SKY or M-FISH assay for all of the chromosomes (Figures 16.4 and 16.5).

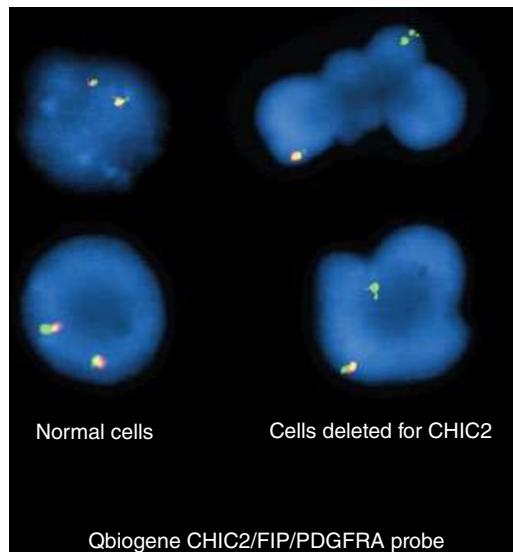


Figure 16.15 CHIC2 deletion probe. This probe set employs an unusual strategy because the chromosome abnormality it is designed to detect is unusual: the *CHIC2* deletion is not the cause of hypereosinophilia (HES); rather, it is the fusion of the *FIP1L1* and *PDGFRA* genes above and below the *CHIC2* that causes the abnormal cell proliferation. This probe labels the *FIP1L1* and *PDGFRA* in green, and the *CHIC2* in red. When the red signal is missing and the 2 green signals fuse, there has been a rearrangement, and the two green signals appear as one green signal or two very close green signals. See insert for color representation of this figure.

16.4.5 Cancer probe panels

An advanced FISH strategy in cancer diagnosis is the use of probe “panels” to query multiple disease-specific loci. For example, a probe panel to detect the diagnostic/prognostic abnormalities of CLL may include *IGH/CCND1* to detect the t(11;14) (q13;q32), the alpha satellite 12 probe for +12, D13S319 with a more distal control probe for the del(13)(q12q22), and *TP53* with a control probe to detect del(17)(p12). Laboratory directors and supervisors should decide, in concert with pathologists, clinicians, oncology groups, and the current literature, which probes are to be offered in panels for each disease or condition.

Deletion/fusion probes are used when there is a fusion of two genes in one single chromosome caused by a deletion of an intervening chromosomal segment. The CHIC2 deletion/fusion probe on chromosome 4q is an example of such a probe. Figure 16.15 shows a probe strategy for detecting this abnormality. The detection of the deletion is surrogate for a fusion of two genes above and below the deletion on chromosome 4 (*FIP1L1* and *PDGFRA*), which causes hypereosinophilia (HES). The normal chromosome shows as a “triple-fusion” signal and the abnormal shows as only double fusion of the flanking breakpoint signals (the middle section is gone and so the second color is also gone).

16.5 Sources and characteristics of probes available to the clinical cytogenetics laboratory

There is a wide range of probe sources, from genomic DNA, cDNA, oligonucleotides (short sequences of DNA, termed “oligos”), special oligos (such as peptide nucleic acid (PNA) probes), and RNA. Commercial probes are usually diluted in a formamide/dextran sulphate buffer, often in a ready-to-use 1× concentration, but also in 5× or 10× concentrations. The latter are used by diluting them to a 1× concentration with buffer or other probes. Commercial probes are also available as a dry residue, bound reversibly on special glass slides with a raised area for each probe mixture (e.g., Cytocell Multiprobe systems). These are used by putting buffer between the probe and the target and co-denaturing them. The advantage is that multiple assays, such as the all-subtelomere screen, can be run on a single slide. To confirm or rule out aCGH variants and abnormalities, BAC clones are available from several suppliers.

Originally, all probes were manufactured by researchers who worked with them. DNA (nucleic acid) probes may still be manufactured fairly easily in the laboratory, and are termed “home-brew” probes. They may be produced by cloning, enzymatic amplification, or chemical synthesis. Most FISH probes are cloned by transferring a DNA sequence into a microorganism,

such as bacteria or yeast. The microorganism can be cultured and will produce new copies of the inserted sequence, thus behaving as a small factory to produce the inserted DNA. The new DNA can be isolated, purified, and used with a fluorescent dye to probe chromosomes and nuclei.

The DNA molecule of the host probe-producing organism is known as the vector, and it can replicate in the bacterium or yeast cells autonomously. A number of vectors are available, including yeast artificial chromosomes, P1 artificial chromosomes, and bacterial artificial chromosomes, as well as cosmids, phages, and plasmids:

- Yeast artificial chromosomes (YACs) are made in *Saccharomyces cerevisiae* (baker's yeast), where they replicate, like normal yeast chromosomes. They are able to contain large human DNA inserts of 100–2000 kb.
- P1 is a (PAC) bacteriophage that grows in *E. coli*. It contains a large, ~45-kb DNA molecule which can be simulated by a synthetic P1 artificial chromosome or PAC. After infecting a bacterium, it replicates, producing large numbers of viruses. Then the infection lyses the bacterium releasing new phage into the surrounding medium. It can be used to clone human DNA up to about 20 kb.
- Bacterial artificial chromosomes (BACs) are vectors which are formed in naturally occurring plasmids found in *E. coli*, and can clone DNA up to 100–300 kb. Plasmids are circular DNA molecules found naturally in bacteria or yeast. Those that are developed for DNA production are usually small and contain a gene, which gives them a growth advantage, such as antibiotic resistance. Plasmids are best used for the production of short sequences up to 15 kb.

Probes may be produced from defined regions of chromosomes by microdissection and cloning of specific segments [24]. The segment of the chromosome, or the entire chromosome, may be scraped off a metaphase spread on a glass slide with a fine glass pipette controlled by a micromanipulator. The pieces are amplified using polymerase chain reaction (PCR), the final cycle of which includes hapten or fluor-conjugated nucleotides for labeling the probe. This method works well for making whole genomic probes, individual chromosome probes (paints), or whole arm probes.

Home-brew probes may be produced in the laboratory in host vectors, or the DNA may be purchased from suppliers, ready to label and use. Cloned sequences for preparing home-brew probes are available from human genome databases, such as those listed at <http://www.ncbi.nlm.nih.gov/genome/cyto/hbrc.shtml>. There are two methods for labeling the DNA obtained from these vectors. First is nick translation, in which a DNA sequence is exposed to DNase to produce single-strand nicks, followed by incorporation of labeled nucleotides into the nicked sites using DNA polymerase. Second is the use of PCR, which is DNA replication using a primer (i.e., short DNA sequence to start replication), a template (i.e., the DNA to be replicated), and cycles of denaturation, primer addition, primer annealing, and replication using special DNA polymerase at high temperatures in the presence of nucleic acid building blocks. The method used for labeling the probe depends upon the size and vector. Commercial kits are available for labeling the DNA with fluorochromes or detectable entities, such as biotin and digoxigenin.

The other source of labeled probes is from commercial vendors (Tables 16.1 and 16.3). These probes are more expensive, but have much of the testing and quality control already done by the manufacturer so that some of them may be FDA-approved. The disadvantage of commercial probes is that they are developed for clinically relevant and commercially profitable targets and are not available for all possible genomic targets. Occasionally the design of commercial probes is not ideal, with the probe either too large or in the wrong locus to be useful for the purpose it is meant for. Home-brew probes can fill the need for esoteric or otherwise unavailable DNA probes.

16.5.1 Probe size

FISH probes are a mixture of DNA fragments that cover a specific region of the genome; the fragments are usually small compared to the target size. Probe size is measured in kilobases, megabases, or base pairs. A base pair is one rung on the DNA ladder with a pair of bases, usually A–T or G–C (see Figure 16.1). One kilobase (kb) is equal to 1000 base pairs (bp), and a megabase (Mb) is equal to one million base pairs. The minimum clinically viable probe size for FISH is around 50 kb, while the largest probes can cover over 1 Mb, but most probes are usually around 200–400 kb. Probes that are smaller than about 50 kb may be too small to visualize reliably. Fragments longer than 300 kb have great difficulty penetrating the nucleus and will surround the cells and stick to the exterior. The size of the target will affect how small the probe can be, since a highly repeated target will appear bright even with a small sequence. Researchers have reported using genomic cDNA single copy probes less than 1 kb, but they have not been sufficiently robust for routine use. Even smaller 30-mer oligo probes have been used effectively when targeting highly repetitive sequences like alpha satellite repeats near the centromere, but the hybridization conditions are so different that they cannot be used simultaneously with traditional FISH probes.

16.5.2 Probe concentration

The concentration of the probe will determine the rate of hybridization and the background. Low concentration of probe will produce little or no signal, and overconcentrated probes will often produce background fluorescence. There should usually be approximately 100 times more probe DNA than target DNA for an optimal reaction.

16.5.3 Probe terminology/nomenclature

Probes are supposed to be and are increasingly named according to the HUGO Gene Nomenclature Committee (HGNC) for genes, and according to international convention for marker regions. For example, the region on chromosome 7q (i.e., 7q11.1-q21.1) most often involved in Williams syndrome deletions is *ELN*, the name for the gene for elastin. The marker region often used to test for a deletion of chromosome 15q13 for Prader–Willi and Angelman syndromes is D15S10. The “D” stands for DNA, the 15 stands for chromosome 15, the “S” stands for a unique DNA sequence (which has no known gene function), and “10” is the sequential discovery number of unique sequences on chromosome 15. Additionally, “Z” stands for repeat sequence, so DYZ1 is a DNA segment on the Y chromosome with repetitive sequences and was the first one numbered for the Y chromosome [25], while D15Z4 is a DNA segment at the centromere of chromosome 15, the fourth unique repetitive DNA sequence to be described on that chromosome. While this nomenclature is for the target of interest, manufacturers often make probes larger, encompassing additional genes or noncoding regions so that they provide sufficient signal.

16.5.4 Probe storage

DNA probes are very stable for 1–2 years and sometimes more, if stored properly. The main consideration is to keep the probes out of light, if they are directly labeled, in order to prevent fluorophore photobleaching. They should be kept cold for long-term storage (4 °C to –20 °C, depending upon the manufacturer’s instructions; all may be kept at –20 °C if desired), but for a few hours to a day or two, room temperatures should not significantly degrade the probe mixtures. Once slides are hybridized with probes, they will last at room temperature for a few weeks in the dark, and much longer in a –20 °C freezer. Eventually the probe signals will fade, but re-hybridizing the slide with fresh probes is usually successful. CAP guidelines require that probes should not be used for clinical use after the manufacturer’s expiration date.

16.6 Special uses of probes

16.6.1 Mixing probes of different brands or kinds

Probes of different designs or from different manufacturers may be successfully combined for certain uses. Paints can be combined with alpha satellite DNA probes or with single copy probes of different colors to better define certain abnormalities (Figure 16.16). If each probe requires different stringent conditions, the lowest stringency is usually chosen for the mixture. For example, to mix a paint and an alpha satellite probe, a 50% formamide buffer would be used for hybridization rather than a high stringency of 65%, which is normally used for the alpha satellite probe, and the post wash conditions would also be those used for the paint probes.

If both probes are concentrated, they can be added to a buffer together, each in the recommended concentration. If one is concentrated and one is preformulated and ready to use, add the concentrated probe to the diluted probe in the correct proportion. If both are ready to use, mix half the volume normally used for each together. Because the probes are in excess, they should still work well. We have mixed together up to three different probes successfully, whether concentrated or dilute. Certain probe types, however, can be diluted more successfully than other types. In our experience, subtelomere specific probes do not dilute well, but paints and alpha satellite DNA probes may be used at lower concentrations than manufacturers recommend, if necessary. Probe buffers may also be interchanged between companies with successful results, as long as the correct stringency is used.

Our laboratory has not had any failures from mixing different types or brands of probes together, and we do this routinely (Figure 16.16). The main consideration is that the probe concentrations may need adjustment. For example, when mixing subtelomeric specific probes with centromere probes, the centromere probes can overpower the small subtelomere probes during hybridization. Doubling the concentration of the telomere probes or halving the concentration of the centromere probe can compensate for this phenomenon.

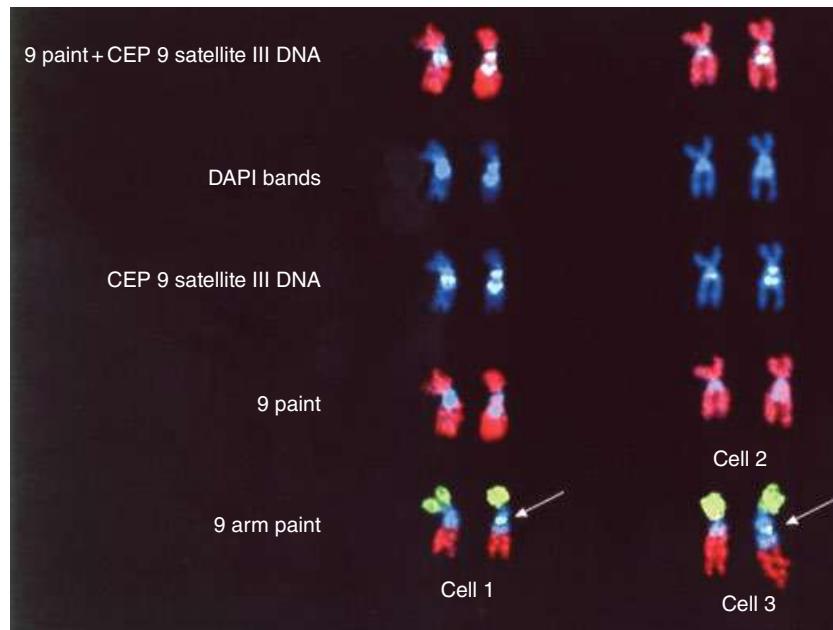


Figure 16.16 Use of probe mixtures and sequential hybridization to delineate a chromosome 9 abnormality. This illustration shows the use of mixing two different types of probe (paint and satellite III pericentric 9 probes) to delineate the makeup of a chromosome 9 that had an extra G-band in the middle of the q-arm heterochromatin region. The top row shows the chromosomes with the Abbott Molecular Laboratories 9 paint in red, and the Cytocell satellite III probe in green. The probes were mixed just before adding them to the slide, and they were hybridized with the target as usual. Note the extra band of red euchromatin in the middle of the satellite III heterochromatin. The second row shows only the DAPI bands from the cells to demonstrate the 9qh regions (bright DAPI staining areas) clearly. The third row is the same cells with only the satellite III probe exhibited, and the fourth row is the same cells with only the 9 paint exhibited. The bottom row is a sequential hybridization of cell 1 on the left, and a different cell from the same slide (right) hybridized with a Qbiogene chromosome 9p (green) and 9q (red) arm specific paint, demonstrating that the extra band (arrows) in the q arm is derived from the 9p. See insert for color representation of this figure.

16.6.2 Off-label use of probes

Probes designed for detecting certain chromosome rearrangements may also be used to investigate other abnormalities. For example, the TUPLE1/ARSA probe, designed to detect interstitial deletions of 22q11.2 in Velocardiofacial/DiGeorge syndromes, may be used to investigate terminal deletions of 22q in the ARSA region. Another example is the use of BCR/ABL1 probes to detect constitutional *BCR* deletions in certain patients [26]. Probes used for interphase chromosome rearrangements can often be used to detect aneuploidy of the same chromosomes.

16.7 Important FISH probe adjuvants

16.7.1 Cot-1® blocking DNA

One of the technical advances that made FISH feasible was the use of repetitive DNA such as Cot-1® DNA to block repetitive sequences present in many FISH probes. Experimental evidence demonstrated that when single-stranded DNA was sheared and allowed to renature, some sequences renatured more quickly than others. When plotted graphically, the time (*t*) a specific DNA sequence takes to reanneal is related to the concentration (*Co*) of DNA present (i.e., the Cot curve). The rate at which a particular sequence will reassociate largely depends on how common it is in the genome (e.g., its concentration); consequently, repetitive DNA families reassociate at lower Cot values than unique or single copy.

Cot-1® describes a DNA family with a reference Cot value of 1, and is enriched for repetitive DNA sequences interspersed throughout the genome. In humans, it is made up mostly of SINEs (short interspersed elements), LINEs (long interspersed elements) 50–300 bp in size. Each species has its own specific Cot-1® DNA sequences, so if one wishes to use mouse or

monkey probes, mouse or monkey Cot-1® DNA sequences must be used in the FISH assay. The result of adding such “blocking DNA” during the hybridization step of FISH is to “trap” repetitive sequences in the target and probe DNA by hybridization with the unlabeled Cot-1® DNA before the labeled DNA from the probe can do so. Hybridization with blocking DNA effectively dampens background fluorescent signal (Figure 16.17) and facilitates very specific hybridization to unique sequences, even when the FISH probe is produced from a heterogeneous mixture of DNA.

Cot-1® DNA is usually included in most commercial probes but must be added to home-brew probes and to some commercial probes (since there is a licensing fee for its use, some companies would rather the user bear the cost). The addition of unlabeled Cot-1® DNA to a FISH assay allows repetitive Cot-1® DNA to bind to the repetitive sequences in the probe, and makes them unavailable to bind the target and probe DNA, thus producing a much cleaner FISH assay (see Figure 16.17). Fifteen to twenty minutes of exposure (preannealing) of unlabeled Cot-1® DNA on a paint probe will effectively block the repeats. Similarly, if the commercial probe comes with unlabeled Cot-1® DNA included, the Cot-1® DNA in the denatured probe will quickly anneal to the probe repeats, which are in a highly concentrated solution, before they anneal to the target DNA, which is available in smaller concentrations. Other DNA sources may also be used to “block” unwanted background hybridization, such as sonicated placental or salmon sperm DNA. However, placental DNA contains other DNAs, such as alpha satellite DNA, and can make some probes appear dim because it may block DNA that is necessary for the assay. Placental DNA, in addition to Cot-1® DNA, can be used to get a cleaner hybridization for probes that have so many repeats that there is always a background, such as TUPLE1. Most alpha satellite DNA probes, however, do not require Cot-1® DNA blocking, as they do not contain Alu repeats. There are some probes available currently that do not require Cot-1® addition because they have been manufactured without repetitive DNA, and are marketed as “repeat-free” probes.

16.7.2 Probe buffers

DNA probes are diluted for use in formamide/2x SSC/dextran sulfate buffers. For high stringency requirements, such as the centromeric alpha satellite repeats, formamide may be 65% by volume, while for almost all other probes, the buffer contains 50% formamide (see 16.8.2, Stringency in FISH assays). Some probes are supplied as 1x concentration, and there is no need to add buffers to them, as they are ready to use. Other probes are supplied in a concentrated 5x or 10x form, so that 1–3 µL is usually diluted up to 10 µL using buffer for each assay. The advantage to the concentrated probes is that it is much easier to mix several probes together.

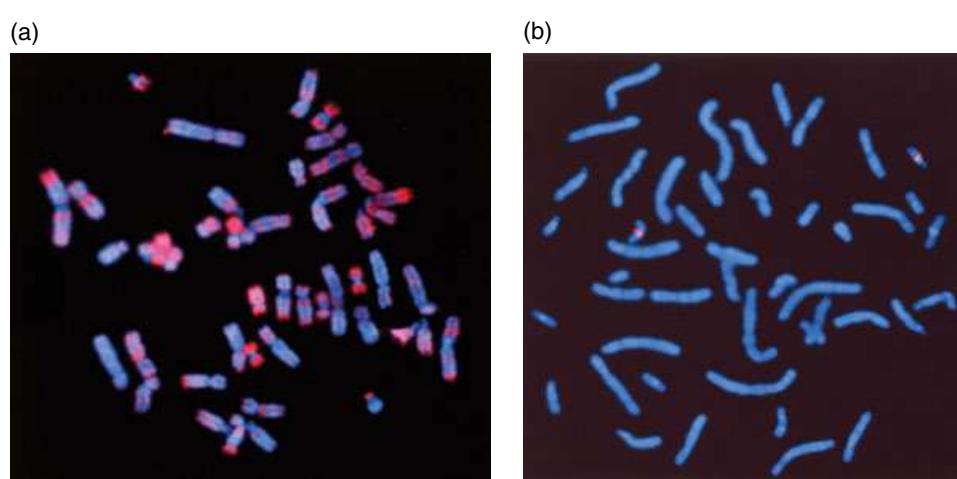


Figure 16.17 Alu banding. Alu repeats are found throughout the genome, and are located mostly in the R-bands. (a) If the FISH assay is not preannealed with Cot-1® or other repetitive DNA to block these repeat sequences, this fluorescent banding pattern is the result. The signals on the 22q are obscured by the background Alu banding. This hybridization is of TUPLE1 in red with no control probe and no Cot-1® DNA in the hybridization mixture. One 22 is located at 11:00 above a chromosome 2. (b) This is the same probe and the same sample, hybridized at the same time with all of the same conditions except that Cot-1® DNA was added to the probe mixture and allowed to preanneal to the probe sequences for 20 minutes before adding it to the slide. See insert for color representation of this figure.

Probe buffers are usually supplied by the manufacturer, or they may be purchased ready-made from various probe companies and suppliers. There are some reagents available, such as Den Hyb, that allow additional dilution of probes. Note: If changes are made to the probe manufacturer's package insert, it is important to validate the method before implementation.

16.8 Principles of FISH

16.8.1 Principles of fluorescence

A fluor is a special dye that responds to exposure to the energy in light by emitting its own light waves. A photon of energy from the light source, if it is of the correct wavelength for that fluor, will excite an electron on the fluor. The electron moves to another energy level (shell) and then back, releasing energy as a photon of lower energy (longer wavelength than the excitation light). The difference between the two wavelengths, excitation and emission, is called the Stokes shift. The wavelength of the filters in the microscope must allow the emitted light of the fluor through, but not the excitation light that came from the light source. Since it is good to use filters that have fairly wide bandwidths to achieve bright signals and capture the image easily, fluors with large Stokes shifts are easier to use, because they allow such bandwidths without leaking the excitation wavelengths (see Chapter 14, section 14.3, Fluorescence microscopy).

Another characteristic of a fluor is photobleaching. Some fluors bleach out, or fade, faster than others when exposed to light. When capturing multicolor FISH probes, a specific capture order is sometimes recommended, with the most sensitive fluors captured first. The counterstains used for staining the chromosomes and nuclei contain antifade to help prevent photobleaching. Certain colors above 650 nm emission wavelengths (e.g., Cy5) are not as visible to the human eye, but are easily seen by the cameras used on modern microscopes. Some fluors do not work as well with a direct label as they do with an indirect label. Cy3.5 and Cy5.5 are among those that work better attached to an antibody.

It is not necessary to understand wavelength characteristics to perform FISH, but it is helpful when ordering new equipment to understand what the requirements are for each test that is performed in the laboratory. The fluor must be matched to the excitation wavelength of the light source. There are three common light sources: mercury arc lamps, xenon arc lamps, and lasers (see Chapter 14, section 14.3.1, Light sources for fluorescence). See Table 16.4 for wavelength characteristics of commonly used fluors. The most commonly used lamps are mercury lamps, which provide emission peaks at 254, 366, 436, and 546 nm. These are a good match for many of the fluors used in the FISH laboratory. The filter sets on the microscope contain a specific excitation filter to excite a given fluor and another emission filter to remove unwanted re-emitted wavelengths (barrier). Some are designed to excite and pass only one fluor ("single band pass") and some are designed to work with two or more fluors ("double" and "triple" band pass). If two fluorophores have similar emissions, there may be "bleed-through" or "cross-talk" between them, making distinction between them difficult. An example of this effect is the similar emission wavelength between Spectrum aqua (DEAC) and Spectrum blue probes from Abbott Molecular Laboratories/Vysis, causing the two fluors to be concurrently visible unless very specific barrier filters are used.

The newer the fluorescent light bulb in the microscope, the stronger the light, and the more the light bleeds through between filters. Bleed-through characteristics can be used to advantage when designing multi-colored probe assays. Since Spectrum Orange is also visible through the Spectrum Gold filter, it is possible to utilize two different red fluorophores, one that will bleed through on the gold filter, such as Spectrum Orange, and one that does not, such as Texas Red or Spectrum Red. They are distinguished from each other by capturing them both using the filter that lets both through, followed by capture using the filter that only allows one wavelength through. The fluorophore that comes through on the most selective filter is subtracted from the first capture image or allowed to make a "fusion" signal with both color assignments showing at once.

16.8.2 Stringency in FISH assays

Stringency refers to the strictness of the conditions of the hybridization that define how much probe will bind and how much will wash off. These conditions are applicable both during the hybridization and during the post-hybridization wash, and can be utilized to optimize the results of the FISH assay. The more closely the probe matches the target sequences, the higher the stringency needed to prevent it from hybridizing or to wash it off after hybridization. Conversely, noncomplementary sequences will be nonspecifically bound and will be washed off at relatively low stringency conditions. Sequences that are intermediate in similarity to the target will be the most problematic, as they will tend to remain as background signal unless the stringency is correct to wash them off, but still allow the desired probe to remain.

There are three main parameters that affect the stringency of a FISH assay: temperature, salt concentration, and formamide concentration. All are important during the hybridization (reannealing) and during the post wash steps (whether SSC or formamide).

Table 16.4 Commonly used fluors and their excitation and emission wavelengths. Some of these fluors work well with direct labeling to nucleotides, while others work best when conjugated to antibodies or avidin

Fluor	Absorption/emission wavelengths for filters	
	Peak absorption/excitation (nm)	Peak emission (nm)
Alexa488	493	517
AMCA	353	442
Cascade Blue	396	410
Cy3	550	570
Cy3.5	581	596
Cy5	649	670
Cy5.5	675	694
DAPI counterstain	359	461
Diethylaminocoumarin (DEAC, or aqua)	432	472
Far Red (Fred)	620–660	700–775
Fluorescein (FITC, or Spectrum Green)	491	515
Oregon Green 488	495	521
Propidium iodide counterstain	520	610
Rhodamine Green	515	530
Rhodamine Red	570	590
Spectrum Aqua	433	480
Spectrum Blue	400	450
Spectrum Gold	530	555
Spectrum Green	485–500	513–542
Spectrum Orange	539–558	574–587
Spectrum Red	592	612
TAMRA	547	573
Texas Red	595	615

"Spectrum" fluorophores are proprietary for Abbott Molecular Laboratories.

1. *Temperature:* Stringency is directly proportional to temperature. Thus, a higher temperature is more stringent, so less probe will bind to the target, and more probe will be washed off. For example, during hybridization, a 37°C temperature will allow more sequences to bind to the target than a 42°C hybridization temperature. Similarly, during the post wash step, a 67°C post wash temperature will allow more probe to remain bound to the target than a 72°C post-wash temperature.
2. *Salt concentrations:* Most probe buffers incorporate 2× SSC (sodium chloride/sodium citrate) as the salt. 2× SSC has a neutral stringency at room temperature, and only becomes a stringent salt solution at high temperatures. The SSC concentration is inversely proportional to stringency. Thus, 2× SSC, which is more concentrated than 0.4× SSC, is less stringent than the 0.4× SSC. Water is even more stringent, and will remove almost all of the probes (denature most DNA) after 1–3 minutes at 72°C. However, for peptide nucleic acid (PNA) probes (currently rarely used in the clinical cytogenetics laboratory), the SSC concentration is directly proportional to the stringency.
3. *Formamide concentration:* Formamide is a solvent that denatures double-stranded DNA and its stringency is directly proportional to concentration. For example, if the probes are mixed with buffer containing 50% formamide, stringency will be lower than if they are mixed with buffer containing 65% formamide (Figure 16.18). Typically, alpha satellite DNA probes are mixed in buffers containing 65% formamide so they will not cross-hybridize with other alpha satellite DNAs with similar but nonidentical sequences. Unique sequence probes are generally mixed with buffer containing 50%

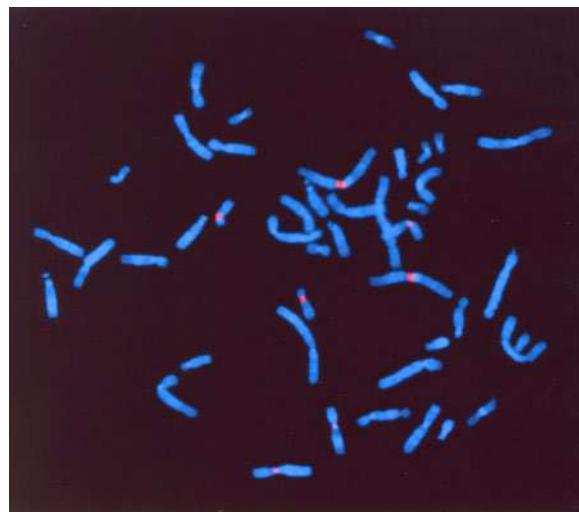


Figure 16.18 Effect of low stringency buffer and post wash. This alpha satellite probe for chromosome 1 was hybridized in 50% formamide instead of 65% formamide, and was rinsed in 2 \times SSC instead of 0.4 \times SSC, so the stringency was too low to prevent cross-hybridization with other chromosomes. Normally one should get only two signals on the two chromosomes 1. However, this low stringency effect can be used as a strategy incase of an unknown centromere region: if the chromosomes that cross hybridize at the low stringency are known, this type of assay can be used to narrow down the possible origin of small marker chromosomes [36]. If it hybridizes to the marker, the marker must have originated from one of the homologues that hybridize with the signal under these stringency conditions. Further FISH can be undertaken to narrow down the candidates until the exact origin is determined. See insert for color representation of this figure.

formamide, because their unique sequence makes them less likely to cross-hybridize with other genomic sequences and therefore requires less stringent conditions. When a unique sequence probe is mixed with an alpha satellite DNA probe, the lower stringency buffer formula is used so that the unique sequence will not be compromised. Depending upon the alpha satellite sequence, some cross-hybridization can be experienced with the lower stringency buffer, but it can often be worked around. Similarly, during the post wash, higher concentrations of formamide wash solution will rinse off more probe than a low concentration of formamide. 70% formamide will also denature much of the DNA at 72°C and is stringent enough to remove most probes after a few minutes.

The pH of a solution can also be used to control its stringency; for example, there is a method to denature slides involving exposure to room temperature sodium hydroxide [27]. If the pH of the post-wash solutions is off, it can affect the quality of the hybridization. A low pH will give a nuclear background glow and may obscure the probe signals.

16.8.3 Fluorescent background in the FISH assay

There are several types of fluorescence that create background signal and can interfere with the interpretation of the FISH test.

1. *Excess unbound probe:* Failure to sufficiently rinse the slide in the correct stringent wash (e.g., 2 \times SSC is less stringent than 0.4 \times SSC) may allow excess probe to adhere to the slide. This problem is particularly encountered using a biotinylated probe, as biotin tends to stick to glass and to other proteins on the slide. Indirectly labeled slides should not be dried during the post-wash, as the unbound probe is more likely to stick to the slide than directly labeled probe. Fluorophores that are not attached to DNA may also stick to the slide.
2. *Alu-bands:* Alu-bands are caused by the presence in a probe of a number of Alu repeats, which bind to Alu-rich regions of all chromosomes, creating an R-banded pattern (see Figure 16.17). Certain probes are more prone to this, such as the *TUPLE1*, because of repeats in the region from which the probe was created. Sometimes the Alu-repeat fluorescence can mimic a weak signal.
3. *Fluorescent debris on the slide:* Many types of debris can fluoresce, including proteins, grease, pencil marks, hair, and bits of insect parts or other biological material in dust.

4. *RNA*: Certain cells, such as preimplantation embryo blastomere cells, may contain pools of RNA that may bind to many probes, and will often fluoresce with colors from every fluorophore used. These may be removed with RNase pretreatment or by use of strong fixatives, such as formaldehyde, instead of 3:1 methanol-acetic acid.
5. *Dirty probes*: Certain probes are predisposed to fluorescent background. *IGH* probes, whether fusion or break-apart types, are examples of “dirty” probes. This may be due to their repetitive nature, as they may hairpin back on themselves and precipitate onto the slide.
6. *Cross-hybridization*: When two regions have similar DNA sequences (sequence homology), probe can bind to both regions. For instance, the similarity of the centromeric region alpha repeats of chromosomes 1, 5, and 19 cause them to cross-hybridize, as do the alpha satellite repeats for 13 and 21, or 14 and 22 (see Table 16.2). Whole chromosome paints for the acrocentric chromosomes will almost always hybridize with some or all of the short arms of other acrocentrics due to their sequence homology (see Figure 16.19); therefore, using whole arm paints for only the acrocentric chromosome long arms will circumvent the problems of short-arm cross-hybridization, because they do not contain the alpha satellite repeats of short arm DNA (Figure 16.20).
7. *Slide-making/spreading issues*: Poorly made slides can create interfering background noise. For example, insufficient hypotonic dilution or fixative washes can leave a protein residue from unlysed red blood cells or red cell ghosts, which can fluoresce. Debris can also cause nuclei to bunch up into dark, three-dimensional cells, as seen under phase contrast, which could accentuate the Alu band-positive material in the chromatin. Background noise created from poor slide-making generally manifests as cells with a “glowing” nuclear background, which may vary on different parts of the slide, depending upon slide-drying conditions. Insufficient spreading of interphase cells may also cause probe signals to bunch together, making interpretation of fusion probes more difficult.
8. *Improper pH of post-wash solutions*: The pH of the post wash solution is usually 7.0 or close to it. If the pH of the post wash solution is incorrect, it can cause a nuclear background glow.

16.9 FISH methods – an overview

With some variations, the basic steps for performing FISH include specimen processing/harvesting, slide preparation, pre-treatments to harden chromatin, denaturation of probe(s) and target(s) (e.g., chromosomes or nuclei on the slide), reannealing the probe and target DNA for a period of hours, followed by the post-wash with stringent conditions designed to remove



Figure 16.19 Acrocentric paint cross-hybridizes in short arm regions. This chromosome 15 paint, as all paints for acrocentric chromosomes, has so many sequences that are common to all of the acrocentrics that all of the pericentromeric repetitive sequences on the other acrocentric chromosomes have hybridized. The best way to get around this problem is to use microdissected arm paints that do not contain the short arm sequences. These sequences generally are not important, as they have no coding sequences except NORs. See insert for color representation of this figure.

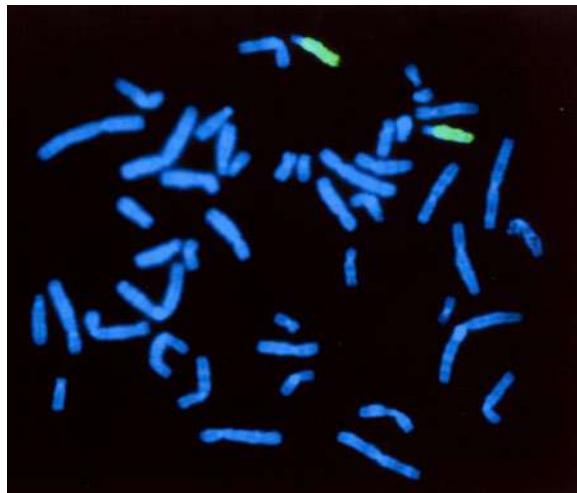


Figure 16.20 Whole arm paints. An example of a microdissected arm paint with only the long arm of chromosome 15 painted; no cross-hybridization is seen with the other acrocentrics. See insert for color representation of this figure.

excess, unbound or weakly-bound sequences. A detection step may be added if indirectly labeled probes are used. The slides are counterstained so that the nuclear boundaries and morphology are visible and then analyzed under a microscope, imaged, interpreted, and possibly re-hybridized with other probes. Other steps may include preparation of slides which are not conventional methanol-acetic acid fixed cells, such as cytopun cells, paraffin-embedded cells, smears, etc., which may further require deparaffinization, protease digestion to remove excess protein, etc. An RNase step can also be added which will remove RNA sequences that may hybridize to the probe and produce background.

16.9.1 Specimen

Any specimen that can be used for chromosome studies, such as suspensions of harvested blood, bone marrow, amniotic fluid, and CVS cultures, may be used for metaphase or interphase FISH. Slides or coverslips from in situ-harvested cultures may also be used for FISH. In addition, interphase FISH may be performed on many other cell and tissue sources. These include cells that are fresh, harvested and acid-alcohol fixed, frozen, flow-sorted, formalin-fixed, or cytopun as well as paraffin-embedded tissues, touch preps, buccal smears, urine sediments, biopsies, and archival fixed tissues. Even dissociated chromatin may be used for FISH, as with fiber-FISH methods.

If the specimen is a fresh solid tissue, it is usually enzymatically dissociated into single cells before processing for FISH. Chorionic villus samples (CVS), products of conception (POC) or tumor samples, for example, may be dissociated using trypsin, collagenase, or both, and a small amount of sample removed for FISH before standard culture methods are performed. Cells from a fresh specimen (either enzyme-dissociated or in natural suspension like amniocytes or bone marrow) are usually centrifuged into a pellet, and “harvested” using standard cytogenetic hypotonic solution and fixative. Amniotic fluid samples have many types of cells, some of which are poor candidates for hybridization due to the tough protein (keratinized epithelium) that makes it difficult for the probe to penetrate into the nucleus (see Figures 16.21, 16.22).

Slides that have been prepared by noncytogenetic methodologies may also be used for FISH in many cases. These include formalin-fixed paraffin-embedded tissues (FFPE tissues), cytocentrifuged (“cytopun preparations”) cells on slides, blood or bone marrow smears, buccal cells taken from inside the cheek, touch preps and unharvested, unfixed cells placed directly on a slide and dried. However, any slide of unfixed cells (e.g., cytopun cells, blood smears, touch preps) should usually be exposed to fixative before use, or cells may detach during hybridization. This may be accomplished by placing the slide in a Coplin jar of 3 : 1 methanol-acetic acid for 10–20 minutes, followed with drying the slide. Alternatively, for cytological preparations, a standard cytology fixative, such as Shandon Cell-Fixx (Fisher), may be used. FFPE tissues must be pretreated to remove the paraffin and much of the protein, and to prepare the fixed chromatin for hybridization. Probes cannot penetrate paraffin or thick layers of protein, and improper pretreatment to make the cells permeable to the probe is the cause of many hybridization failures for this type of tissue. On the other hand, small tissue samples may be fragile and may require pretreatment

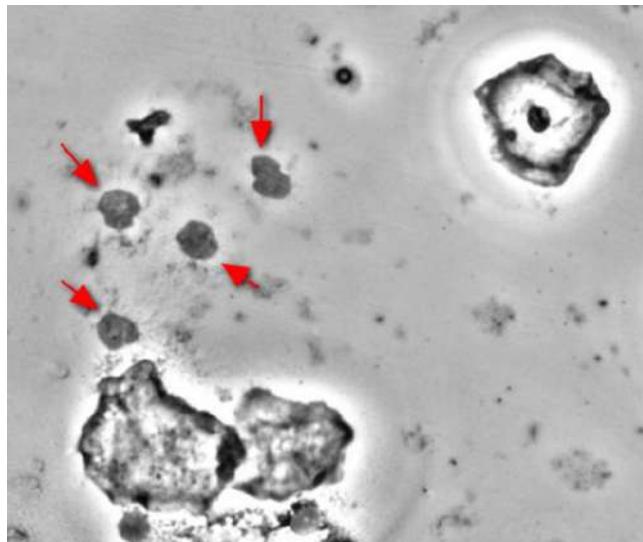


Figure 16.21 Phase contrast microscope image of uncultured amniocytes. These samples are commonly used for interphase FISH for aneuploidy studies for chromosomes 13, 18, 21, X, and Y. Note that the upper right cell has a thick, highly keratinized cell membrane, indicating that it is highly differentiated. These cells are difficult to hybridize because of the thick protein layer over the nucleus that prevents the probe from entering. The four gray nuclei to its left are good candidates for hybridization because they have very little cytoplasmic material. Nonetheless, for the best results, pepsin pretreatment is important to remove protein from these cells.

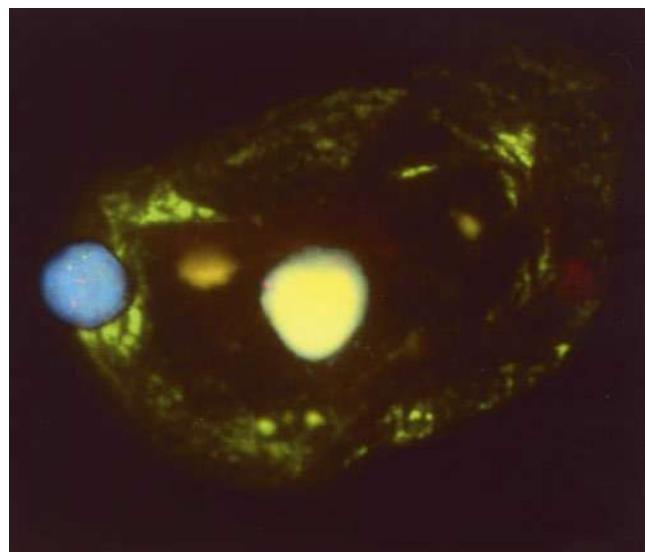


Figure 16.22 Comparison of amniocytes for FISH aneuvision. Two amniocytes hybridized with the Abbott Aneuvysion LSI 13 (green) and 21 (red). The nucleus to the right is highly keratinized, and even the DAPI stain did not get through very effectively. The proteins in this cell are auto-fluorescing. The nucleus to the left is a less differentiated cell and is not covered with as much protein, so it was able to hybridize with the probes and stain with the DAPI counterstain. See insert for color representation of this figure.

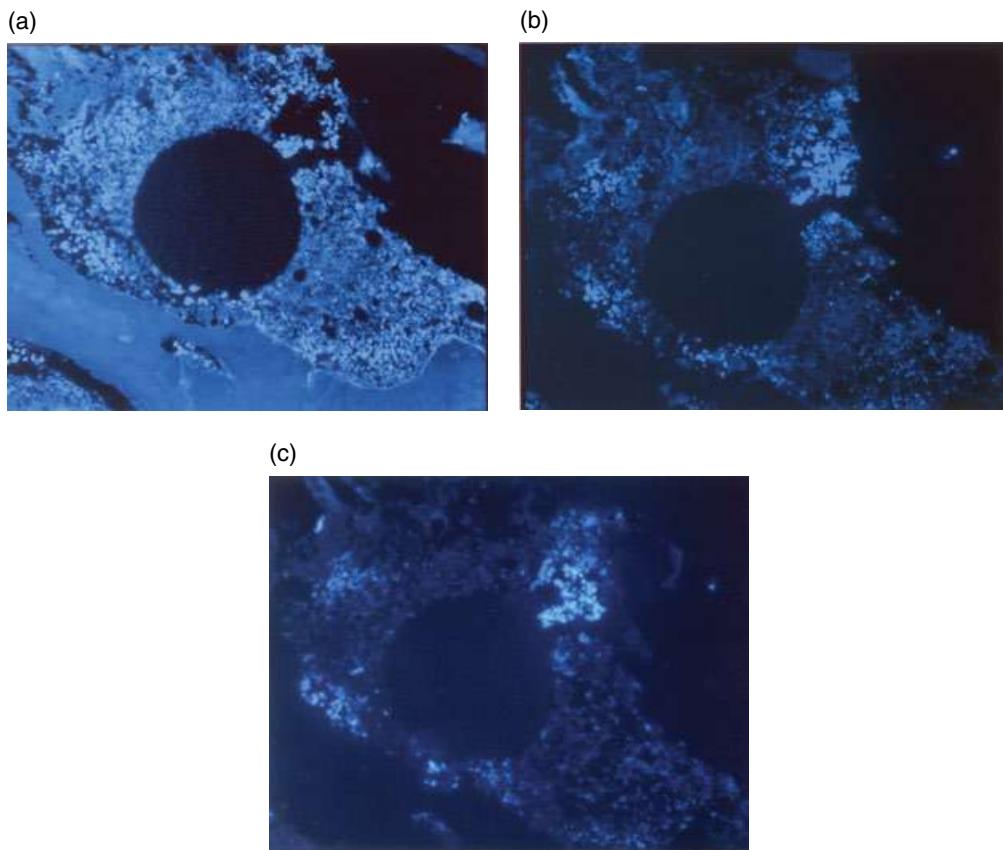


Figure 16.23 Comparing pretreatment time for HER2/neu FISH on breast tissue. Three sections of breast tissue submitted for HER2/neu FISH were treated exactly the same except that the time in sodium isothiocyanate pretreatment solution was increased from 10 minutes (a) to 20 minutes (b) to 30 minutes (c). Protease digestion was identical for all three experiments. Note that the DAPI counterstain becomes lighter as the tissue is exposed for longer times. The pretreatment solution removes protein cross-links caused by the formaldehyde fixative, making the proteins more available to the protease digestion, and allowing removal of excess cytoplasmic proteins to allow the probe to enter the cells. The best hybridization was seen in (b). (c) was overdigested (loss of cells and signal) and A was underdigested (too much auto-fluorescence from residual proteins). See insert for color representation of this figure.

time adjustments (see Figure 16.23). Other problems with FFPE tissues are due to pre-analytical issues, such as overfixation in formalin or treatment of bone marrow sections with decalcifying agents that can destroy the DNA for FISH purposes.

Of critical importance is that the tissue type used for FISH is appropriate for the test requested. For example, if the patient is being tested for one of the leukemia or lymphoma probe panels, blood may be an inappropriate source if no abnormal cells are present in blood; bone marrow may be the tissue of choice in such a case. The caveat for the use of CVS or placental tissue for FISH studies is that the syncytiotrophoblast layer of the villi originates from a completely different tissue layer from the fetus, so interphase FISH on placental tissues may exhibit disparate results from FISH on fetal tissues. FISH studies of placenta may reflect confined placental mosaicism (CPM) and/or may be contaminated with maternal cells. For determination of the fetal genotype, fetal tissue is the best choice. Placental FISH studies may, however, be important for comparison with fetal cells to detect CPM.

16.9.2 Harvest/cell preparation

If metaphase cells are to be studied, Colcemid® treatment should be incorporated into the harvest method. Otherwise, if only interphase cells will be used for FISH, Colcemid® is optional, although often metaphase cells yield valuable information such as probe signal location. Noncultured cells will only yield metaphases if the tissue is spontaneously dividing, such as bone marrow or chorionic villi. For certain types of metaphase FISH studies, long Colcemid® times may be helpful.

For example, if a large number of metaphase cells is necessary for extensive studies, such as an all-telomere panel or M-FISH, a one-hour exposure to Colcemid® (for blood studies) can yield a good mitotic index and facilitate the multiple-probe study on small areas of multiple slides. Specimens with very low mitotic indexes may also be more successful after longer Colcemid® exposure times.

Cells used for FISH can be from harvested or nonharvested, fixed cells. Cells exposed to hypotonic and fixation are best, even in interphase, because the cells become more transparent and flattened, giving the probe better access. It is good quality control to check the location of probe signals on metaphases from the patient slide whenever possible to verify the probe that was used was the correct one and to clarify unusual results or signal patterns. However, nonharvested samples have the advantage of losing fewer cells during the centrifugation and resuspension steps, and can be helpful when there are few cells or when there is a STAT case and time is of the essence (see 16.9.3, Slide preparation, for a description of a method of preparing very small samples using this method). A control slide with metaphases is acceptable for verification of probe location and hybridization efficiency.

Very harsh or overly prolonged fixation, such as formaldehyde tissue fixation for more than a day, may interfere with the hybridization. Decalcification, which is used on formalin-fixed, paraffin-embedded bone marrow specimens, is very destructive to chromatin, and may prevent the assay from working properly. Acid-based decalcifying agents degrade DNA structures more than EDTA-based decalcifying agents. Most FISH assays (more than half) performed on acid-decalcified bone marrow cores fail, in our laboratory. It is best to try to use unstained aspirate smears, touch preparation imprints or clot sections (not decalcified) to perform FISH. In some situations, there is no choice because of the unavailability of these undecalcified portions of the specimen, and the decalcified paraffin block would then be used as a last resort for FFPE tissue FISH.

16.9.3 Slide preparation

Slides prepared optimally for conventional cytogenetics are also suitable for metaphase and interphase FISH. “Flat” nuclei/metaphases that appear light gray, rather than dark gray or black on a phase contrast microscope, are best for FISH, because they do not require as much focusing to detect all of the signals present. Also, they tend to yield cleaner preparations, with less protein for the probe to penetrate. Dark, cytoplasmic preparations may still be used, and can be much improved by pretreatment with proteases such as pepsin, to decrease the protein barrier and improve the probe binding kinetics. Interphase nuclei which do not disperse well on the slide can be problematic for interphase FISH as well, due to bunching of signals.

Overcrowded slides may be difficult for any FISH assay because of increased concentrations of RNA, which may cause background due to cross-hybridization, and protein, which physically blocks the probe from entering the nucleus and may autofluoresce. In addition, overcrowding makes it difficult for the microscopist to determine which cells have been scored in a given field. Overdiluted preparations may not yield enough cells for a complete study. Overdilution also requires laborious and time-consuming effort, even when the complete study is attained. Prenatal interphase preparations of uncultured amniotic fluid can appear overcrowded when they are at optimal concentrations, because of the presence of highly differentiated epithelial cells that will fall off during the pretreatment of the slides. These lost cells are not optimal for FISH, since they have a very thick layer of keratin (see Figures 16.21, 16.22), so making concentrated preparations of amniotic fluid interphase cells is actually better than a dilute preparation. *In situ*-harvested cells may show too much cytoplasm in the middle of colonies, and the peripheral cells are usually best for scoring. If *In situ* preparations are very cytoplasmic, a pretreatment with pepsin or proteinase K is very helpful.

For interphase FISH of very small, nonmitotic samples, our laboratory has developed a method in which the cells may simply be prepared directly on slides without hypotonic or fixative steps to prevent cell loss during processing. Cells are centrifuged and resuspended in a very small amount of specimen supernatant – just the volume needed for the number of assays to be performed. A tiny drop of the unfixed suspension is placed on a clean, glass slide coated with fresh 3 : 1 methanol–acetic acid fixative, and fixative is carefully dropped on the bead of fluid until a crater appears in the cell suspension. Additional drops of fixative are dropped on the cell suspension droplet until the aqueous solution draws back to the slide edges, and the slide is then drained and dried. Further fixation may be accomplished by placing the slide in a Coplin jar of 3 : 1 methanol–acetic acid for 10–20 minutes. Proceed with FISH as usual, preferably using a protease step to be sure the nuclei are accessible to the probe. This method can retain most of the cells in a very small sample, and sufficient cells may be present for a complete study, whereas processing the sample through several steps of hypotonic and fixation may have yielded few or no cells for FISH.

Storing slides before hybridization

Slides that will be used within one week may be stored at room temperature. Slides that will not be hybridized within a few days or weeks should be protected from dehydration and oxidation; they can be stored in the freezer and used later. To keep the cells from oxidizing, they may be stored frozen in a Coplin jar of ethanol until ready to use. Alternatively, they may be

stored frozen in Ziploc bags. The bag may be filled with nitrogen, if desired, to minimize oxidation. When stored slides are removed from the freezer, they tend to fog over from condensation in ambient air, and thus may be run through a series of alcohol dehydration steps. Paraffin-embedded sections do not need to be frozen; if they are not deparaffinized, they are usually stable at room temperature almost indefinitely. FFPE tissue blocks are also good for several years, and may be sectioned and used for FISH.

Old slides left at ambient conditions, and slides that have been baked to prepare for G-banding purposes may be problematic for FISH, because the probe cannot penetrate a very dry cytoplasmic membrane. Such slides can be salvaged by rehydrating the slides in 95%, 80%, and 70% alcohol followed by water. Then the FISH method is performed as usual including the warm 2× SSC step and alcohol dehydration series.

16.9.4 Pretreatments for FISH slides

Aging and baking slides

For conventional G-band analysis, slides with nuclei and chromosomes fixed in methanol-acetic acid are generally aged in some fashion, such as allowing them to sit at room temperature for a day or more or baking them in a warm (37°C) oven for 3 hours to overnight, baking them in a hot (90–95°C) oven for 5–20 minutes or leaving them 3 hours to overnight on a hotplate or in an oven at 36–60°C. For FISH, baking slides for long periods (more than 10–20 minutes) at high temperatures such as 90°C may dry out the chromatin and result in poor or no hybridization; however, baking for short periods (5–10 minutes, an optional step) at 90°C can help condense the chromatin and thus help retain good chromosome morphology. Baking also condenses scattered signals into more discrete signals, and helps cells stick to the slide better (e.g., epithelial cells on interphase amniotic fluid samples). Our laboratory routinely bakes all conventional FISH preparations for 5 minutes at 90–95°C before performing the FISH steps. Baking is optional, however, and slides that have not been baked (or treated with SSC) will still work. Ethanol has been used to age slides as well [28]. Slides can be aged at room temperature for a day before use for FISH to improve the quality of the hybridization if there is no rush to obtain a result. Slides should be aged just before hybridization for best results; if slides are to be stored for more than a week, they should not be heated or exposed to SSC until the day they are to be hybridized, if possible, because longer times at room temperature can have the same effect as baking the slides, and overaging slides will diminish the signal intensity.

Salvaging overbaked slides

Slides that have been baked too long (e.g., baked as for G-band studies) may be salvaged by rehydrating through 95%, 80%, 70% ethanol followed by water. Then slides are put into 2× SSC (see later) and dehydrated through 70%, 80%, and 95% ethanol again before hybridization as usual. Rehydration also helps slides that have been stored at room temperature for several weeks, and have dried out too much for optimal FISH studies.

2× SSC treatment

An optional, but useful step is to pre-soak slides to be used for FISH in 2× SSC. Exposure to 2× SSC has a condensing effect upon the chromatin, giving more discrete FISH signals, and hardens the chromatin to prepare it for the harsh treatment during denaturation, yielding sharper, more morphologically distinct chromosomes. The 2× SSC step is performed on standard cytogenetic preparations after the (optional) slide baking or 24-hour aging at room temperature (see 16.9.4, Aging and baking slides) for best results. Slides that have been aged at room temperature for at least a day may not need to be treated with this 2× SSC step, although our laboratory always uses 2× SSC pretreatment. Once slides have been exposed to 2× SSC, baking no longer helps with morphology. Thus, if slides will be both baked and treated with 2× SSC, the baking step must be done first. The 2× SSC step may be performed at room temperature for just a few (e.g., from 2 to 5) minutes, 37°C for 30–60 minutes, or 72°C for 2 minutes. The higher temperature may be more successful when used with unbaked slides. Our laboratory prefers 30 minutes 2× SSC at 37°C for best morphology. Usually, the pretreatment is followed with ethanol dehydration, and then the slide is dried before hybridization is performed. An exception would be for slides that require protease treatment (see later), which is usually performed just after the 2× SSC step. FISH may also be performed on cells without any pretreatment, with some loss in signal quality (fuzzy, indistinct signals are more common). Once slides have been exposed to the SSC treatment, they can be stored for up to a week before continuing on with the hybridization steps.

Protease pretreatment for standard slide preparations

Certain types of slides, such as uncultured amniotic fluid cells, or cells that did not get hypotonic and fixation steps, such as blood smears, will be easier to hybridize if a protease treatment is performed just after the 2× SSC pretreatment step. The usual protease is 0.05 mg/mL pepsin in 0.01 N HCl (5% w/v) at 37°C for 10–15 minutes. Usually the slides are then put through a post-fixation step involving formamide to re-harden the nuclei before they are dehydrated, dried, and hybridized.

RNase treatments

The 2× SSC treatment arose historically from an RNase/2× SSC incubation step at 37°C (for as long as one hour) that used to be routine in the early days of FISH. RNase is generally diluted in a 2× SSC solution, and so historically, when RNase was deemed to be an unnecessary and labor-intensive step, it was replaced with the shorter 2× SSC incubation. There are certain assays, however, where RNase is still recommended, such as all-telomere panels and M-FISH, because background noise from extraneous RNA sometimes makes the assay difficult to read. To remove any RNA that could cross-hybridize with the probe and to clean the specimen enough so that the smaller probes can gain access to the chromatin, the RNase step can be substituted for the 2× SSC incubation, since it is also performed at 37°C in a 2× SSC solution.

Pretreatment for formalin-fixed paraffin-embedded tissue preparations

FFPE cells or tissues that are cut 4–5 µm in thickness and placed on positively charged slides can be used for FISH if the paraffin is removed and the cells are treated with agents that will remove enough protein to expose the nucleus for probe penetration. For best results, tissue should be fixed with 10% neutral buffered formalin or paraformaldehyde. Other fixatives, such as Bouin's or gluteraldehyde, are not recommended as they have been known to negatively affect hybridization. Overfixation (longer than 24–48 hours) with formaldehyde prior to paraffinizing and sectioning tissue may also affect the ability of the DNA to hybridize effectively [29].

The 4–5-µm-thick sections are applied to the slides by floating them in a warm water bath and sliding the glass slide under the section to lift it out of the bath. The slide is dried, but some residual water can stay under the section, which could cause the tissue to detach from the slide during the pretreatment or rinse steps if the sections are not properly baked on. The paraffin wax can be removed using xylene, which is said to reduce signal intensity, or xylene substitutes such as Histoclear or Propar. Most procedures will then rinse off the xylene or its substitute in ethanol washes. Slides may be stored at this point for a few days, if necessary. The slide is then treated with hydrochloric acid, usually 0.2 M, to hydrolyze the cytoplasmic protein covering the nuclei (optional).

After rinsing off the acid, the slide is immersed in a “pretreatment” solution, either with 1 mM sodium thiocyanate at 80°C for 20–30 minutes, 30% sodium bisulfite in 2× SSC pH 7.0 at 45°C for 20 minutes, or 10 mM pH 6.0 citric acid buffer at 80°C for 30 minutes to 2 hours. This latter procedure, using a citric acid pretreatment, has also been reported to preserve tissue adhesion to the slide [30]. The pretreatment step is necessary in order to reduce cross-linking induced by the formalin fixative so that the protease will be able to digest off some of the protein in the next step. Sodium thiocyanate is harsh enough to remove the tissue piece from the slide if it is very small; therefore for small pieces, the time in sodium thiocyanate may be reduced (see Figure 16.23).

The next step is to expose the slide to a 37°C protease, such as pepsin or proteinase K. Protein is unwanted because not only does it prevent the probe from getting into the nucleus, but it can also autofluoresce and obscure signals. The goal is to remove enough protein to allow the probe in but not so much protein that the cell architecture is lost. Architecture is very important for differentiating normal tissues from tumor cells so that tumor cells may be targeted for scoring.

It is possible to determine the quality of the slide pretreatment protocol before moving on to the hybridization steps; for tissue types being newly validated in the laboratory, this is a good practice. First, the pretreated nuclei may be evaluated with a phase microscope, and with some experience, it will be apparent which cell preparations are good and which are marginal. If they appear clean, well defined, and sharp, the preparations are probably good, and can be then hybridized in the FISH assay. If the nuclei are difficult to see, chewed up, or covered with dense material, they are most likely problematic specimens.

To better define the problematic slides, apply 15 µL of propidium iodide (PI) counterstain (0.6 µg/mL) in antifade or DAPI in antifade (0.1 µg/mL) to the slide and coverslip it. View the counterstain under the appropriate filters and evaluate the tissue sections for under-/overdigestion:

- *Overdigested* tissue has ill-defined or indistinct cell borders. Nuclei may appear ghostly or be lost. This slide should be discarded, and another slide should be attempted with less time in pretreatment solution (or less concentrated pretreatment solution) and/or less time in protease (see the Troubleshooting chart at the end of this chapter).

- *Underdigested* tissue shows large areas of red and green autofluorescence (protein) and poor counterstaining of nuclei. If the background fluorescence is very high, the pretreatment step may not have been long enough, and the protease was unable to digest the protein because of extensive residual cross-linking. A small amount of background fluorescence with somewhat ill-defined, dull counterstaining may indicate that the protease treatment was insufficient. This slide may be re-treated using longer protease times or more concentrated protease, and then hybridized. Slides may be re-treated until they appear optimal, as many times as needed, using this method.
- *Optimal* slides appear with little or no background fluorescence, nuclei that are evenly and brightly stained, with sharp, well-defined borders.
- Counterstains are removed by soaking off coverslip in 2x SSC and dehydrating through alcohols. Three to one parts methanol–acetic acid will also remove residual counterstain. Then proceed to hybridization.

New advances

Because of the inherent irreplaceable nature of paraffin-embedded material, FISH may be able to identify tissue-specific DNA changes, if the probe is capable of penetrating the protective sealant of these preserved nuclei. It is therefore understandable that new pretreatment methods are periodically being introduced to improve a probe's access to these embedded nuclei. For example, one innovative method, reported by Ventura et al. (31) uses a pressure cooker to deparaffinize, permeabilize, and reverse the cross-linking induced by fixation.

A dispersal method to create a cell suspension from paraffin tissue blocks, cores, or shavings has also been introduced. Tissue is deparaffinized, and cells are enzymatically digested apart into a cell suspension, that ultimately can be dropped or cytospon onto a slide and used for FISH [32–34]. The main advantage to this method is that the sectioning artifact (i.e., nuclei truncation as a result of tissue slicing) is mostly eliminated, since the only cut nuclei are at the edges of the sections. This is especially important when testing for deletions, because truncation can allow for a significant level (10–30%, depending on nuclear shape) of signal loss, making cutoff values difficult to determine. The main disadvantage to this method is that tissue architecture is no longer intact, and it will thus be difficult to distinguish between tumor and normal stromal cells. To reduce this potential misrepresentation, cores are often removed from specific areas of the paraffin block using a needle, in order to select only the desired cells and to enrich the tumor population in the final preparation.

16.9.5 Denaturation of probes and target

There are two methods commonly used to accomplish hybridization: separate denaturation and codenaturation. The best method depends upon what the assay is testing for. Separate denaturation can yield sharper chromosomes with less distortion and better DAPI banding patterns, and codenaturation can be faster and more efficient, especially when more than a few slides are run at one time.

Separate denaturation is accomplished by incubating the slide in hot, usually 72 °C/ 70% formamide/2x SSC solution for about 2 minutes, usually followed by an ice cold alcohol dehydration series to preserve the denatured state, and air drying. Ice-cold alcohol better preserves the open, denatured DNA configuration, but some protocols call for room temperature alcohol. Slide denaturation may also be accomplished in a sodium hydroxide solution at room temperature [27].

The probe is also denatured separately in a small conical tube in a water bath, usually at 70–75 °C, for 5–10 minutes. The tube may contain unlabeled Cot-1® DNA, if indicated. The probe may be used immediately by adding it to a slide, or it may be incubated in a 37 °C water bath for 20–60 minutes in a microfuge tube in order to allow preannealing of the repetitive DNA in the probe to the Cot-1® DNA before it is added to the slide (see 16.7.1, Cot-1® blocking DNA).

Another possible choice is to place the probe on ice before use to prevent it from continuing to reanneal before the slide is ready to use. Either way, the next step is to pipet the probe onto the appropriate area of the slide, coverslip, and then seal with rubber cement or other sealants.

In contrast, codenaturation is performed by simply adding an undenatured probe to an undenatured slide, coverslipping and sealing the slide, and placing it onto a hotplate or hybridization device which will denature both the probe and the target at once. This may be accomplished by heating them to 72–80 °C for a period of time, usually 2–10 minutes, and then removing the slide (or ramping down the temperature of the hybridization device) to reanneal at 37 °C for 4–20 hours depending upon the sample, the probe manufacturer, and the size of the probe. If preannealing with Cot-1® DNA is required before slides are codenatured, the probe denaturation and reannealing steps can still be performed in a microfuge tube before adding it to the slide. Even though the Cot-1® will be re-denatured during the codenaturation, the preannealing seems to work well. Codenaturation on a commercial hybridization device or programmable hotplate can be accomplished by either ramping up and down to the desired temperature or by having the temperature constant during the denaturation step. There are several useful commercial

hybridization instruments, such as Abbott Molecular Laboratories' HYBrite or Thermobrite, the DAKO cycler, the Statspin Thermobrite, or various thermocyclers made for PCR of DNA. A plain hotplate can be adapted for use, as well.

It is possible to get the best of both methods using a modified codenaturation method. First, a solution of 70% formamide/2× SSC solution (the same as would be used in a Coplin jar in a water bath for separate denaturation) is placed on the slide to be hybridized, coverslipped, and placed on a 72 °C hotplate to denature the target nuclei. After 2 minutes, the coverslip is removed and the slide is dehydrated through a succession of ethanol washes and dried. After the probe and coverslip is added to the slide and the coverslip edges sealed, the slide is codenatured as usual. Pre-denaturing the target in this way has the effect of giving formamide-denatured chromosome quality using only the hotplate/hybridization instrument without the need for a water bath and Coplin jar to separately denature the slides.

16.9.6 Coverslips, sealants, and alternatives

It is extremely important to keep the probe mixture from drying out during the denaturation/reannealing steps. Drying will create a pernicious fluorescent background, and may overpower the probe signal (Figure 16.24). Coverslips are usually used to cover probes on the slide. They are sealed to prevent drying, most often by using rubber cement (termed "cow gum" in some methods). The high temperatures of codenaturation and reannealing can cause rubber cement to become very hard. Reannealing in a humidified chamber will solve this problem and make the rubber cement soft and easily removed. Rubber cement may be used full strength or diluted. Alternatively, if the coverslip is small enough to allow a sufficient border around the perimeter, it can be sealed with a piece of Parafilm, which seals itself to the slide on the hot plate during codenaturation. When reannealing is complete, the parafilm can be pulled off fairly easily with a pair of forceps. Besides these two sealing methods, a "Gene Frame" may be mounted on the slide. Gene Frames consist of a gasket with glue on both sides that is placed over the hybridization spot or over the coverslipped probe, and a plastic coverslip is placed over the gasket that adheres to it tightly, keeping moisture in. It requires larger volumes of probe mixture than a standard coverslip to fill the chamber with probe.

Coverslips may be composed of glass or plastic. Plastic is said to absorb less probe than glass and can be cut to special dimensions with a pair of scissors. Glass may be siliconized to enhance performance. Siliconized glass absorbs less probe and creates fewer bubbles, but unsiliconized glass works quite well.

Coverslip dimensions may be 22 × 22 mm square, 18 mm square, or round coverslips with diameters of 5, 8, 12, or 15 mm. The latter sizes require less probe but hybridize a smaller area and are more prone to drying out; 24 × 50 mm coverslips can be used when hybridizing the entire slide. Two 22 × 22 mm coverslips can be used on a standard microscope slide, each with a different probe. However, it is important to make sure that the probe does not leak from one coverslip to the other before the sealant is added to the edges. The volume of probe needed for each of these coverslips is, approximately 10 µL for 22 × 22 mm square, 2–3 µL for 12–15 mm round, and 20–30 µL for 24 × 50 mm rectangular coverslips.

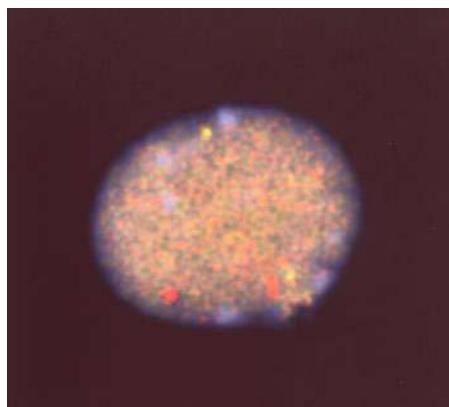


Figure 16.24 Effect of probe mixture drying out. This nucleus was hybridized with subtelomeric probes for 8p and 8q, but the signals are obscured by background fluorescence that was caused by improper sealing of the coverslip during denaturation and reannealing so that the probe mixture dried out. See insert for color representation of this figure.

16.9.7 Renaturation (reannealing) times and temperatures

Once the probe and target are denatured, they must be given some time at a specific temperature to reanneal or hybridize with each other. In separate denaturation protocols, after the denatured slide and probe have been combined, they are allowed to reanneal at 37–42°C either on a hotplate or in an incubator, occasionally in a floating chamber in a waterbath. Most probes hybridize well at 37°C, although some protocols call for reannealing of alpha satellite DNA probes and other highly repetitive sequences at 42°C in order to decrease cross-hybridization. For codenaturation protocols, slides are either ramped down from the denaturation temperature to reanneal or are removed from the hot plate and placed in an incubator with the appropriate temperature, usually 37°C. Use of a humidified box in the incubator helps keep the slides from drying out. To prevent drying for codenaturation methods, strips of paper towels with moisture may be placed in the hybridization device.

The incubation time required to reanneal the probe and target is quite variable. Most methods call for 4–20 hours, but some methods call for less than 4 hours and some call for longer than 20 hours. For example, Children's Oncology Group (COG) FISH protocols for acute lymphoblastic leukemia include an assay for triple trisomies of chromosomes 4, 10, and 17 using alpha satellite DNA probes. The chromosome 4 alpha satellite probe cross-hybridizes with the centromere of the 9, and in some cases it can appear as a very strong signal. Using a 15–30-minute renaturation time can decrease the cross hybridization significantly (Matt Folsom, personal communication). Some other alpha satellite probes work well at a decreased reannealing time also. Locus-specific probes take longer since the DNA they are composed of is not as repetitive. Paraffin-embedded slides may be more difficult to hybridize, and some methods call for a 2-day renaturation time. If necessary, for holidays or weekends when personnel will not be available to rinse the slides the following day, the probes should work well after being reannealed for two days as long as they are kept in a moist chamber and do not dry out.

16.9.8 Post-hybridization wash

After the probe has been hybridized to the target for a few hours to overnight, the coverslips are removed and the slides are put into a Coplin jar with post-hybridization wash to remove the unbound and nonspecifically bound probe so that the signal of interest is bright and the background is clean. There are two main methods for post-washing probes: formamide washes (usually 50% formamide in 2× SSC) at 45°C followed by a rinse in 2× SSC at 45°C, or SSC solutions (0.4x to 2× SSC depending upon desired stringency) at 72°C followed by room temperature 2× SSC. The formamide washes take longer, so the SSC washes have become popular. The stringency of the post-wash solution is adjusted to accommodate the probe type and the desired effect. For example, a paint may be washed in 2× SSC or 0.4x SSC. With the former, the paint will appear brighter, but will have a little more cross-hybridization; therefore, if the target (chromosome segment to be hybridized with the probe) is very small and likely to appear dim, as in a very small translocation, the 2× SSC would be a good choice. Low stringency can be utilized as a tool in some cases. For example, if an unknown marker chromosome is being probed with an alpha satellite probe for identification of its origin, for example chromosome 1 alpha satellite probe, it may be desirable to use a low stringency wash to cause the probe to cross-hybridize with three or four chromosomes other than the 1 alpha satellite probe. If the marker exhibits signal, it either originated from the chromosome that would be targeted using normal stringency or one of the chromosomes that show cross-hybridization signals with lower stringency [35]. The origin of the marker can be narrowed down with subsequent FISH assays using probes that are specific to all of the chromosomes that hybridized in the low stringency FISH assay (see Figure 16.18). This way, one does not need to use every alpha satellite DNA probe that exists to identify the unknown marker chromosome.

The SSC washes usually contain a nonionic detergent (does not ionize in water) such as NP-40 (nonadet), Tween 20, SDS, or Igepal. These are surfactants that make unbound probe rinse off better. Use of a post wash without surfactants will work, but some probes will exhibit more background; for example, if the centromeric (DYZ1) Y probe is not washed with a surfactant, some nonspecifically bound probe may remain, making some negative cells appear to have a signal. In such a case, it would be appropriate to re-rinse the slide in SSC that has a surfactant added.

Some methods call for agitation of the slide in the post-wash SSC solution. The reason for this is that the probes are diluted with a 50% formamide buffer, and DNA easily becomes denatured at high temperatures in formamide solutions, leading to loss of signal. Agitating the slide removes the formamide quickly before it denatures the probe and chromatin, preserving brighter signals.

16.9.9 Detection

If a probe is directly labeled, no detection is necessary; however, for indirectly labeled probes tagged with biotin or digoxigenin, there will be no fluorescent signal until the probes have been “detected” with a fluor-conjugated detection molecule. For a digoxigenin-labeled probe, this involves exposing the post-washed slide to an anti-digoxigenin with a fluorescent tag. In the case of a biotin-labeled probe, it is exposed to avidin or streptavidin with a fluorescent molecule incorporated into it. Avidin

has a strong affinity for biotin and sticks to it. Streptavidin is avidin from a bacterial source. The detection is usually performed under a coverslip in a room-temperature to 37°C environment for 5–10 minutes, followed by 2× SSC rinses and slide mounting with counterstain. The slide should not be dried after post-washing and detection steps, and is mounted wet, because the detection molecules stick to the glass slide and will not rinse off well if dried. In the early days of FISH, if the signal obtained with indirectly labeled probes was insufficient, the slides could be amplified using antibodies to the detection agents with more fluorescence attached to the antibodies. However, this method produced so much background that it is rarely used today.

16.9.10 Slide mounting and counterstains

Nuclei and chromosomes are usually counterstained with a dye that allows concurrent visualization of the probe and the chromatin of the cell. The exception to this is when the counterstain would interfere with one or more of the fluorescent labels on the probe, in which case there is usually enough fluorescence visible in the nuclei from nonspecific probe binding to allow identification of individual cells. The counterstain is usually diluted in an antifade solution that retards the quenching of the fluorescence of the probe label.

Although the counterstain allows the visualization of the nuclei and chromosomes, it does not always need to be visualized concurrently with the probe, though it should be available for capture and documentation in images. For example, using a triple pass red/green/blue filter to score a red and green probe set with a DAPI (blue) counterstain may make the green signal difficult to see, so the technologist may choose to score the slide with a dual bandpass red/green filter so that the green signal is easier to score accurately, but capture blue, green, and DAPI together on the saved images. The caveat for use of the dual bandpass filter is to be sure that the nuclear boundaries can be visualized.

Slides may be counterstained in a Coplin jar and then mounted with an antifade solution, or they may be mounted in an antifade-counterstain mixture that takes a few minutes to counterstain the cells and is then left on the slide permanently. The antifade usually consists of pH 8.8-buffered glycerol with 0.5% p-phenylenediamine. DABCO antifade is 1,4-diazabicyclo(2.2.2)octane. The counterstain is usually DAPI (4',6-diamidino-2-phenylindole), a blue counterstain that allows visualization of most probe colors, or propidium iodide (PI), which fluoresces red and is best for visualizing green probes. Do not use PI with red probes or DAPI with blue probes such as Vysis Spectrum Blue, since the colors of the emitted light from each pair of dyes is too similar and the probe signal will be obscured. PI is a good counterstain for FITC-labeled paint probes when looking for abnormalities, such as very small segments derived from a translocation, because the segment might be missed with the DAPI due to its subtle nature, while PI is very bright and contrasts well with the green FITC.

The mounting medium is usually placed on the slides after the post-wash step, and a coverslip added. The excess mounting material is gently pressed out between paper towels. Failure to press the excess material out will cause some fluorescent flare and will tend to exude medium into the microscope oil and blur the image. Overstaining with PI or DAPI (e.g., use of a high concentration) can cause the fluorescence of the nucleus/chromosomes to overpower the probe. However, certain preparations, such as paraffin-embedded materials, need more concentrated counterstain than cytogenetic preparations since they have been exposed to harsh pretreatments. Slide preparations that have been re-hybridized can become difficult to counterstain, and a more concentrated counterstain can be helpful. Accidental use of the wrong counterstain is remedied by soaking off the coverslip in 2× SSC with NP-40 for a few minutes, and then dipping slide in methanol-acetic acid to remove the counterstain. Slides may then be stained with the correct counterstain and coverslipped for viewing.

The best coverslip sizes are 24×60 mm (for slides with probe on the entire slide) or 24×50 mm, even with small samples, since there is better protection with a large coverslip from accidental mixing of the antifade/counterstain and the immersion oil. Coverslips may be thickness 0 or 1. Some preparations may be viewed without any counterstain at all, such as preimplantation blastomeres, which often use five probe colors, precluding the use of any counterstain, which could make probes of a similar wavelength disappear. Such preparations are simply mounted in antifade solution without any stain in it. Preimplantation FISH from commercial suppliers may include a slight amount of fluorescence-tagged antibody stain against the embryonic cells to outline the nucleus. Figure 16.25 shows a scheme for handling different types of specimen for FISH and Figure 16.26 shows probe preparation guidelines.

16.9.11 Storage of hybridized slides

Hybridized slides may be stored in a -20°C freezer until ready to use, and the fluorescence will last much longer at cold temperatures. Some feel that freezing the slide for a short period of time before performing the microscopy makes the signals better. The most important storage requirement is to keep the slides in the dark until ready to use to prevent quenching of the fluorophores. A few minutes to an hour in the ambient light is not likely to affect the fluorescence, but hours in a bright light setting will certainly affect the brightness of the signal.

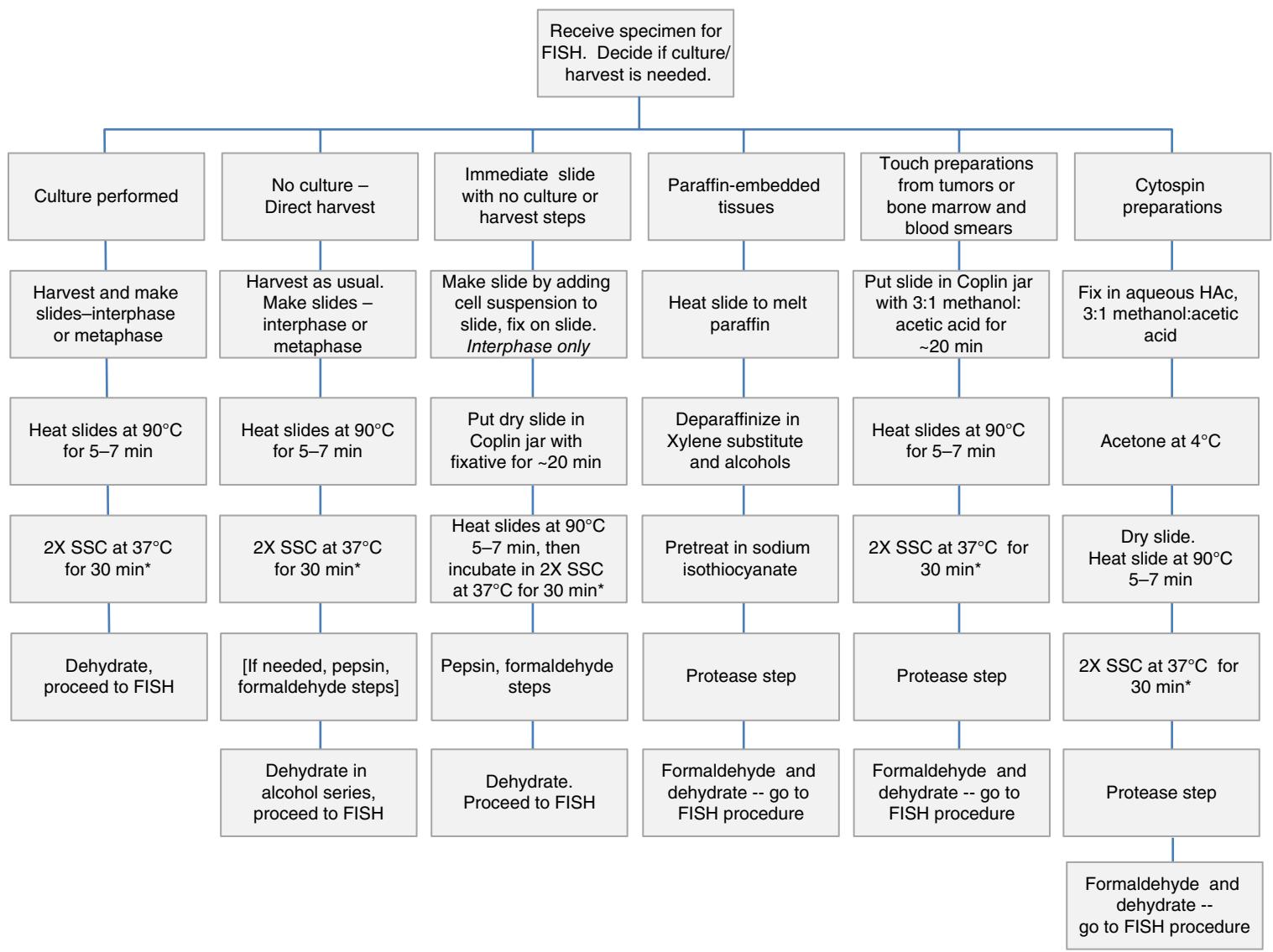
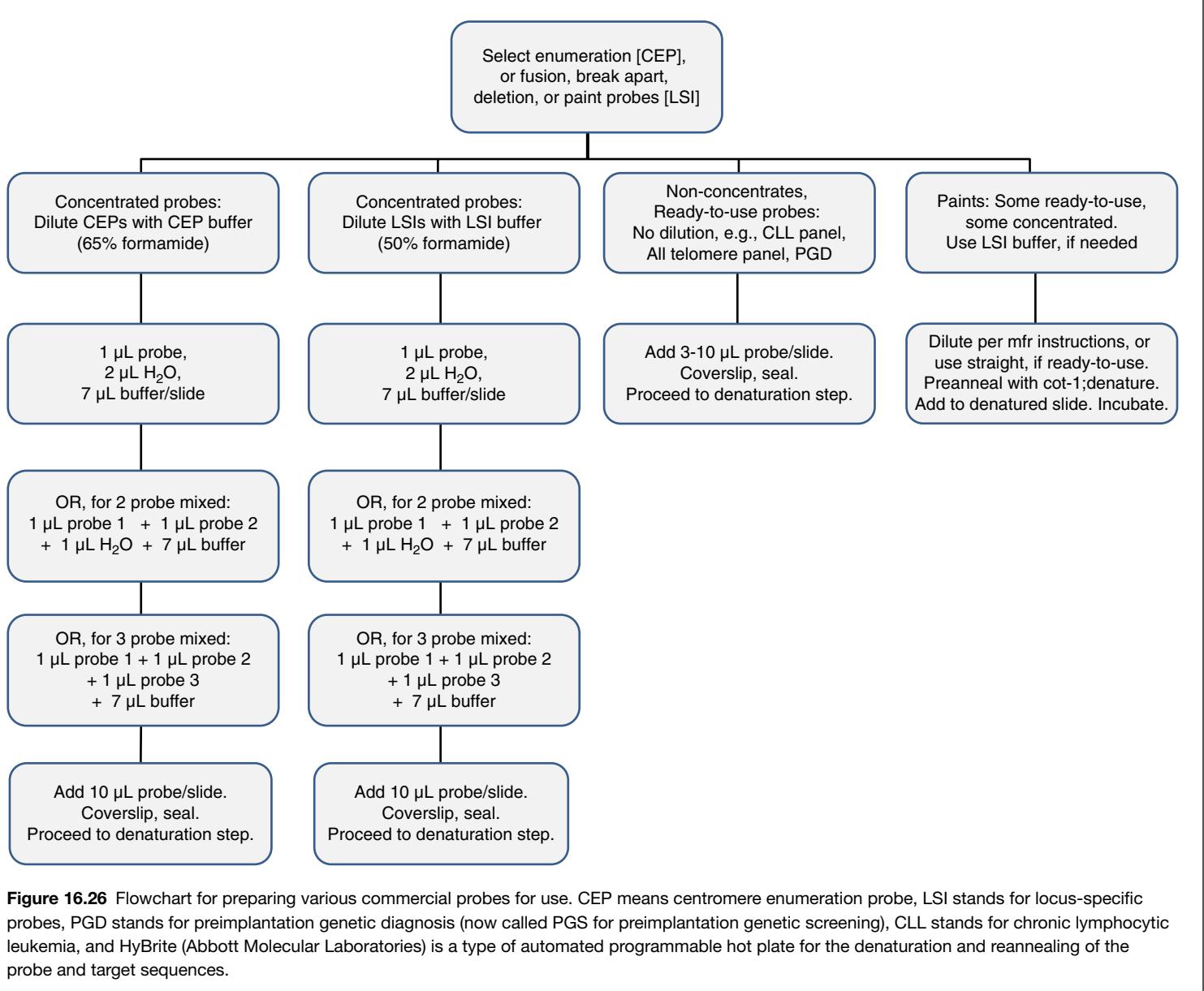


Figure 16.25 Flowchart for preparing slides for FISH.

*Denotes optional step. Timing of this step is also variable.



16.9.12 Safety precautions

Some reagents used for FISH have safety issues, and every technologist working with them should review the SDS for each one (see Chapter 20, Chemical safety). Coverslips can be broken when they are removed from the slide, and the fragments must be thoroughly cleaned up and put in the sharps container. Tape can be used to pick up the smaller pieces of glass. There are other concerns with materials used for FISH, so check the SDS sheets for all components.

16.10 FISH analysis and reporting

16.10.1 Microscopy

The microscope is usually an epifluorescence (light from above the sample) model with a 50–100-watt high-pressure mercury lamp, and equipped with the necessary filters for the counterstain and probe fluorophores to be visualized, and at least 10 \times low power lens and 60–100 \times oil high power lenses. If applicable, make sure the lamp is properly centered so the field of view is evenly illuminated (Figure 16.27), and that the recommended number of hours has not been exceeded on the lamp (check manufacturer's guidelines for lamp hours).

When visualizing FITC and rhodamine or Texas Red together with a DAPI counterstain, use a DAPI filter to scan on low power to find the cells, a “triple bandpass” filter (DAPI/FITC/Texas Red) to visualize the probes and counterstain together, and a “dual bandpass” filter (FITC/Texas Red) set to obtain the brightest view of the probe signals. For Spectrum Orange probes, the regular dual bandpass filter may be fine for some probes, while others are best viewed using a special dual bandpass filter with wavelengths specific for FITC and Spectrum Orange. With the latter, the green FITC signal may not be as clear as with the regular dual bandpass filter set. The Spectrum Orange probe signal is also clear using the Spectrum Gold filter. However, the green FITC is not visible using this filter, and so it would be inappropriate for scoring fusion or break-apart probes.

The immersion oil should be a low-fluorescence type (e.g., Cargille type DF, formula 1261) so that objects scanned on low power are not obscured by autofluorescence of the immersion oil layer. Fluor low power lenses are best for a bright image while scanning for metaphase cells. Interphase cells may be scored with oil immersion lenses without changing to low power unless they are sparse. Most laboratories use computer-assisted imaging systems to record the cells instead of photography. Using photography, all signals are captured simultaneously. Using imaging systems, a major advantage is the individual capture of different fluorescent channels that allows individualized enhancement. Therefore, dim images may be enhanced; fluorescent debris may be omitted from the image; the object of interest can be easily isolated from the surroundings and/or made into a composite with other images; a computer print can be made in much less time with less effort than a photograph; images are easily annotated; and digital images are more easily stored.

For certain applications, such as the 3D visualization of nuclear FISH signals, a z-stack mechanism or a confocal laser scanning microscope is often used. The microscope at high power focuses on a very narrow depth of field, excluding all other

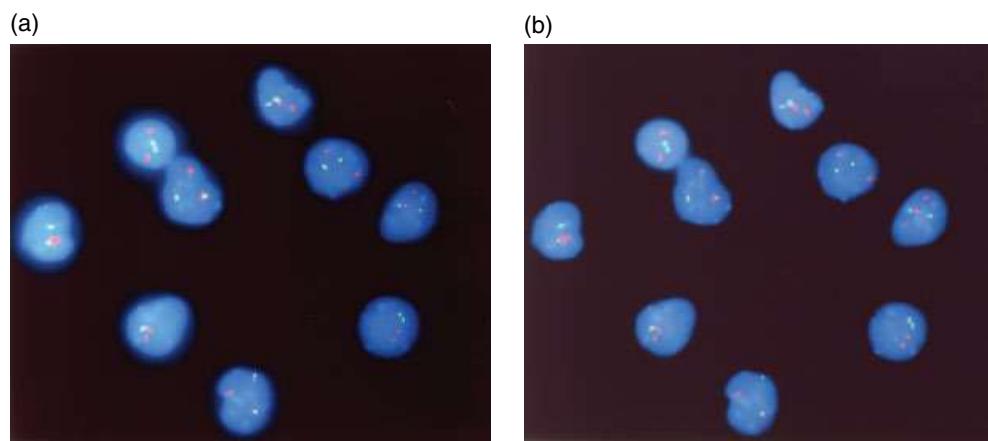


Figure 16.27 Proper centration of the microscope lamp. Proper centration of the microscope lamp is important for good visualization of the probe signals. Left: light not centered. Note that cell on left side is too bright and cells on right side are too dim. Right: same cells with light properly centered. Note the even illumination and signal size. See insert for color representation of this figure.

signals not in the plane of focus, so that serial images, called light optical sections, are collected at different focal planes. The compiled images are termed an image stack, or z-stack (from x, y, and z dimensions on a graph). The confocal microscope scans sequentially through the cell point by point, and is recorded by a sensor called a photomultiplier tube. The computer software assembles the data into an image. A three dimensional array of data is produced. For imaging cells with signals in different planes, there are also tricks that can be utilized (see 16.10.3 Capturing and working with FISH signal images).

16.10.2 Scoring cells

Cells are scored methodically across the slide, either scanning right and left or up and down, moving one field before returning the other direction, similar to scanning G-banded slides. The number of cells to score for each type of study is laboratory specific, and the American College of Medical Genetics has published guidelines (<http://www.acmg.net>) to help laboratories make their own scoring protocols. Probe signals should be bright, distinct, and easily evaluated. Background should appear dark or black and clean (no fluorescence between cells, and no haze). Probe should be specific to the chromosome/region and not cross-hybridized with other chromosomes/regions. More than 90% of viable cells in a control specimen should show adequate/expected signal patterns. Abnormal hematology/oncology specimens with 100% of cells showing the abnormality may represent a constitutional finding, and should be investigated with chromosomes from a tissue not involved in the neoplasm. Avoid scoring suboptimal cells, overlapping or crowded nuclei, uninformative cells, or cells with artifacts (Figures 16.28 and 16.29).

Interphase specimens with low percentages of aneuploidy should be interpreted with care, because mosaicism is difficult to rule in or out near the 10% level. It is also important to take into consideration possible chimerism due to bone marrow or stem cell transplants, maternal cell contamination (see Figure 16.30), lost twins (prenatal), or true patient chimerism. Equivocal results may need to be verified using other probes for the loci involved. Metaphase cells should always be used as verification of interphase results whenever feasible because they may add to the interpretation [36]. Metaphase cells that are scored should be recorded on an analysis sheet, which may be specific for each probe. Interphase cells are usually only recorded when captured. The coordinates of captures are recorded, along with a note about what the FISH assay exhibits on that cell. Tallies are made to show the number/percent/signal ratios of abnormal cells. Straightforward numbers and percentages are sufficient for most metaphase and interphase studies; however, for certain deletion/amplification interphase studies, such as those on paraffin-embedded tumors for deletions of 1p and 19q for gliomas, and amplification of HER2/neu for breast carcinomas, a ratio of the test probe to the control probe can sometimes be more meaningful because of the high ploidy of tumor cells. This method involves scoring a set number of cells for both the test, (e.g., red) probe, and the control (e.g., green) probe signal number. The number of red signals is added up for all the cells scored, and the number of green signals is also added up. Then the number of green signals is divided into the number of red (test) signals to arrive at a ratio. In many laboratories, this is the method of choice for loss of 1p/19q studies for gliomas, and for amplification of HER2/neu in breast carcinomas. Typically, deletions are seen with a ratio of less than 0.8, and over-representation/amplifications greater than 1.2 (gliomas) or 2.0 (HER2/neu).

Scoring interphase cells properly requires some skill and experience, and knowledge about the performance of the specific probe on normal cells and abnormal cells, as well as the specific breakpoints of the patient's rearrangement. For example, probes that are used to detect translocations in interphase come with a number of designs (see Figure 16.7). Examples of probe designs for interphase translocations include fusion probes and fission or break-apart probes (see 16.2.2, Probe designs). Fusion probes can be single fusion (showing the fusion signal pattern only on one derivative), or dual fusion (showing fusion signal patterns on both derivatives) (Figure 16.7).

Further confounding the interpretation of interphase fusion probes is a tendency for certain translocations, such as the t(9;22)-mediated *BCR/ABL1* gene rearrangement seen in chronic myelogenous leukemia, to have small deletions near the breakpoints, causing variant abnormal patterns in certain patients (Figure 16.31). When there is a deletion just distal to *BCR* on chromosome 22 and just proximal (centromeric) to *ABL1* on chromosome 9, there will be no material left to make the fusion signal. Such cells appear to have one red, one green, and one fusion, with the second fusion invisible by FISH due to the deletions. If there is only a deletion in *BCR* but not in *ABL1*, or vice versa, the pattern will appear RGGY or RRGY since the complementary color for the second fusion is deleted [37, 38]. This has prognostic significance, as well. Typically, a deletion for either proximal *ABL1* or distal *BCR* will produce a pattern with either red/red/green/fusion or red/green/green/fusion when a dual-color dual-fusion probe is used instead of the usual red/green/fusion/fusion. Patient breakpoints will determine whether a given probe design will work for that patient. Deletions for both 9q34 and 22q11 that result in a red/green/fusion signal pattern are problematic because the pattern is identical to that of overlapping *BCR* and *ABL1* signals in normal cells. Overlapping signals due to close proximity of chromosomes is called juxtaposition artifact. Use of a third color probe in the 9q34 region (for the *ASS* gene region) can distinguish between juxtaposition artifact and a true single fusion. If there are two signals for *ASS* in a single fusion cell (red/green/fusion), it is juxtaposition artifact, while if there is only one signal under the corresponding normal *ABL1* signal, it is a true single

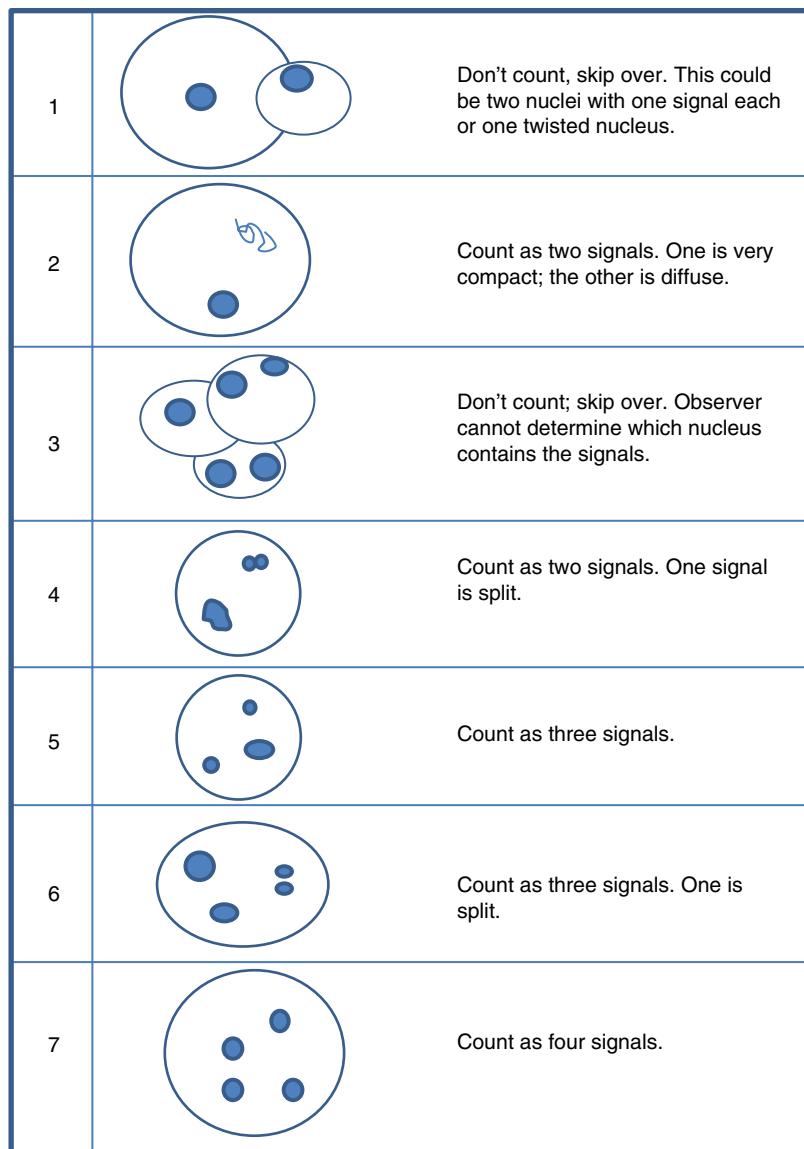


Figure 16.28 Single color FISH counting guidelines. Courtesy of Abbott Molecular Laboratories.

fusion. Some laboratories use a single fusion probe for BCR/ABL1 plus the 9q34 to cover all possibilities. If there is a third chromosome involved in the 9;22 translocation, one of the fusion signals is disrupted, for example, using a dual-fusion probe, a characteristic two red, two green, one fusion signal variant positive pattern is seen (Figure 16.31). Always try to get an abnormal metaphase cell on any FISH study to confirm the abnormality, whenever possible. Note that the percent of abnormal cells may differ between metaphase and interphase due to a variety of reasons, such as patient treatment, differing activity of different cell types, and disease type (indolent diseases may not have mitotic activity). Also, the *BCR/ABL1* rearrangement can be, like some other translocations, cryptic using G-bands, and only visible with FISH.

A dual-fusion probe will not show any fusions if a variant translocation partner is present. For example, in rhabdomyosarcoma a variant translocation t(1;13)(p36;q14) sometimes occurs instead of the typical diagnostic translocation t(2;13)(q35q14). Both translocations involve the *FOXO1* gene on chromosome 13 at 13q14.1. Therefore the best strategy is a break-apart design using flanking probes for the constant chromosome/gene partner, in this case *FOXO1* on chromosome 13. Ewing sarcoma may show a t(11;22)(q24;q12) or a variant t(21;22)(q22;q12), so the constant partner is the 22q, and

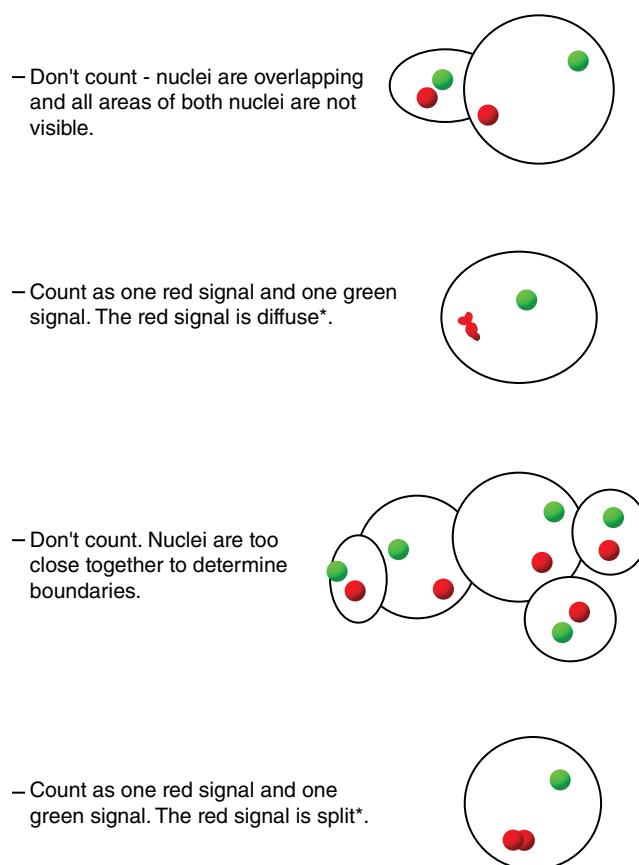


Figure 16.29 Two color (e.g., X and Y) counting guidelines. Courtesy of Abbott Molecular Laboratories. See insert for color representation of this figure.

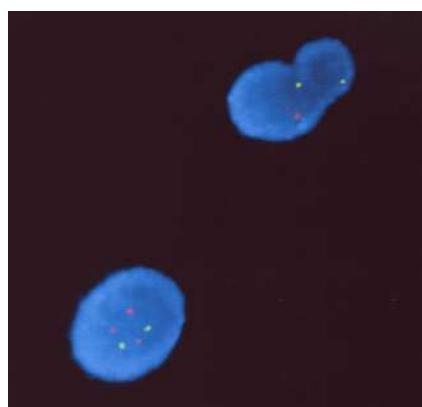


Figure 16.30 Maternal cell contamination in amniotic fluid interphase FISH study. These two cells are from an uncultured amniocyte preparation that was hybridized with a locus specific probe for 13 (green) and 21 (red). The upper cell is from maternal blood, and has 2 signals for both probes. The lower cell is from the fetus, and has 3 signals for chromosome 21. Note that the morphology of the maternal cell nucleus is lobed, which is characteristic of white blood cells. See insert for color representation of this figure.

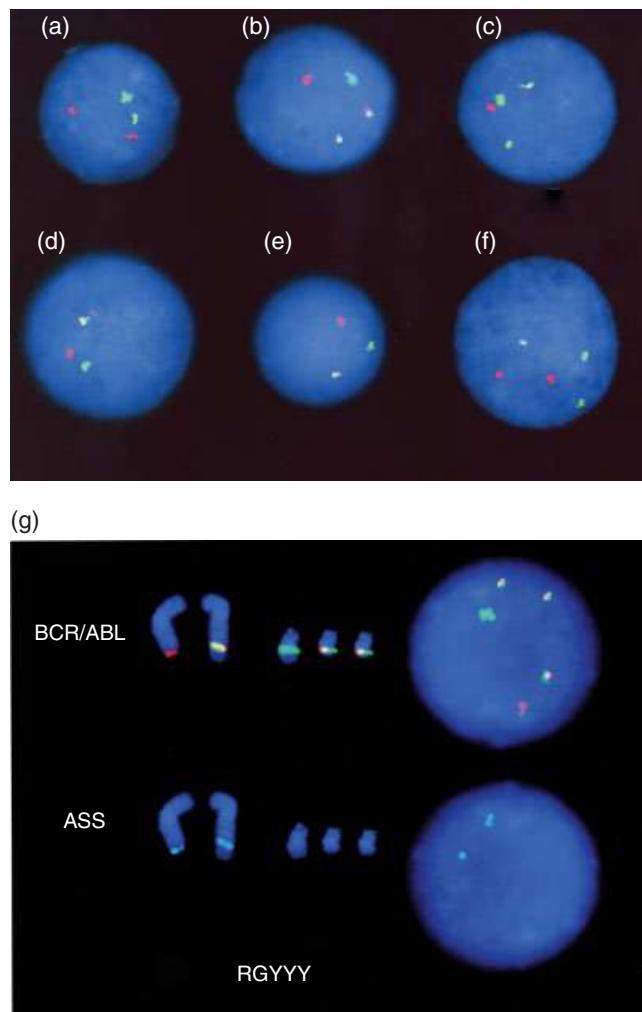


Figure 16.31 BCR/ABL1/ASS metaphase and interphase variations. Metaphase and interphase cells were hybridized concurrently with an Abbott Molecular Laboratories dual fusion BCR (green)/ABL1 (red) probe and the ASS 9q34 probe (argininsuccinate synthase) (aqua). The same cells are represented twice, once to show the red and green signals, and below each cell to show the aqua signal patterns. (a) 2R2G2A Normal interphase with the usual classic signal pattern of two red, two green, two aqua signals. (b) 1R1G2F2A BCR/ABL1 fusion: classic abnormal dual fusion abnormal signal pattern: one red, one green, two fusions, and two aqua signals caused by the t(9;22) rearrangement. (c) 1R2G1F1A BCR/ABL1 fusion cell with a variant abnormal signal pattern of one red, two green, one fusion signal caused by a *deletion* of the red (ABL1) portion of the fusion on the der(9) and also the nearby aqua ASS just proximal to the ABL1. The der(9) lacks native red proximal ABL1 signal and the aqua ASS signal. (d) 2R1G1F2A BCR/ABL1 fusion cell with a variant abnormal pattern of two red, one green, one fusion, two aqua signals caused by a *deletion* of the green distal BCR portion (from the 22) of one fusion signal, usually the der(9) fusion. Only the BCR chromosome 22 material is deleted, so both ABL1 and both ASS signals are present. (e) 1R1G1F1A BCR/ABL1 fusion cell with a variant abnormal pattern of 1 red, 1 green, 1 yellow, 1 aqua ASS signal caused by a *deletion* of both the red and green portions of one fusion signal, usually the der(9). This is accompanied by a deletion of the ASS gene; therefore, by using the BCR/ABL1 with the ASS, a true single fusion variant cell can be differentiated from a normal cell with juxtaposition (overlap) of the 9 and 22, which can appear as if it were a fusion signal. Cells with one red, one green, one fusion, one aqua ASS are scored as true BCR/ABL1 fusion cells, while cells with one red, one green, one yellow, two aqua are scored as juxtaposition artifact of normal signals. (f) 2R2G1F2A BCR/ABL1 fusion cell with a variant abnormal pattern of two red, two green, one fusion, and two aqua ASS signals. This pattern is caused by the involvement of a 3rd chromosome [t(9;22;?)] that separates the red and green from the putative second fusion. It is rarely caused by aneuploidy of the 9s and 22s. It does not involve a deletion of ASS in most cases. (g) 1R1G3F1-2A Another common BCR/ABL1 fusion pattern seen with disease progression is one red, one green, three fusions, with the third fusion often indicating the presence of a second der(22) Ph chromosome. The classic 1R1G2F patients with progression to an extra Ph will also have two aqua signals; however, a patient with a one red, one green, one fusion variant pattern will show the classic one red, one green, two fusion signal pattern when a second Ph chromosome is acquired but would have only one aqua signal. It is important to find metaphase cells in interphase BCR/ABL1 FISH studies to be sure of the interpretation of the interphase signal pattern. See insert for color representation of this figure.

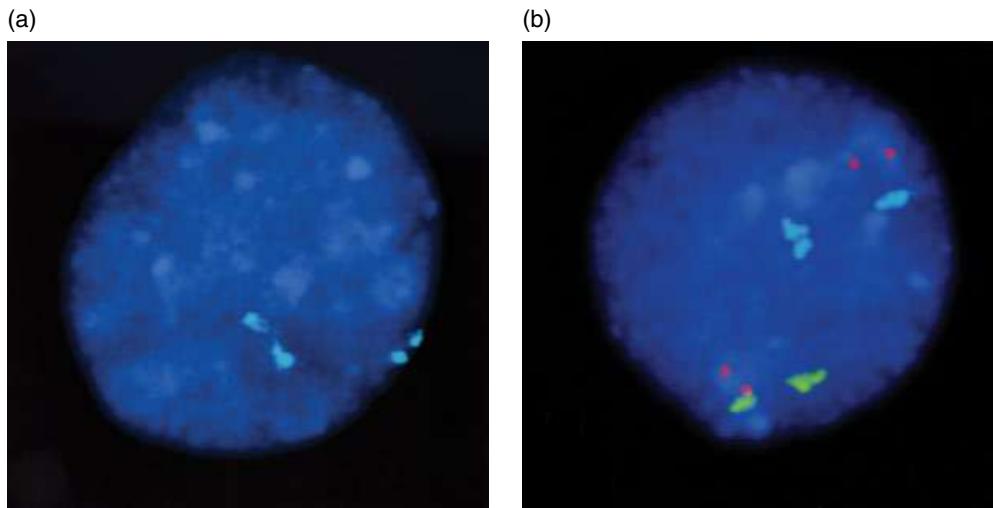


Figure 16.32 Scoring replicated signals. When chromosomes replicate, there are four chromatids available to hybridize with a probe, and this can result in 4 signals from two chromosome pairs. (a) Alpha satellite probe for 18 showing replicated signals. (b) Cocktail of D13S319 (red), 13q34 (aqua), and CEP 12 (green) showing signal splitting due to replication in the aqua and red fluorophores. See insert for color representation of this figure.

the commercial probes for this translocation are usually designed as a break-apart for the 22q *EWSR1* region. The normal break-apart probe pattern shows two fusions because the breakpoints have not separated, and the abnormal pattern usually shows one fusion for the normal chromosome homologue and separated red and green signals for the translocated homologue (Figure 16.10). The challenges are great for these probes as well, because deletions often occur in the translocation breakpoint regions, and may yield a fusion/red pattern, or a fusion/green pattern. There may be polyploidy, further confusing the interpretation, especially in interphase only studies of paraffin-embedded tumor tissue. An interpretation dilemma is whether to say in the final report that the break-apart signal pattern is evidence for the specific translocation, when actual evidence only implies the translocation, but does not truly demonstrate it. Perhaps the best way to report it is to say that there is a disruption of the breakpoints of the test chromosome that is consistent with the disease translocation. A real dilemma occurs when the gene rearrangement is inconclusive because it may be consistent with more than one type of tumor. An example is the break-apart probe for Ewing sarcoma. A positive result may indicate Ewing sarcoma/ PNET, desmoplastic small round cell tumor, or clear cell sarcoma.

Another challenge for scoring interphase FISH signals is that if the DNA in the nucleus is undergoing synthesis or if the cell is in G2 of the cell cycle, each chromatid will have a signal, appearing together as a doublet (Figure 16.32a). Usually, if the doublet is not more than a probe signal width apart, it is scored as a single replicated signal (see Figure 16.32b). It is possible for one homologue to be out of synchrony with the second homologue, so that one signal appears as a singlet and one as a doublet in the same nucleus. It is also fairly common to find one probe synthesized and another not yet synthesized in the same cell; for example, the two p53 signals appear as a pair of doublets but the centromeric control probe is still appearing as two singlets.

Repetitive probes may appear as segmented signals (alpha satellite DNA, Figure 16.33) or as a cloud of tiny signals (subtelomeric specific probes). This “spatter” effect can be minimized by briefly (5 minutes at 90–95 °C) baking the slides just before performing the FISH procedure.

If the cells or tissues used for FISH are not completely flat on the slide, the microscopist will need to focus through the depth of the cell to find all of the signals. This is especially important when viewing signals in paraffin-embedded material. Poor slide-making of standard 3 : 1 methanol–acetic acid fixed cells may also result in excessively three-dimensional cells. It may be necessary to focus very carefully when analyzing such specimens to detect all signals, most especially when looking for deletions in interphase.

Another consideration when scoring a FISH study is the purpose for which the probe is being used. For example, the *SNRPN*/subtelomeric 15q probe may be used to look for a deletion, as in Prader–Willi/Angelman syndromes, or it may be used to look for a duplication of the *SNRPN* (small nuclear ribonucleoprotein polypeptide N at 15q12) region in patients with

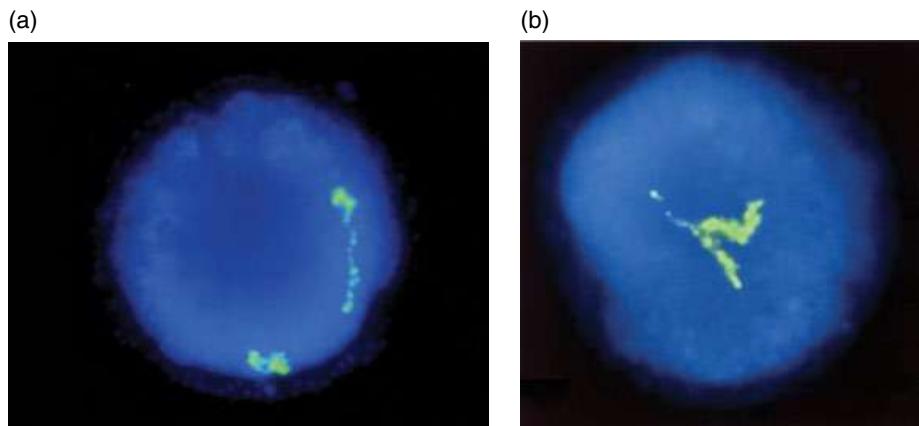


Figure 16.33 Scoring repetitive DNA signals. (a) Owing to their repetitive nature, alpha satellite probes may, due to their repetitive nature, unravel into long stringy structures in interphase. This can make scoring them difficult, as the attachment threads may not be visible, and one signal can appear as two, or two signals may be indistinguishable from a single signal. (b) This cell is not scorable. See insert for color representation of this figure.

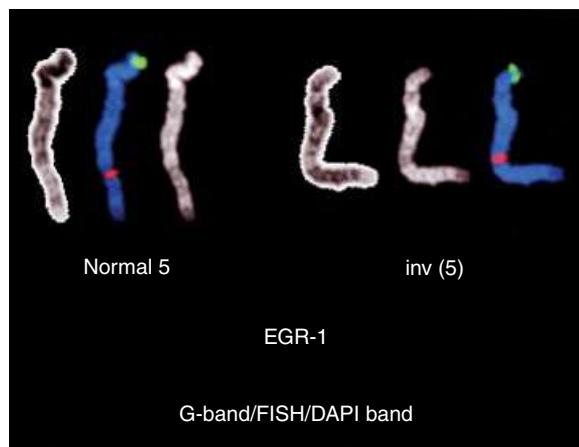


Figure 16.34 Using DAPI bands to determine location of a probe. Shown is a pair of chromosome five homologues from a PHA-stimulated blood culture of a child with anomalies. The left hand image in each pair is the reversed DAPI bands, corresponding to G-bands. The center image is the FISH capture of the same cell, with the control probe on 5p in green, and the *EGR1* probe at band 5q31 in red. The farthest right image in each set shows the DAPI bands, which were captured on the fluorescence software as if they were Q-banded. The right-hand homologue has a paracentric inversion of 5q, and the 5q31 band can be seen in its new position above where it is located on the normal 5q. This confirms the G-band interpretation of an inversion rather than a derivative chromosome. See insert for color representation of this figure.

autism. Each requires a different scoring method. For Prader–Willi/Angelman, a number of metaphases would be scored to see if one of the chromosomes 15 is missing a SNRPN signal, signifying a deletion of that region. For the autism indication, in addition to the metaphases, a number of interphase cells would also be scored, since it is much easier to detect duplications in interphase when the chromatin is opened up and elongated (Figure 16.12).

Metaphase cells are usually scored for rearrangements, and the chromosomes can be identified using an identifier probe on the same chromosome, DAPI bands, inverted DAPI bands (requiring software in an imaging system), sequential FISH after G-bands, or sequential FISH after a FISH assay using different probes (Figures 16.34 and 16.35). DAPI bands are very similar to Q-bands and can be karyotyped if captured as a fluorescent image. Karyotyping a FISH counterstain can be helpful to identify rearrangements or markers. DAPI bands can be made more distinct by the use of BrdU substitution during culture [39].

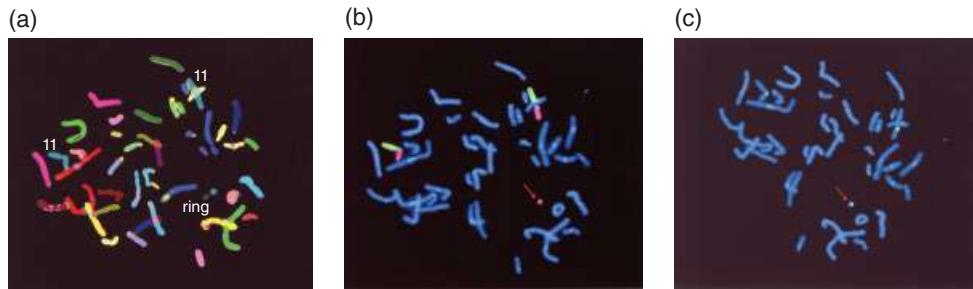


Figure 16.35 Sequential hybridizations of the same cell to identify an unknown chromosome. Stimulated peripheral blood cultures from a patient with a small ring were hybridized three times in sequence. (a) M-FISH showing the ring as having a probable 11 origin by the color and the spectral characteristics. (b) A whole-arm 11p (red) and 11q (green) paint. The ring exhibits red signal and is therefore derived from 11p. (c) Centromeric alpha satellite DNA probe for chromosome 11 to verify the origin of the ring. The same cell was used for all three hybridizations, with destaining in between by exposure of the slide to 72 °C hot water for 2 minutes. See insert for color representation of this figure.

Scoring paraffin-embedded tissue sections requires some consideration of the technical artifact from the sectioning process, as some nuclei will be partially sectioned away. Some tissues will appear three dimensional, and the signals may be in very different planes of focus. FFPE samples are heterogeneous, and require distinguishing between neoplastic and normal tissues. It is often important to obtain the assistance of a pathologist to mark the region to be scored.

When scoring preimplantation FISH nuclei, sometimes there are multiple nuclei on a given embryo from one biopsy. If they seem to be fragmented bits from one nucleus, with signals unequally divided among the parts, the signals can be added together, with the report stating that the result is from a “fragmented nucleus.” If there are clearly two nuclei present, each with a normal or near-normal signal complement, they should be reported separately, as nucleus 1 and 2 from that blastomere.

16.10.3 Capturing and working with FISH signal images

Capturing FISH signals requires a good video camera and special imaging software from one of the major automated karyotyping computer companies. There is no way to directly adjust the intensity of the emitted light. To get the brightest image, the fluorescence microscope must be properly adjusted. Make sure the bulb is centered and focused and balanced with the mirror image, if the lamp housing has a mirror. Center and focus the diaphragms that control intensity (aperture stop) and the size of the field to be illuminated (field stop). Most image capture software packages include tools to enhance the brightness of the image for capture. Usually, one color is captured at a time monochromatically in black and white, and a color is assigned to the raw image by the software. The component images are then compiled into full color image. The user can change the assigned color, which sometimes helps with the clarity of the image. For example, blue and green probe signals can be obscured if captured against a DAPI blue counterstain. In this case, DAPI can be assigned a light gray color against which the probes will be very distinct. Also, if there are two blue colors (e.g., Spectrum Aqua and Spectrum Blue), one can be assigned, for example, pink, and the resulting photograph will be much easier to interpret.

Some systems include a post-capture threshold slider that allows the user to omit all small background signals from the image. However, this may obscure some actual signals. For example, with MYCN probes, if MYCN is highly amplified, and there are dozens of tiny signals, thresholding may delete many of them. In such a case, it may be best to leave the threshold slider all the way open and capture without thresholding at all.

When capturing signals in multiple planes, such as in FFPE tissues, if the microscope is not equipped with a Z-stack to capture several layers, a trick is to capture each color at least twice in different planes. This is done by loading the “rhodamine” or “FITC” designations more than once in the capture window. They may be given new names such as “red” and “green” if needed, using the same filter assignments.

Commercial software programs usually provide a tool to move the signals on the image, which is useful when enumeration probes of two colors overlap one another within a nucleus. However, it is important not to move signals on images of fusion or fission probes, since this may cause misinterpretation of the results.

16.10.4 Reporting results

FISH reports should include, besides the usual and referral patient information, the type of probe, source, number of cells examined, whether the cells were interphase or metaphase, numbers of normal and abnormal cells, and the diagnostic findings and interpretations. Additional information may include probe design (e.g., double fusion, break-apart, etc.), locus/gene information, test cut-off values and normal ranges. Results can be given using ISCN nomenclature (see Chapter 8, The universal language of cytogenetics). However, in cases where the ISCN may be too complex or confusing, the interpretation can explain the results and clinical implication. Comments may be made about the suitability of the sample or quality of sample or assay. The American College of Medical Genetics has composed a disclaimer that, by their guidelines, should also be included in the report (<http://www.acmg.net/Content/NavigationMenu/Publications/LaboratoryStandardsGuidelines/default.htm>). Laboratory-specific disclaimers may also be added. For example, some laboratory reports will state that the interphase FISH results for an uncultured CVS sample may not represent the fetal cells if maternal cell contamination is present or if there is confined placental mosaicism.

16.10.5 FISH and mosaicism

Interphase FISH is often used to look for mosaicism or chimerism, for example using the BCR/ABL1 or other oncology probe set to detect residual abnormal cells after treatment, or using the centromere enumeration probes for chromosomes X and Y to detect transplantation donor/host proportions in opposite sex transplants. These assays are robust; therefore, with good validation studies, they may be used routinely in the cytogenetics laboratory. FISH may also be used to look for mosaic constitutional abnormalities, such as Turner syndrome mosaicism, in tissues other than blood, when blood chromosome studies are normal. Buccal smears, which represent cells derived from the ectoderm, may be used for FISH in interphase (see Chapter 3.1, Using peripheral blood for cytogenetic analysis, for a discussion of ectoderm, mesoderm, and endoderm germ layers). Urine cells may also be used, as they represent endoderm. Since blood represents mesoderm, all three tissue layers may be studied for a given abnormal finding in interphase. If a female patient is undergoing fertility assistance, follicular fluid obtained during oocyte harvest may be used for interphase FISH to examine cells in the reproductive tract for certain abnormalities. Without robust controls, however, interpretation may be difficult, especially for low-level mosaic findings. Be aware too that some patients have variant patterns with no phenotypic effect, and their normal interphase pattern may show one or three signals for the variant. These variants are most common in using alpha satellite DNA probes or subtelomeric specific probes [19, 20].

Interphase amniocytes and chorionic villi cells that have been harvested without culturing are often used for determining the ploidy of chromosomes 13, 18, and 21, as well as the sex chromosomes. Even when there is no visible blood present in the prenatal sample, they may be contaminated with maternal blood cells and cells from missed twins. Amniotic fluid from pregnancies with oligohydramnios are often mainly of maternal origin. Chorionic villi are known to exhibit confined placental mosaicism, and the result may not accurately reflect the fetal karyotype. Mosaicism in these types of samples should be confirmed with chromosome analysis before any irreversible action is taken. All reports should be accompanied by a disclaimer to explain the pitfalls of the method.

16.11 FISH probe testing and validation

The majority of probes used in the clinical cytogenetics laboratory are analyte-specific reagents (ASR), meaning that the user is responsible for determining their efficiency (see 16.2 Clinical applications of FISH probes). Both the probe itself and the specific method for using the probe must be validated in the laboratory before it can be used in the clinical setting. Probe validation is thus a process for testing each probe or set of probes intended for clinical use to ensure that they produce repeatable, reliable results that detect the target most of the time and do not bind to another spurious target. It is also a mechanism to determine what parameters will be defined as normal and abnormal, so that patients who are not affected do not receive treatment or incorrect information, and patients who are affected, get the care they need. FDA-approved probes, however, do not require a thorough validation, as long as the FDA-approved method is adhered to strictly. If there are any changes to the FDA method, it must be revalidated using the new conditions in the laboratory.

16.11.1 Sensitivity, specificity, and efficiency

There are a number of excellent references about the design of FISH tests and their validation [40–45]. The analytical sensitivity (how well it detects the targets) and specificity (how often does it detect or cross-hybridize with another chromosome than the one it was designed for), both expressed as a percentage number, should be established for all probes used in a clinical setting. For probe sensitivity, one might score 200 interphase cells and use the formula:

$$\text{Sensitivity} = \# \text{ interphase nuclei with the expected signal pattern} / \text{total number of interphase nuclei} \times 100$$

For example, if 199 cells of 200 showed two signals for a centromere probe and one cell showed 1 signal, the formula would be:

$$\text{Sensitivity} = 199/200 = 0.995 \times 100 = 99.5\%$$

For probe specificity, it is common to analyze 100 metaphase cells for signal patterns and with reverse DAPI (or chromosome specific centromere probes) to confirm location, and use the formula:

$$\begin{aligned}\text{Specificity} &= \frac{\text{number of FISH signals at the expected chromosomal locus}}{\text{total number of FISH signals}} \\ &(\text{or number of signals at expected loci} + \text{number of signals at unexpected loci}) \times 100.\end{aligned}$$

For example, if 100 nuclei showed two signals for a centromere probe on the correct homologues, the formula would be:

$$\text{Specificity} = 100/100 = 1 \times 100 = 100\%$$

If 100 metaphases showed two signals at the correct centromeres, but five additional metaphases showed signals on a third chromosome, the formula would be:

$$\text{Specificity} = 100/100 + 5 = 100/105 = 0.95 \times 100 = 95\%$$

The efficiency of a probe is the percentage of available cells that it hybridizes to compared with the number of cells with no signals, and can be affected by the sample type. For example, dead or dying cells will not hybridize, so a necrotic or apoptotic sample will show a lower efficiency than a healthy normal blood sample. Probes used to detect minimum residual disease in interphase should have a high specificity and sensitivity. Also important is reproducibility, or how well a probe performs (how closely the sensitivity and specificity matches) in repeated assays on different days with different technologists performing the hybridization and post wash, and slide making conditions.

The number of normal specimens required to validate a probe has been suggested to be twenty [41]. The number of cells scored per test control should reflect the number that will be scored in the clinical test. For example, if 200 interphase cells will be scored for each BCR/ABL1 interphase FISH test, then for each slide used for validation, 200 cells per tech should also be scored. Some laboratories mix together 20 normal specimens and count 4000 (200×20) cells on that specimen. However, mixing cells together could be confusing if there are any variant hybridization patterns in the normal population, as are often seen with centromeric or subtelomere specific probes. To become familiar with the probe, the technologist should analyze different individuals separately.

The precise chromosomal location of a new probe (and new probe lots) should be determined on banded metaphases (reverse DAPI bands or sequential G- or Q-bands work well) or by incorporating another probe as a known identifier such as an additional differently colored centromeric probe. This assures that the probe hybridizes to the appropriate chromosome and region, that there is no cross hybridization or contamination of the probe mix, and that the protocol stringency was optimal for the probe (see 16.11.2 Special considerations). Peripheral blood cultures of normal males (containing all 23 chromosomes) are generally used for this part of the validation process because the quality and quantity of metaphases and nuclei will provide a clean and well-hybridized preparation, making it possible to verify the precise location where the probe binds. Tissue-specific testing, however, should be performed when establishing normal/abnormal ranges for a specific tissue type (see 16.11.2 Establishing cutoffs).

It is important that all technologists involved with FISH testing are proficient for all categories of FISH. Before using the various probe types (see 16.2.2 Probe designs), the novice technologist should score normals and abnormalities, and the results should be compared with what other, experienced technologists have reported for the same slides. This may require splitting samples with another laboratory, with technologists who are already familiar with the probes. The validation process is helpful for developing technologist proficiency with the probe.

It may also be important to have slides chosen for the validation process that have been made by more than one technologist, as hybridization quality can be influenced by the spreading of cells, even in an interphase study. Crowded, poorly spread cells yield preparations with autofluorescent debris and poor hybridization, due to residual proteins and RNA between nuclei, and to thick cytoplasm over the nuclei, forming a barrier to probe penetration.

16.11.2 Establishing cutoffs

Each laboratory needs to decide what validation methods and cutoff values will be used, with attention to the guidelines published by various regulatory agencies (see Chapter 21, Process management; Chapter 25.1.7, Credentialing and guidelines). The hybridization behavior of each probe should be well understood for every tissue in which it will be utilized;

therefore, both normal and abnormal samples should be assessed in order to gain familiarity with the new probe's characteristics. The technologist should familiarize himself with the different spurious patterns that occur, and decide which patterns are significant for scoring and validating. Several technologists should participate in the scoring process of new probes so that variations in interpretation can also be taken into account.

Normal cutoffs need to be calculated, along with reportable abnormal ranges (e.g., the percent of abnormal cells in the typical untreated specimen). Signal patterns may be different for various tissue types and for various diseases; therefore, the best tissue for establishing cutoff values is the tissue type on which it will be used. If both bone marrow and blood will be used, therefore, they both may need to be validated separately. Statistical tests should be performed for each possible parameter that could have diagnostic significance in the clinical setting. For example, the *EGR1* probe binds to chromosome 5q, and its control probe binds to 5p; therefore, possible abnormal results may reflect a deletion of 5q, a deletion of 5p, monosomy for the entire chromosome 5 and trisomy for chromosome 5. Scoring during the validation process, therefore, should keep track of all four possible interpretative parameters, and cutoffs should be established for each.

In addition to normal cutoff values, an abnormal database can help ensure that the probe is working and help find what variations in signal patterns can be seen with a given probe. Even a set of data from five abnormal individuals will help familiarize the technologist with the probe. Some abnormalities are too rare to allow for a comprehensive database.

Statistical methods for calculating cutoffs vary from the use of two or three standard deviations from a mean to the use of a Microsoft Excel spreadsheet to calculate the beta inverse function (see Chapter 19, Determining the normal cutoff for FISH validation) or binomial distribution. If there will be different numbers of cells scored under different circumstances, each number needs to have its own cutoff value, as the cutoff value will decrease as the total number of cells increases. Regardless of the method used, it is important to keep in mind that the cutoff value is a guideline, and values that fall near the cutoff need to be interpreted with caution, in the context of other patient information and other laboratory test results.

Special considerations

There are often several probes that will hybridize to the same region(s) of a chromosome, so proper localization does not ensure that the probe is the right one. For example, there are two translocation probes for t(14;18) with the same breakpoints but different loci on the 18 – IGH/BCL2 and IGH/MALT. The only way to ensure that the probe is the correct one is to test it on a known abnormal, or preferably several abnormalities. This would also familiarize the technologist with any variant patterns that might be encountered.

One caveat is to try to make sure that the probe being sold for a given purpose is in the correct region to detect the abnormality. For example, the commercially available Smith–Magenis probes can miss up to 15–20% of 17p deletions, because the probe companies were able to avoid paying royalties on the *RAI1* smallest region of overlap by moving the probes slightly away from this region [46].

For validation of preimplantation blastomere FISH probes, it is still important to score a large number of interphase cells to obtain validation statistics, even though there is only one cell per slide in the clinical study.

Cutoff values for formalin-fixed paraffin-embedded tissue must be calculated from tests on FFPE tissue, preferably from tissue and nuclei similar in type and morphology to those that will be scored, because of microtome-slicing. Interphase nuclei can vary in shape from round to oval, and the direction of the microtome cut can change the results dramatically in an elongated nucleus, depending upon whether it was parallel or perpendicular to the knife. For this reason, deletion probes are very difficult to interpret in FFPE tissues, and unless the tumor is very concentrated, and good control probes are used, interphase deletion studies should be performed with caution. Calculations of probe sensitivity and specificity in metaphase cells are also not necessary for paraffin-embedded cells, although it is still important to check the binding location of the probe on a metaphase to be sure the probe is what it claims to be.

Probes used to confirm copy number in array CGH or other microarray analysis need to be validated for localization, and a normal control specimen should be run with each FISH test to compare the performance of the probes in metaphase and interphase. It is critical to make sure that the probe fits within the region of the copy number change. For example, a deletion of 1p36 might not be detectable with a commercial probe for that deletion, since the manufacturer wants to be sure the signal is bright, and may make the probe larger than the deleted region. BACs are available that may be a better choice for confirmation of the smaller copy number changes.

If there is any consistent cross-hybridization, the method should be modified to correct the problem or the probe should be discontinued. Cross-talk between probe signals (e.g., fluors which will show through two different primary filters, such as Spectrum Blue and Spectrum Aqua) also needs to be adjusted by changing the fluor, getting a more specific filter, or discontinuing the problematic fluor.

16.11.3 FISH controls

Some laboratories run a normal control with each probe run. This can be used to confirm that the probe used binds to the correct targets and to determine that all of the probes in the mixture are working when there is no signal on a patient sample (e.g., both D13S319 signals are deleted on chromosome 13). It should be a slide from a normal peripheral blood sample so that metaphases are present to check the loci of the probe signals. It is important to run a normal informative control in cases where complete absence of target sequence is a possibility. This will control against a hybridization failure that would appear the same as if there were no target sequence present, as might be the case with the Y chromosome SRY constitutional probe in an individual who has an XX chromosome constitution or an abnormal Y chromosome.

Use of an abnormal control would show that the correct probe was used and would make sure the technologist knows the abnormal pattern. Checking metaphase cells present on the patient slide is very helpful as an internal control when there is a confusing or variant pattern seen in interphase cells.

Some FISH probes have internal controls, such as a centromeric probe, unique sequence probe or subtelomere-specific probe that identify the chromosome in question. For metaphase studies, this is often sufficient. However, for interphase FISH studies on which no metaphase cells are present, it is possible to put the wrong probe set on the slide and not realize it, possibly leading to a false negative result. For such studies it is important to run a normal control containing good quality metaphases for checking the probe locations. However, for certain probes, the location alone is insufficient. For example, N25 (TBX1) and TUPLE1 probes look the same on 22q, *SNRPN* and D15S10 are in the same position on chromosome 15, and IGH/BCL2 and IGH/MALT are both translocations for acquired t(14;18), so they would be indistinguishable on metaphases. Only positive abnormal cells could be used as a control against the use of a wrong probe in such cases. Another example is the location of various probes for detecting deletion of 6q in CLL, which are not the same from one company to another, each detecting some deletions and missing others.

One important interphase pre-analytic control is to perform baseline FISH at disease presentation when post-therapy leukemia/tumor surveillance analysis will be done. This is because the post-treatment sample is liable to show a very low percentage of abnormal cells or none at all. Performing the FISH assay on the pre-treatment sample is important to be sure that the abnormality is detectable using the probe that was ordered by the physician and to be sure that there is no unusual variant pattern in the patient. Metaphase cells should be documented as well, if possible, in the original study to show the location of the probes in the rearrangement. This is especially important if the probe selection was based on the G-banded metaphase study, since, for example, a “deletion” can turn out to be a translocation. Some rearrangements may be cryptic, as well [36].

It is very important for interphase aneuploidy testing that the probes are also checked in metaphase to rule out a patient-specific variation in the FISH probe behavior. For example, some centromere specific probes can be divided between the short and long arms, and a translocation involving the centromeric area could result in an extra signal due to separation of the two sections. Also, some subtelomere specific probes can hybridize with chromosomes other than the normal target in people with no discernible phenotypic abnormalities. Sometimes, only interphase cells are available in a given specimen. If abnormal signal patterns are found, the report should recommend metaphase FISH to rule out a variant pattern. For putative subtle constitutional FISH rearrangements, such as subtelomeric specific rearrangements, parental metaphase FISH should always be performed to be sure the rearrangement is an unbalanced product and not an inherited variant. It is important to know the common variations possible for a given probe, such as the benign presence of DYZ1 (the probe on Yq that detects the variable region of the Yq) on autosomal chromosomes, notably the 15 and 22 p arms, in individuals of both sexes. In normal males the DYZ1 region can be variable in size, sometimes almost absent, and may cause a false diagnosis of loss of Y. These variants are common enough that DYZ1 should never be relied upon for interphase sexing (See Chapter 7, section 7.28, for Y chromosome Q-band variations, and Chapter 21, section 21.1.7, Process management). See Stupca [47] for additional control recommendations.

16.12 FISH for special investigation

16.12.1 Sequential staining methods combined with FISH

There are a number of stains that can be used in conjunction with FISH to elucidate the study. For example, if rare abnormal cells are seen by G-banding, it is often easier to destain and perform FISH on the G-banded slide to find the same abnormal cells again than it is to perform FISH on a new slide and find these rare cells. Before hybridization, the G-banded cell is captured, and the microscope coordinates are recorded. If the fluorescent microscope to be used for FISH has widely different stage coordinates, an England cell finder can be used to relocate the cells, or a “marker slide” can be made. To make a marker slide,

- place white tape on a blank slide (marker slide);
- locate the cell on the G-banded slide, and carefully removed from the stage;

- insert the marker slide on the stage, and using a fine-tipped marking pen, mark the location where the light passes through, using a closed-down condenser for a small, accurate mark.

When this marker slide is placed on the second microscope, the epi-illumination light beam is positioned at the mark, and the G-banded/FISH slide is then put on without moving the stage. (When finding the mark on the epifluorescence, use a filter that does not blind the viewer, such as DAPI.) This should put the cell of interest in the field of view on the second microscope.

Often it is useful to rehydrate G-banded slides after destaining them in 3 : 1 methanol–acetic acid. This is accomplished by running the slides from concentrated ethanol through less concentrated and finally in distilled water for a few minutes. This makes the slide easier to use for FISH because it makes the chromatin more available for hybridization.

The main consideration when performing FISH on previously G-banded cells is that they may be more sensitive to denaturation after trypsin treatments; therefore, the time for denaturation may need to be cut down to a quarter or half of the usual time. One trick to determine the denaturation time without sacrificing the slide is to take another slide from the same patient which was previously G-banded, run it through 72 °C formamide with a given denaturation time, but do not put any probe on it. Then counterstain it with DAPI, and check it on the fluorescence microscope. If the chromosome morphology is good and the p and q arms show good staining, the denaturation time was good. If only the pericentromeric regions are well stained with the DAPI, then denaturation time was too long and should be reduced.

Sequential Q-bands to FISH works well in many laboratories, because the cells have not been enzymatically digested by trypsin, and so destaining the slide with fixative is all that is needed to prepare the slide for FISH methods. It is not necessary in most cases to do DI-DAPI banding sequentially to FISH, since DAPI-positive staining areas can be seen on FISH slides that are counterstained with DAPI (Figure 16.34).

C-band pretreatments are harsh, and preclude subsequent FISH. C-banding should be undertaken after the FISH step. Often, staining denatured slides with Giemsa will yield C-bands without any further treatment. The first C-banding was discovered (and 2x SSC became a cytogenetics reagent) while trying to perform early in situ hybridization in the 1970s.

Another sequential stain is the use of an immunohistochemical (IHC) stain to detect plasma cells in a bone marrow specimen that will then be used for FISH to detect multiple myeloma [48]. This method is termed “T-FISH” for “targeted” FISH. If the plasma cells are rare in the marrow, they may be targeted with the IHC and only the positive cells scored for FISH signals. When this targeting is not done, the rarity of the cells can make the FISH abnormality appear to be below the cutoff value for abnormal results. One type of simultaneous detection of FISH and immunohistochemical stains is termed FICTION (Fluorescence immunophenotyping and Interphase Cytogenetics as a Tool for the Investigation of Neoplasms) [49].

Sometimes the pathologist will ask the cytogenetics laboratory to use the only slide available for FISH, which may be a slide previously stained with PAP or H&E. These stains may require harsh treatments to remove them, such as acid washes; however, FISH can be performed.

FISH can also be followed by a second FISH assay on the same slide (Figure 16.35). If the first probes are locus-specific type probes, it is not usually necessary to destain or remove probes from the slides before rehybridization. Simply remove the coverslip, clean with 2x SSC with NP-40, dehydrate, and rehybridize. If some of the first probes used are centromere enumeration type probes, the denaturation step will not remove them completely, and they must be destained in 72 °C hot water or 70% formamide for 1 or 2 minutes before dehydration. Up to three hybridizations are feasible on a single slide. Rehybridization is also useful for old, faded FISH slides that need refreshing for another look at the same probe. One interesting observation we have made in our laboratory is that signals can move quite a bit during re-hybridization within the nucleus, probably from the effects of the high heat. Metaphase chromosomes sometimes curl a little bit between hybridizations as well.

16.12.2 Rx-FISH

Rx-FISH, a color banding method, was developed during investigations into the evolution of the gibbons and siamangs by using human chromosome paints on the ape chromosomes and also by using gibbon-generated paints on human chromosomes. Because the genome is so complexly rearranged in the gibbon family, the paint for a single chromosome in a gibbon paints bands on several different human chromosomes. This finding was used to create a set of multicolored probes that paints the entire genome in a single assay resulting in a unique bar code-type pattern for each homologue. The current system uses combinatorial labeling to create seven colors in the banding pattern. This can be used to identify both inter- and intra-chromosomal rearrangements and markers (Figure 16.36). It requires software to analyze the patterns and combine the data to make the image. Currently this software is available from Genetix/Applied Imaging. Advantages of Rx-FISH over SKY and M-FISH are that intrachromosomal changes can be identified, the chromosomal region represented by a marker chromosome can be better pinpointed (e.g., 2pter rather than chromosome 2), and because the gibbons do not have the same repetitive sequences that humans do, no Cot-1® suppression is necessary. For a description of other types of multicolor FISH, see chapter 17.

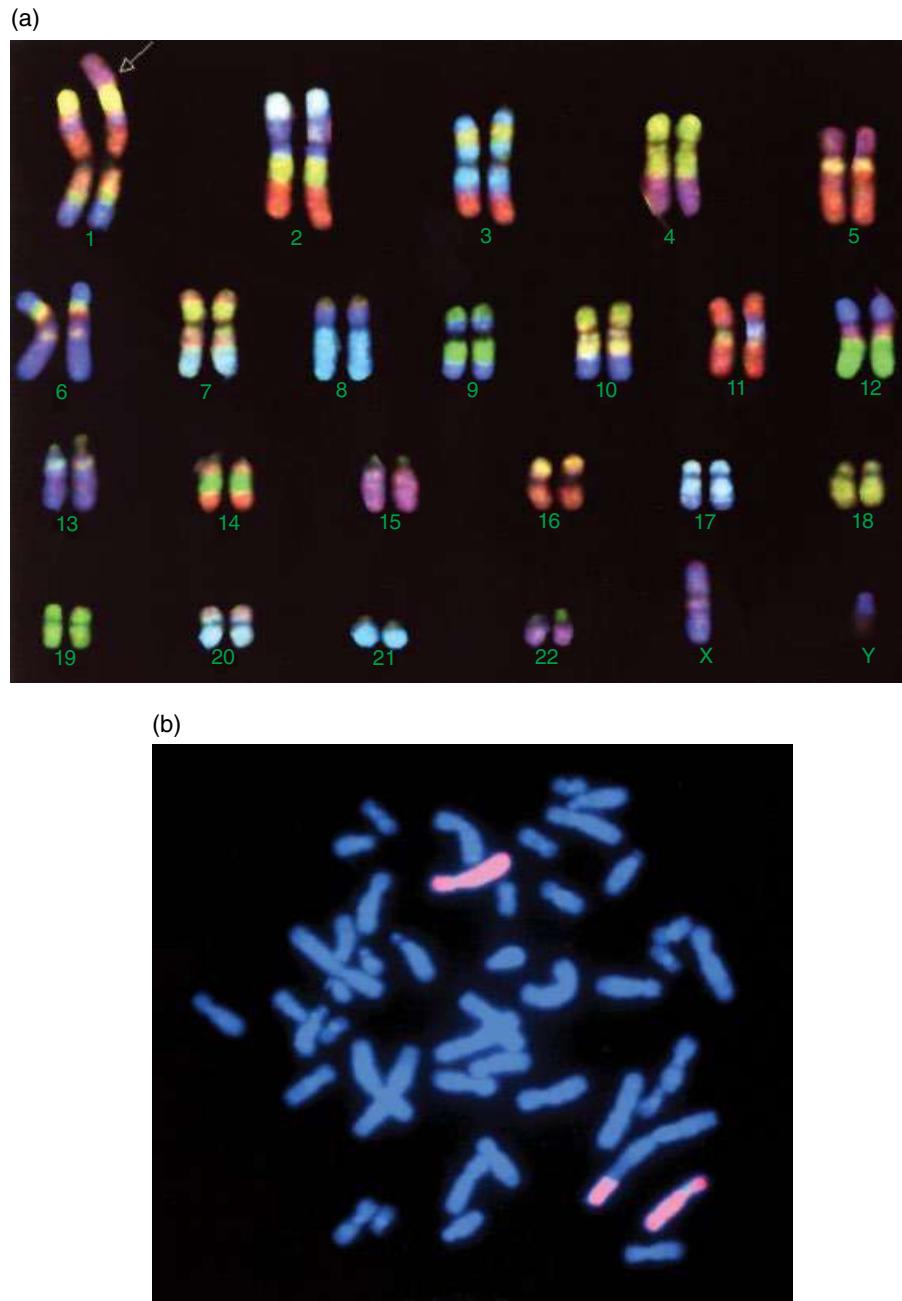


Figure 16.36 Rx FISH. (a) RxFISH performed to investigate additional material on chromosome 1p, showing it to be derived from chromosome 4q. (b) Chromosome 4 paint used to confirm the RxFISH findings. See insert for color representation of this figure.

16.12.3 Fiber FISH

Fiber FISH was developed [50] as a research tool for visualizing the sequence of probes along the chromatin with a precision not obtained on metaphases or even interphase nuclei. The nuclei are lysed, or the protein is extracted, releasing the DNA fibers in a halo around the cell. Alternatively, free DNA in a solution may be applied to a slide for FISH methods. It is used in research for the physical mapping of DNA, establishing contigs, mapping ESTs, and determining what gaps remain in the Human Genome Project, among other applications. It is also useful to study gene amplifications, duplications, deletions, insertions and translocations

in the clinical setting. Many protocols are available for chromatin fiber FISH [51]. The fibers are obtained by using chemical treatments to decondense the chromatin, lysing the nucleus with alkaline treatments or extensive hypotonic exposures, use of late G2 or early G1 nuclei, or cytocentrifugation to the point of physical stretching of the chromatin. Once the chromatin is opened up, the DNA fibers can be released using alkaline/detergent and physical stretching by use of a coverslip, protein extraction, releasing DNA from a gel block by mechanical or electronic forces, or by use of an air-water phase interaction. From this point, the FISH methods are standard. The slide is first exposed to RNase to clean the fibers for the FISH assay, and then slides may be either separately denatured or co-denatured, reannealed, and post-washed. The fibers are counterstained with DAPI for visualization. Computerized capture with sensitive video or CCD cameras is important for best results.

16.13 Preimplantation genetic FISH

FISH has been used to improve outcome in assisted reproductive technologies [52]. Preimplantation genetic diagnosis (PGD) is the general term used for any preimplantation genetic test, including FISH and PCR-based testing. PGD requires the use of in vitro fertilization (IVF) to obtain embryos for testing. The American Society for Reproductive Medicine (ASRM) guidelines [53] indicate that PGD is used when a genetic parent or parents carry a gene mutation or a balanced chromosome rearrangement. Testing is performed to determine whether the abnormal genetic finding has been transmitted to an embryo so that embryos may be implanted with a better chance of developing normally. Preimplantation genetic testing is sometimes termed preimplantation genetic screening (PGS) when used to refer to testing performed when the parents are not carrying any known genetic abnormalities.

There are two main indications for preimplantation FISH:

- Aneuploidy testing for maternal age, previous history of aneuploidy, multiple miscarriages and recurrent implantation failure.
- Translocation status when one parent is a carrier of a balanced translocation.

Aneuploidy screening is usually performed using a set of probes for the most common abnormalities seen at term (trisomies 13, 18, 21, and aneuploidy for X and Y), in spontaneous abortions (trisomies 15, 16, 21, 22) and in day 3 embryos (aneuploidy for 15, 16, 17, 21, 22). Various centers use different mixtures that may include probes for chromosomes 1, 7, 8, 13, 14, 15, 16, 17, 18, 20, 21, 22, X, and Y. These probes are usually used in series of three to five probes at a time with sequential rehybridizations of the same cell. Labeled peptide nucleic acid probes (PNA probes) may be used instead of DNA probe. These probes make sequential FISH easier because the temperatures may be lower and the cells are better preserved between hybridizations [54]. The use of probes for chromosomes X, Y, 13, 14, 15, 16, 18, 21, and 22 has been reported to potentially detect 70% of the aneuploidies found in spontaneous abortions [55]. However, the specific aneuploidies found in preimplantation embryos may differ from those seen in abortion material. Some laboratories re-examine cells with subtelomeric-specific probe sets to verify questionable results. CGH has also been used for PGS to screen all of the chromosomes [56] and a method called cenM-FISH has also been used to screen for all 23 aneuploidies at once [57]. Screening for translocation status requires the testing of probes on parental cells to work out a set of probes which will detect all of the permutations of nondisjunction (Table 16.5; Figure 16.37). For many couples with balanced translocations or inversions, preimplantation testing is much preferable to amniocentesis and selective termination. Different studies indicate [53, 58] that preimplantation FISH testing increases the implantation rate and lowers the subsequent spontaneous miscarriage rate. However, there are still some questions about the efficacy of PGD [59]. PGS may also be performed for sex selection, termed “family balancing,” an indication that is being debated regarding its ethical acceptability.

Usually, the cytogenetics laboratory receives cells on a slide from an embryologist, and FISH is performed on the prepared cells. However, it is advantageous to understand the procedure from the beginning. Several factors contribute to a successful FISH assay and viable implantation: the IVF laboratory conditions – media, incubator, and blastocyst growth; the skill of the embryologist – the technique used to extract the blastomeres can be damaging to the embryo if done improperly; fixation of the blastomere, which is crucial to the success of the FISH analysis; the room humidity, fixation concentration, hypotonic solution and timing of these components, which affect how well the cell will adhere to the slide and whether cytoplasm is removed properly; and the successful implantation of the selected embryo(s).

16.13.1 Obtaining samples for PGD FISH

IVF patients first undergo controlled ovarian stimulation using gonadotrophin-releasing hormone (hMG), follicle stimulating hormone (FSH) or some other regimen. Human chorionic gonadotropin (hCG) is then administered when three or more healthy follicles can be seen on transvaginal ultrasound. Transvaginal ultrasound-guided oocyte retrieval is performed 36 hours

Table 16.5 Segregation products of a 3;13 translocation using two telomere probes and a centromere probe

Disjunction (segregation)	Chromosomes		Interphase signals		
	Paternal	Maternal	3qter (red)	3cen (yellow)	13qter (green)
2:2 alternate – normal	3, 13	3, 13	2	2	2*
2:2 alternate – balanced	der(3), der(13)	3, 13	2	2	2*
2:2 adjacent-1	3, der(13)	3, 13	3	2	1
	der(3), 13	3, 13	1	2	3
2:2 adjacent-2	3, der(3)	3, 13	2	1	2
	13, der(13)	3, 13	2	3	2
3:1	3, der(3), 13	3, 13	2	2	3
	3, 13, der(13)	3, 13	3	3	2
	der(3)	3, 13	1	1	2
	der(13)	3, 13	2	2	1
	3, der(3), der(13)	3, 13	3	2	2
	der(3), 13, der(13)	3, 13	2	3	3

* Denotes balanced segregation products: note that the balanced carrier state is indistinguishable from the normal noncarrier state.

Bolded numbers highlight the unbalanced signal patterns.

This probe set is sufficient to detect all of the unbalanced products of segregation using just the three probes, according to this table.

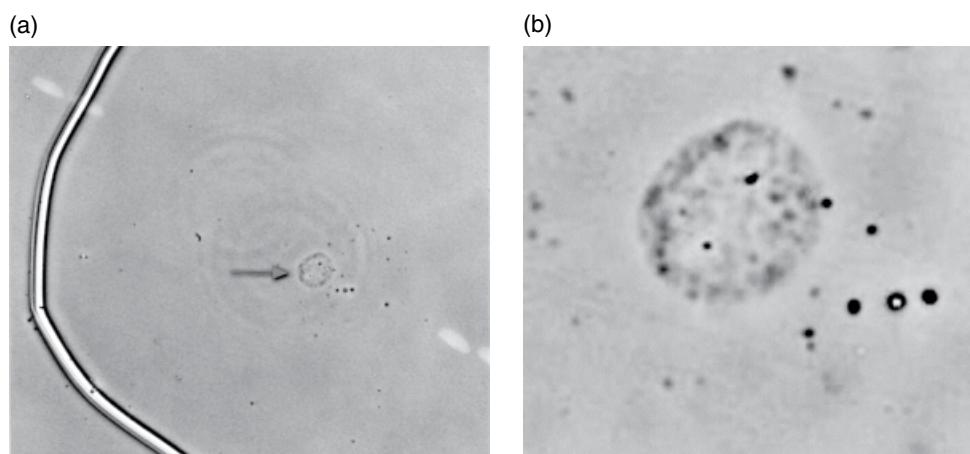


Figure 16.37 Single blastomere nucleus from a 3-day embryo. (a) Low power image to show the diamond pen circle the embryologist marked on the slide to indicate the location of the cell (arrow). (b) High power image to show the same nucleus. Typically, no pretreatment is necessary on these preparations, and since there is a need for quick results, this helps to save time.

after hCG administration, and oocytes are fertilized in Petri dishes after first removing the cumulus cells. Sometimes, the sperm is injected directly into the egg (intracytoplasmic sperm injection, or ICSI), if motility is low or for other reasons. The biopsy may be performed at all preimplantation stages, with three that are most common:

- Polar bodies may be removed from the unfertilized and fertilized oocytes.
- The three-day cleavage-stage embryo may have one or two cells (blastomeres) removed.
- Blastocysts may be biopsied for trophectoderm cells (most often used for PCR).

The biopsy is usually performed by use of suction through a drawn-out glass pipette. In some countries, legislation requires that only polar bodies are used. However, this can lead to errors since it only describes the maternal contribution to the

embryo. Cleavage-stage biopsies are performed on the third day after fertilization when the embryo is at a six- to eight-cell stage. Both parents' genetic contributions are present in the embryo at this point. The zona pellucida is disrupted and one or two blastomeres are aspirated and placed on a slide. A large proportion of cells from human embryos at this stage can be removed without apparent damage to the embryo. The cells are placed in a microdrop of hypotonic solution, placed on a slide and flooded with fixative, and then sent to the cytogenetics laboratory to be used for FISH. The embryos will be cultured until day 4 or 5 post fertilization, so the FISH testing must be completed within that period of time, usually 48 hours from biopsy. Embryos usually progress to the blastocyst stage on day 5, so if the FISH shows multiple embryos available for transfer, the physician may choose to wait until day 5 to make sure the chosen one or two embryos have shown that they can progress. Not all normal embryos develop to the blastocyst stage, with about one third arresting at day 4. Extra embryos that blastulate may be frozen on day 5 or 6 for future use. The patient's serum hCG is tested at day 12 to establish whether the pregnancy is ongoing, and if so, an ultrasound is performed at 7 weeks to confirm a fetal heartbeat. Although couples are often counseled to undergo prenatal diagnosis to confirm the diagnosis, the increased risk of pregnancy loss often discourages the patients from doing so.

16.13.2 PGD technical issues

PGD FISH relies on the study of only one or two cells. The small sampling can lead to several problems. Finding the cell(s) can be very difficult, especially since the assays often involve multiple probe colors, including blue, that may preclude use of a counterstain. Our method calls for finding the cells on a phase microscope and making a "marker" slide to line up with the light beam on the FISH microscope. It is also possible to find the plane of focus under high power by looking for the fluorescent debris, proteins, and other materials present on the slide using a dual or triple band pass filter.

Normally, interphase FISH studies require scoring tens or hundreds of nuclei to insure statistical reliability. In PGD, the small cell sampling is not optimal for good quality control and can lead to error due to misinterpretation of the signals in a single nucleus, with no confirmation possible on other cells. For example, a replicating signal may be scored as two signals, or a small signal may be missed or interpreted as background fluorescence.

Another issue is that embryos are frequently "chaotic," meaning that the cells have undergone multiple nondisjunctions, and each cell has a different abnormality. This means that looking at one embryonic cell does not insure that all other cells from that embryo are genetically identical to it. The future fetus is represented by only one of the cells in the 3-day embryo, and the extra-embryonic cells can tolerate some chromosomal aneuploidy when the fetus is chromosomally normal.

In addition, only parts of certain chromosomes have been included in the FISH assay (e.g., chromosome 18 is detected using the centromere alpha satellite probe so trisomy 18, by virtue of an iso(18q), would be missed), and other aneuploidies and rearrangements cannot be excluded. Therefore, FISH testing of embryos is at best an inexact science. It may yield false-negative (normal FISH with an abnormal embryo) or false-positive (abnormal FISH and a normal embryo) results, leading to the rejection of a normal embryo. All PGD reports should include disclaimers to this effect.

16.13.3 Designing translocation probe sets for balanced carrier patients

Most translocations are amenable to interphase FISH analysis to distinguish unbalanced embryos from normal/balanced embryos. There is currently no feasible method to distinguish between normal (no translocation present) and balanced translocation embryos. The design depends upon the type of translocation. For Robertsonian translocations, it is sufficient to make a two color subtelomeric specific probe set to distinguish all of the unbalanced segregation permutations, because there are only two chromosome arms that are involved. However, for translocations involving two nonacrocentric chromosomes or for both an acrocentric and a nonacrocentric chromosome, a third probe color is required. Usually, two of the probes needed for the latter case will be from regions distal to the translocation breakpoints on each chromosome and one probe will be a centromeric alpha satellite probe from either of the two involved chromosomes. Even though the centromere of the second involved chromosome is not tested for, all of the unbalanced embryos will show an abnormal probe signal pattern with just the three probes. Table 16.5 shows the segregation products from a translocation and the signal patterns that would manifest from this type of probe design.

16.13.4 Preimplantation FISH abnormalities

Bielanska [60] showed that out of 216 embryos studied with interphase FISH, approximately 30% of them were normal for the 11 probes used, 22% were abnormal, and the rest were chaotic. In addition, 48% were mosaic. Chaotic embryos may be caused by centrosome abnormalities leading to abnormal spindles and an inability of the cells to segregate the

chromosomes properly [61]. Since the centrosome for the embryo is responsible for proper chromosome segregation, centrosome instability could account for the fragmented nuclei that are common in blastomeres because micronuclei are a manifestation of anaphase lag, a phenomenon often caused by spindle malfunction. Monosomies are a very common abnormality found in PGD for FISH [62].

When two cells are biopsied from a single embryo, they are often discordant [63], with only 25% of 102 pairs being the same for all eight probes used. This is further evidence for the unreliability of testing only one cell.

Patients referred for translocation status have even higher abnormality rates than those sent for maternal age, with 70–80% of embryos being unbalanced [64]. This phenomenon is the manifestation of a mechanism called “meiotic drive,” which causes certain combinations of genes to be over-represented compared to expected Mendelian segregation. Sampson et al. [65] report results of translocation PGD from a series of 56 embryos from six patients: one embryo was balanced (1.7%), 43 embryos were unbalanced (76%), eight were aneuploid, and four were uninformative.

16.13.5 Technical issues for preimplantation FISH

Just as slide-making is critical to good chromosome preparations, it is critical to FISH preparations, as well. The cytogenetic technologist often has no control over the slide-making methods used by the embryologist. The cells must be allowed to flatten out for a few seconds before the fixative is dropped onto the slide. If the fixative is added too soon, the nucleus may be compact to the degree that probe signals will overlap, causing difficulty in scoring cells. Another problem is that preimplantation nuclei commonly form micronuclei, representing perhaps a single chromosome that was not included in the nuclear envelope during the previous division. When fixative is dropped onto the slide, these micronuclei are often lost, leading to false monosity.

The use of detergents during cell preparation may interfere with the FISH assay, and is best avoided.

Some embryologists like to use positively charged slides for preparing the biopsy FISH slide. The cells do not spread as well on these slides, and the nuclei can be very condensed and difficult to score. It is best to use a good cytogenetic type slide for preimplantation FISH studies.

It is important to have one slide for each embryo to avoid mix-ups. The slide should be labeled with the patient name, embryo designation, and one other identifier.

During addition of probes, it is important to avoid touching the slide with the micropipette, as it will often scratch the cell. When the coverslip is added to the slide with the probe on the cell, it is critical to avoid even tiny bubbles that can interfere with binding of probes. When post-washing slides, it is important not to allow a slide to touch another nearby slide in the Coplin jar, as cells may be scraped off at this stage, as well.

Validation of probes is more difficult than with other types of FISH because of the relative unavailability of blastomeres. Therefore, initial validation studies may be performed on chorionic villus, products of conception, and/or blood samples. Additional validation may be performed on donated blastomeres from discarded embryos. For translocation probes, it is very important to validate the translocation probe set on blood chromosomes from both partners in the couple (not just the carrier), because often subtelomere or alpha satellite probes are involved, and these may show variation in the population, showing duplications and deletions or unusual patterns. Even centromeric probes can show aberrant patterns in normal individuals [19, 20, 66]. Then, after the parental probe validations, the same probes should ideally be tested on donated blastomeres to be sure they perform well.

16.13.6 PGD FISH methods

Upon receipt in the cytogenetics laboratory, after the specimen has been logged in, each slide is marked with the accession number. The embryologist should already have labeled each slide with the embryo number and how many cells are present, as well as the patient name and other identifier, such as birth date. The embryologist also will have marked the area of the slide where the cells were deposited, usually using a diamond pencil (Figure 16.37). If the probe set(s) to be used does not allow for a counterstain, as with the Vysis PGT set for 13, 18, 21, X, and Y, it is critical to look at each cell on the phase microscope to see what it looks like. It is good practice to note the size, shape, number of nuclei seen (this may vary from the number the embryologist saw), whether fragmented nuclei are present, density, and the presence of a cytoplasmic background. A marker slide can be made for each blastomere, or an England cell finder may be used to find the cells on the FISH microscope.

If the cell appears to be covered with cytoplasm, flooding it with 3:1 methanol: acetic acid may improve it enough for good FISH signals. Pepsin treatment, as performed for uncultured amniocytes, can also be used if cytoplasm is very thick, but sometimes it can remove the cell from the slide.

Slides are usually used immediately, without any aging or heating. The probe mixture (usually 3 µL) is added to the marked region, and the coverslip (usually 12-mm circle) is carefully added. The coverslip is sealed using either rubber cement or Parafilm, although Gene-frames (a coverslip/sealer combination) are popular in some parts of Europe. Probes are usually

codenatured at 68–73°C for 3–5 minutes, and reannealed at 37°C for 1–18 hours. Some laboratories have worked out a microwave method for denaturing and/or reannealing probes (often using standard methods for the first round of probes and then using the microwave method for the subsequent rounds, although some reports are also using the microwave for all rounds [67] that shortens the times to as little as one minute for denaturation followed by 5 minutes for reannealing [68–71]. This method works best with centromeric alpha satellite probes but may be used for other, locus-specific probes, as well [68]. After reannealing, the coverslip is gently removed, and the excess probe is washed off in SSC salt solutions (0.4× SSC to 0.7× SSC) at 71–73°C. Then slides are briefly cooled in another SSC salt solution at room temperature, and slides are mounted with just antifade for the five color probes, or DAPI for three or four color probe mixtures. After each slide has been scored and images captured, the slides may be destained at 72°C in distilled water, 70% formamide in 2× SSC, or 0.4× SSC with NP-40 for a few (~10) seconds to 2 minutes. Slides can then be dehydrated and dried, and re-hybridized with another set of probes in a subsequent round (or more) of FISH, so that the maximum number of chromosomes can be assayed.

16.13.7 Scoring PGD samples

The slide is first placed on the microscope with the ground-glass label under the low power lens to make sure that the microscope is in the plane of focus of the cell. Then the slide is moved to the region that was marked by the embryologist, where the cell has been deposited, or in the light path which has been lined up with a mark on a “marker” slide. A high-power oil-immersion lens is used to find the nucleus. A 60× lens is easier to use, with a wider field of view, but a 100× lens will do. Usually, a dual band pass lens is a good choice for searching for the cell if it is not counterstained. When the cell is encountered, it should be scored by eye first to determine how many of each signal is present; then the cell can be captured. If the captured signals disagree with the original count, the cell should be re-examined on the microscope to be sure how many signals are present and re-captured, if necessary.

One problem encountered with some blastomeres is that there are autofluorescent spots present which are clearly not probe signals because they fluoresce with all or most of the filter sets used. These spots may represent pools of RNA, and are not usually a problem when using formalin-fixed blastomere cells.

Small doublets separated by less than a probe width can be scored as single, split signals representing replicated loci. On the other hand, when two true signals appear very close, they may be interpreted as a single split signal instead. This may be the largest source of error in preimplantation FISH testing.

When two or more cells are available on a single embryo, they must both/all be normal to score the embryo as normal.

The most important consideration about scoring a single cell is to be conservative, and if there is any doubt about the signal count, to err on the side of caution. Some laboratories have a second observer to confirm the findings of the first person. If this approach yields no usable embryos, some of the marginal cells may be re-tested using the same or other probes to verify the ambiguous probe result. Some laboratory protocols call for routinely re-hybridizing all monosomies to rule out poor hybridization in the first round.

16.13.8 Sperm FISH

Infertility clinics often wish to perform interphase FISH on sperm nuclei to study the reasons for male infertility (e.g., there may be a high percentage of aneuploidy present in the sperm), to assess the cytogenetic quality (e.g., post-cancer therapy) of sperm, or to determine the percentage of unbalanced gametes in male balanced translocation carriers (Figure 16.38). Slides may be made from fresh or DMSO-frozen samples, and fixation is optional. The preparation of sperm cells is quite different from that of other cell types. The nuclei of sperm are so condensed that their interphase cells would be unscorable without some pretreatment. After the sperm are washed several times in Tris buffer, their nuclei are decondensed using dithiothreitol (DTT) and lithium diiodosalicylate (LIS). Both the decondensation and FISH methods may be accomplished in a microwave oven [71], with the advantage that the method works better and less volume of toxic chemicals are needed. One method involves denaturation using NaOH instead of formamide, as it maintains nuclear integrity [35]. Generally, several thousand cells are scored, because the abnormality rate is usually low and requires large data sets for meaningful interpretations.

16.13.9 PRINS

Primed *in situ* labeling (PRINS) is an alternative to conventional FISH that is cheap and has low background fluorescence. The denatured target is hybridized to short designed oligonucleotides using polymerase in the presence of labeled nucleotides. It is essentially an *in situ* polymerase chain reaction or PCR that is modified to produce a FISH signal on a cell. The disadvantage of PRINS is that only certain highly repeated probe targets work well unless numerous cycles are performed.

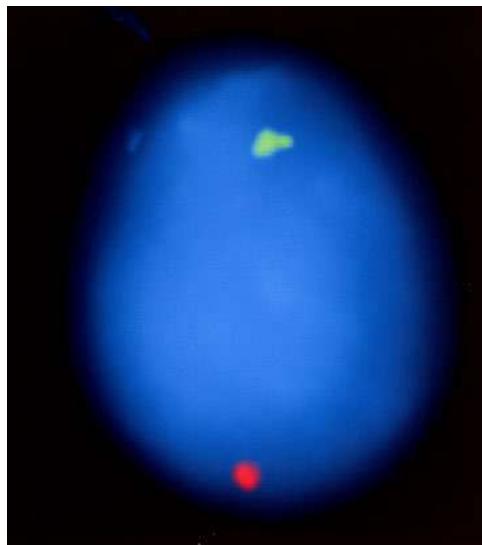


Figure 16.38 Sperm FISH. Sperm nucleus hybridized with probes for 13 (green) and 21 (red). See insert for color representation of this figure.

16.14 Other applications

16.14.1 Array CGH confirmation FISH

Usually this is done using BACs or specific commercial probes from the region of interest. BACs may be better for confirming small deletions because they can be tailored to the size of the deletion, while commercial probes may be manufactured large enough to be as bright as possible. Constitutional abnormalities are usually studied in metaphase cells, since fewer cells need to be analyzed in most cases; however, for certain types of abnormalities, such as microduplications, interphase cells may show the abnormality much better than metaphases, because the chromatin is extended and the duplicated signals will be more discrete. For example, Prader–Willi region deletions of 15q11–13 are well known to cause the obesity seen in this condition, but there can sometimes be a duplication of this region in patients with certain kinds of autism (Figure 16.12). The chromosome regions involved in the mismatch-repair-caused constitutional deletion syndromes (e.g., DiGeorge/ Velocardiofacial syndrome) may also undergo mismatch-repair duplications causing other syndromes for which the probes are also useful [72].

For FISH analysis of a microarray-detected deletion, 10 metaphase cells should be enough to document whether it is present. For duplications seen on microarray tests it is often more subtle than an extra signal inserted into another chromosome, so it may require an interphase study with 50 or more nuclei checked for the duplication. The duplication should be apparent in the majority of interphase nuclei before reporting. Further testing, such as fiber FISH or PCR, may be necessary to detect the duplication if the FISH is equivocal. Parental FISH studies should be done in the case of an abnormal finding to see if a parent has the same abnormality. The laboratory that does the parental FISH study should be the same laboratory that did the original study for consistent scoring and proper control of hybridization variables.

16.14.2 FISH for bladder cancer

Specimens received for bladder cancer include urine, cytospin slides, thin-preps, and bladder washings from persons with hematuria, who are suspected of having bladder cancer. FISH probes on these urine specimens are designed to detect aneuploidy for chromosomes 3, 7, 17, and loss of the 9p21 locus. Nuclei are scanned for abnormal morphology (tumor) before scoring signal patterns. However, if no abnormal nuclei are present, normal cells are scored. Problems with urine specimens can include a flocculent pellet caused by the lubricant used when catheterizing the patient. The physician can be contacted to use less of the lubricant, and the pellet can be washed in fixative several times until clean before use.

16.15 Variants in FISH signal patterns

Chromosome variation is manifested not only in G- and Q-band staining, but in all types of FISH probes. It is important to understand how variants can cause misleading results on FISH tests, especially in interphase cells. Some examples of common variations in FISH signal patterns are described here.

16.15.1 Alpha satellite DNA probes

Blocks of alpha satellite DNA, usually found near the chromosome centromere, are quite variable in size, position, and number of sub-blocks, and may even be missing or located in an unusual locus. Very small alpha satellite DNA blocks, therefore, may be difficult to visualize and can lead to a misdiagnosis of monosomy. For example, very small pericentromeric blocks of alpha satellite DNA in the X chromosome may lead to the misdiagnosis of Turner syndrome, if only interphase nuclei are being studied, but metaphase cells will clarify the presence of the second X [19]. Chromosome 12 alpha satellite probes, used for interphase FISH on CLL samples to look for trisomy 12, may yield incorrect results due to a very small block of alpha satellite DNA on one homologue, so that the second homologue, or even the third copy of chromosome 12 can be missed in the interphase study. Alpha satellite DNA may even, although rarely, be totally missing from a chromosome. Marker chromosomes and neocentromeres, for example, are commonly missing an alpha satellite DNA component. Very large blocks of alpha satellite DNA may also be misinterpreted as trisomies due to segmentation, especially in interphase cells. For example, the pericentromeric alpha satellite probe signal on chromosome 18 may be large and subdivided into two sections, and may appear as two separate signals in interphase. In a similar way, translocations that occur within the actual centromere region can transpose part of each alpha satellite section to the partner chromosome, so that there will be three alpha satellite DNA probe signals in interphase nuclei of the normal, balanced cell for each of its reciprocal partners [20]. Examining the positions of these additional alpha satellite signals in metaphase cells with FISH would be essential for interpretation in these situations.

16.15.2 Subtelomere-specific probes

These probes, immediately adjacent to the telomeres of most of the chromosome arms, are subject to cross-hybridization with other chromosomes, as well as deletions in normal populations. If an abnormality is found in a proband being tested due to phenotypic abnormalities, the parents should be studied to be sure the aberration(s) are de novo in the patient. If these probes are to be used for purely interphase testing, such as for preimplantation genetic analysis of translocation status, the probes must be validated in both parents before using them on cells from embryos.

16.15.3 Locus-specific probes

These probes are used for detecting deletions, duplications, and chromosomal rearrangements in metaphase and interphase. The most common variation in locus-specific probes is found in probes designed to detect translocations, especially for those used in interphase studies. Dual-fusion probes may show variant signal patterns depending on the presence of extra derivative chromosomes, the involvement of a third chromosome partner in the translocation, or deletions at the breakpoints (see Figure 16.31). There may also be insertions of one locus onto another instead of a classic translocation. Break-apart probes may exhibit a deletion of part of the probe signal concurrent with the rearrangement; for example, the *MLL* break-apart probe may have the telomeric 3' region lost during the *MLL* rearrangement, and the abnormal pattern will be zero red, one green, and one fusion signal. This pattern is still compatible with a rearrangement, but should be verified in a metaphase cell from the FISH preparation, when possible.

16.16 Conclusion

In conclusion, cytogeneticists should keep in mind that any scientific endeavor is subject to variation, and take care when interpreting a new or unexpected finding, especially when dealing with interphase nuclei. The most important factor is to understand the entire process. Then troubleshooting and interpreting, while it is still a challenge, is possible. With the power of FISH it has become and remains an essential, critical tool of the cytogenetics laboratory for prognosis, diagnosing and monitoring various genetic syndromes and diseases that would otherwise be misunderstood or undetected.

Acknowledgments

Our deepest thanks to the following people who made comments, additions, and changes to this chapter: Charles Dana Bangs, Martin Lowrie, Michelle Lenzi, Marilyn Arsham, Thomas S. Wan, Stephen Moore, Susan Olson, Betty Jackle, Octavian Tavi, and Amy Hanlon-Newell. A special thanks also to Gret Boyd of BoyDog Design (gret@boydogdesign.com) for the probe design cartoons in this chapter. And endless thanks for help formatting all those contributed methods to Turid Knutsen.

Glossary

ALL: acute lymphoblastic leukemia.

AML: acute myelogenous leukemia.

AMMoL: acute myelomonocytic leukemia.

BAC: bacterial artificial chromosome, used to manufacture DNA segments 100–300 kb.

Base: molecule that accepts a proton in solution – often referring to purines and pyrimidines in DNA and RNA.

bp: base pair – two bases, one in each of the two strands of DNA that are complementary to each other, such as A and T, or G and C.

CEL: chronic eosinophilic leukemia.

CentiMorgan: a unit of measure of genetic recombination frequency. One cM is equal to a 1% chance that two markers will be separated from each other due to crossing over in a single generation. Named after Nobel Prize winning geneticist Thomas Hunt Morgan.

CGH: comparative genomic hybridization.

CLL: chronic lymphocytic leukemia.

CML: chronic myelogenous leukemia.

COBRA FISH: multicolor *combined binary ratio* fluorescent *in situ* hybridization.

DAPI: 4',6-diamidino-2-phenylindole, used as a counterstain for FISH in order to be able to see the nucleus/chromosomes behind the probe signals. Appears blue in color using the proper filter set on a fluorescent microscope. Best used with probes labeled with red and green fluorophores.

Denaturation: the separation of a double-stranded nucleic acid or DNA into two single strands. This can be caused by alkaline pH or heat treatment. The hydrogen bonds are broken between the strands during denaturation, and will realign into double strands when cooled or restored to neutral pH conditions during reannealing.

Digoxigenin (DIG): hapten used to detect probes (indirect label).

Disassociation: denaturation of DNA is sometimes called disassociation of the DNA strands.

DOP: Degenerate (having multiple combinations of nucleotides that code for the same amino acid) oligonucleotide-primed PCR reaction.

EST: an expressed sequence tag is a cDNA made from a small part of the active sequence of a gene. It can be used to locate the gene using radioactive or fluorescence detection.

FFPE tissue: formalin-fixed paraffin-embedded tissues in blocks of paraffin or in thin sections on a slide.

FICION: fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms.

FISH: fluorescence *in situ* hybridization. Use of fluorescently labeled DNA or RNA probes to detect sequences in metaphase or interphase cells as well as in chromatin fibers and DNA on substrates such as microarray chips.

FITC: fluorescein isothiocyanate, a commonly used fluorophore in FISH that appears green when viewed through FITC band pass filters.

Fluor: a biological dye with fluorescent emissions upon exposure to UV light.

Fluorescence: a luminescence in which the molecular absorption of a photon triggers the emission of another photon with a longer wavelength. Usually, the absorbed photon is in the ultraviolet (UV) range and the emitted light is in the visible range. Thus, exposure to a UV lamp will cause light to be emitted by a fluorophore that is visible to the human eye and can be distinguished as different colors using different dyes or fluorophores. Many natural substances will emit fluorescence, such as the marks from a china marker crayon, the mineral fluorite (calcium fluoride, from which the word fluorescence obtains), and many proteins.

Fusion: close juxtaposition of two FISH probe signals in a nucleus denoting a rearrangement in a translocation probe, no rearrangement in a break-apart probe, or false-positive finding with two overlapping chromosomes (juxtaposition artifact).

Hapten: a small molecule that reacts to a specific antibody, sometimes called an incomplete or partial antigen. Used for indirect detection of FISH probes, as with digoxigenin and anti-digoxigenin antibodies with a fluorescent dye, or biotin with avidin with a fluorescent dye attached.

HES: hypereosinophilia.

Hybridize: the reannealing of two strands of denatured DNA to form a double stranded DNA.

In situ: in the original position; performed in place without dissection or dissociation of cells or tissues. In situ harvesting involves the growth, harvest and analysis of colonies of cells on a coverslip or slide. In situ hybridization involves hybridizing probes to DNA from cells on slides without removing the DNA from the cells or the cells from the slide.

kb: kilobase, or 1000 base pairs – sometimes written as kbp for *kilobase pairs*. For RNA, it refers to 1000 nitrogenous bases, since they are not paired.

Locus-specific probe: commercially available probes for specific loci are designed for the detection of many different types of chromosome rearrangement such as deletions, duplications, inversions, and translocations. Probe designs include the locus-specific probe with an identifier or control probe, break-apart probes for inversions and translocations that occur with multiple partners, and fusion probes for specific translocations.

-mer: refers to the number of nucleotide bases in an oligonucleotide—for example, a fragment of 25 bases would be called a 25-mer.

M-FISH: multicolor or multiplex FISH, a FISH method that uses combinations of paint probes to assign each chromosome pair of a karyogram a different color, and is used to identify interchromosomal exchanges and extra material. Does not identify intrachromosomal changes. Similar to SKY in appearance. Using the capital M in M-FISH refers to the specific software/probe combination while lower case m-FISH may refer to the general method of using multiple-colored probes to obtain different colors and patterns on the genome.

Mb: megabase, or 1 million nucleotides, roughly equal to 1 centiMorgan (cM).

MDS: myelodysplastic syndrome.

MPD: myeloproliferative disease.

Multiplex FISH: the use of several fluorescence dyes to identify each target in a complex assay, as in M-FISH and SKY.

Nucleic acid: complex compounds made up of purine and pyrimidine bases, carbohydrates, and phosphoric acid that make up DNA and RNA and are responsible for cellular functions and the genetic code.

Oligonucleotide: a short (oligo = Greek for “few”) chain of up to 20 nucleotides. Automated sequencers allow the synthesis of oligonucleotides up to 160-200 bases. Often used as probes because they bind well and can be synthesized to order.

PAC: *P*1 artificial chromosome, used to manufacture DNA segments up to about 20 kb.

Paint probe: library of overlapping sequences designed to cover a whole chromosome or arm with signal. Paints for each human chromosome and most arms are available commercially.

PCR: polymerase chain reaction is used to amplify (copy) regions of DNA by multiple rounds of polymerization, each followed by a brief heat treatment to separate the two strands.

PI: propidium iodide – used as a counterstain for FISH in order to be able to see the nucleus/chromosomes behind the probe signals. Appears red in color using the proper filter set on a fluorescence microscope. Best used with probes labeled with other colors than red.

PNA: peptide nucleic acid – an artificially constructed nucleic acid analog that may be used as a probe in which the pentose phosphate backbone of the nucleic acid is replaced by polyaminoethyl glycine.

PNET: primitive neuroectodermal tumor.

Probe: defined fragment of DNA, used to identify the presence of that sequence in unknown target sequences such as chromatin or DNA. For FISH, the probe is labeled with a chemical tag or fluorescent molecule allowing its detection visually on a fluorescence microscope.

Purine: a type of base found in nucleotides including adenine and guanine. ATP, uric acid, and caffeine are also purines.

Pyrimidine: a type of base found in nucleotides including thymine and cytosine, as well as uracil in RNA.

Reanneal: verb meaning to re-form a double-stranded DNA from two denatured, single-stranded DNA molecules. Also called reassociation, renaturation.

Reassociation: see reanneal.

Renaturation: see reanneal.

Satellite probes: tandem repeat DNA probes found primarily around the centromeres of the chromosomes and on the distal Y long arm.

SKY: spectral karyotyping – a FISH method that uses combinations of paint probes and an interferometer to capture, analyze, and assign each chromosome pair of a karyogram a different color. It is used to identify interchromosomal exchanges and extra material. Does not identify intrachromosomal changes. Similar to M-FISH in appearance.

SSC solution: a sodium citrate and sodium chloride salt solution that is used in various concentrations to control the stringency of the FISH method. The lower the concentration of SSC, the higher the stringency. 2 \times SSC has a fairly neutral stringency, and is often used in rinses as a substitute for normal saline. 0.4–0.5 \times SSC is of a moderate stringency, and is often used as a post-hybridization wash to remove unbound and weakly bound probe from the target. Detergents such as NP-40 (noniodet), SDS, and Triton-X are often added to the SSC to help remove sticky probe and background debris from the slides.

Subtelomeric specific probe: probes for sequences 70–300 kb long that are just proximal to the telomeres and are specific for each chromosome arm. These probes are all commercially available (except for the short arms of the acrocentric chromosomes).

Telomere probe: repeats for a (TTAGGG) DNA base sequence are present on the telomeres of all chromosomes with the number of repeats varying from 400–1000. There is a critical number of these repeats necessary for chromosome stability. Commercial probes are available but they are primarily for research use.

UPD: uniparental disomy, or the inheritance of both homologues from only one parent. Seems to have a phenotypic effect only if the involved chromosome contains methylated genes, such as with Prader–Willi/Angelman genes on chromosome 15 (see Chapter 10, Genomic imprinting).

VNTRs: variable number tandem repeats, used for DNA fingerprinting due to the highly variable number of repeats. They correspond to “minisatellite” DNA.

Wavelength: the distance between the crests of a wave. Measured in nanometers. Visible wavelengths range from approximately 400–750 nm.

YAC: yeast artificial chromosome, used to manufacture DNA segments of 100–2000 kb.

References

- Levsky JM, Singer R. Fluorescence in situ hybridization: past, present and future. *J Cell Science* 2003;116:2833–2838.
- Gall JG, Pardue ML. Molecular hybridization of radioactive DNA to the DNA of cytological preparations. *Proc Natl Acad Sci USA* 1969;64:600–604.
- Harper ME, Ullrich A, Saunders GF. Localization of the human insulin gene to the distal end of the short arm of chromosome 11. *Proc Natl Acad Sci USA* 1981;78:4458–4460.
- Bauman JG, Wiegant J, Borst P, van Duijn P. A new method for fluorescence microscopical localization of specific DNA sequences by in situ hybridization of fluorochrome-labeled RNA. *Exp Cell Res* 1980;128:485–490.
- Pinkel D, Landegent J, Collins C, Fuscoe J, Segraves R, Lucas J, Gray J. Fluorescence in situ hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. *Proc Natl Acad Sci USA* 1988;85(23):9138–9142.
- Donlon TA. Practical approaches to in situ hybridization. *Karyogram* 1986;12(1):3–10.
- Landegent JE, Jansen in de Wal N, Dirks RW, Baao F, van der Ploeg M. Use of whole cosmid cloned genomic sequences for chromosomal localization by non-radioactive in situ hybridization. *Hum Genet* 1987;77:366–370.
- Hopman AH, Wiegant J, Raap AK, Landegent JE, van der Ploeg M, van Duijn P. Bi-color detection of two target DNAs by non-radioactive in situ hybridization. *Histochemistry* 1986;85:1–4.
- Manuelidis L, Langer-Safer P, Ward D. High-resolution mapping of satellite DNA using biotin-labeled DNA probes. *J. Cell Biol* 1982;95:619–625.
- Fauth C, Speicher MR. Classifying by colors: FISH-based genome analysis. *Cytogenet Cell Genet* 2001;93:1–10.
- Tanke HJ, Wiegant J, van Gijlswijk RP, Bezooikove V, Pattenier H, Heetebij RJ, Talman EG, Raap AK, Vrolijk J. New strategy for multi-colour fluorescence in situ hybridization: COBRA: COnbined Binary RAstro labelling. *Eur J Hum Genet* 1999;7:2–11.

12. Brown J, Horsley SW, Jung C, Saracoglu K, Janssen B, Brough M, Daschner M, Beedgen B, Kerkhoffs G, Eils R. Identification of a subtle t(16;19)(p13.3;p13.3) in an infant with multiple congenital abnormalities using a 12-color multiplex FISH telomere assay, M-TEL. *Eur J Hum Genet* 2000;8:903–910.
13. Forozan F, Karhu R, Kononen J, Kallioniemi OP. Genome screening by comparative genomic hybridization. *Trends Genet* 1997;13:405–409.
14. Gersen SL. Instrumentation in the cytogenetics laboratory. In: Gersen SL, Keagle MB, eds. *The Principles of Clinical Cytogenetics*, 2nd ed. 2005; Totowa, NJ: Humana Press.
15. Lawce H, Olson S, Wolf D, Magenis RE. Comparative mapping of human cosmid probes in Rhesus monkey (*Macaca mulatta*) using fluorescence in situ hybridization. *J Assoc Genet Tech* 1998;24(2):37–43.
16. Weinberg J. Fluorescence in situ hybridization to chromosomes as a tool to understand human and primate genome evolution. *Cytogenet Genome Res* 2005;108:139–160.
17. Scherthan H, Cremer C, Arnason C, Weier H, Lima-de-Faria A, Frönicke L. Comparative chromosome painting discloses homologous segments in distantly related mammals. *Nat Genet* 1994;6:342–347.
18. Robbins WA, Vine MF, Truong KY, Everson RB. Use of fluorescence in situ hybridization (FISH) to assess effects of smoking, caffeine, and alcohol on aneuploidy load in sperm of healthy men. *Environment Molec Mutagen* 1997;30(2):175–183.
19. Wyandt HE, Tonk VS. *Human Chromosome Variation: Heteromorphism and Polymorphism*. Springer, 2011.
20. Lawce H, Buckmaster D, Magenis E, Olson S. Split centromeric alpha-satellite FISH signals in a whole arm translocation 5p;10p: consequences and implications for interphase FISH studies. *J Assoc Genet Tech* 2006;32(1):5–7.
21. Boyd LJ, Livingston JS, Brown MG, Lawce HJ, Gilhooly JT, Wildin RS, Linck LM, Magenis RE, Pillers DM. Meiotic exchange event within the stalk region of an inverted chromosome 22 results in a recombinant chromosome with duplication of the distal long arm. *Am J Med Genet* 2005;138A:355–360.
22. Shaffer LG, Lupski JR. Molecular mechanisms for constitutional chromosomal rearrangements in humans. *Annu Rev Genet* 2000;34:297–329.
23. Wan TS, Marten UM, Poon SS, Tsao SW, Chan LC, Lansdorp PM. Absence or low number of telomere repeats at junctions of dicentric chromosomes. *Genes Chromo Cancer* 1999;24:83–86.
24. Meltzer P, Guan XY, Burgess A, Trent J. Rapid generation of region specific probes by chromosome microdissection and their application. *Nat Genet* 1992;1:24–28.
25. Wain H, Bruford EA, Lovering RC, Lush MJ, Wright MW, Povey S. Guidelines for human gene nomenclature. *Genomics* 2002;79(4):464–470.
26. Mikhail F, Descartes M, Piotrowski A, Andersson R, Diaz de Stahl T, Komorowski J, Bruder C, Dumanski J, Carroll A. A previously unrecognized microdeletion syndrome on chromosome 22 band q11.2 encompassing the BCR gene. *AJMG part A* 2007;143A(18):2178–2184.
27. Aridgides LJ, Stacey M, Brihn L, Scott D, Osgood C. Fluorescence in situ hybridization on sperm using alkaline denaturation. *Bio Techniques* 2002;33(2):266–267.
28. <http://info.med.yale.edu/genetics/ward/tavi/>
29. Selvarajan S, Bay B-H, Choo A, Chuah K-L, Sivaswaren CR, Tien S-L, Wong C-Y, Tan PH. Effect of fixation period on HER2/neu gene amplification detected by fluorescence in situ hybridization in invasive breast carcinoma. *J Histochem Cytochem* 2002;(12):1693–1696.
30. Chin SF, Daigo Y, Huang, HE, Iyer NG, Callagy G, Kranjac T, Gonzalez M, Sangan T, Earl H, Caldas C. A simple and reliable pretreatment protocol facilitates fluorescent in situ hybridization on tissue microarrays of paraffin wax embedded tumour samples. *Mol Pathol* 2003;56(5):275–279.
31. Ventura R, Martin-Subero JI, Jones M, McParland J, Gesk S, Mason D, Siebert R. FISH analysis of lymphoma-associated chromosome abnormalities in routine paraffin-embedded tissue. *J Mol Diag* 2006;8:141–151.
32. Paternoster SF, Brockman SR, McClure RF, Remstein ED, Kurtin PJ, Dewald GW. A new method to extract nuclei from paraffin-embedded tissue to study lymphomas using interphase fluorescence in situ hybridization. *Am J Pathol* 2002;160(6):1967–1972.

33. Gelpi E, Ambros IM, Birner P, Luegmayr A, Drlcek M, Fischer I, Kleinert R, Maier H, Huemer M, Gatterbauer B, Anton J, Rössler K, Budka H, Ambros P, Hainfellner JA. Fluorescent *in situ* hybridization on isolated tumor cell nuclei: a sensitive method for 1p and 19q deletion analysis in paraffin-embedded oligodendroglial tumor specimens. *Mod Pathol* 2003;16(7):708–715.
34. Schurter MJ, LeBrun DP, Harrison KJ. Improved technique for fluorescence *in situ* hybridisation analysis of isolated nuclei from archival, B5 or formalin fixed, paraffin wax embedded tissue. *Mol Pathol* 2002;55(2):121–124.
35. Plattner R, Heerema N, Yurov Y, Palmer C. Efficient identification of marker chromosomes in 27 patients by stepwise hybridization with alpha-satellite DNA probes. *Hum Genet* 1992;91(2):131–140.
36. Wan TS, Ma SK, Li CK, Chan LC. Atypical fluorescence *in situ* hybridization pattern in chronic leukemia due to cryptic insertion of BCR at 9q34. *Leukemia* 2004;18:161–162.
37. Lawce H, Durum C, Unsworth N, Olson S, Magenis RE. BCR-ABL FISH: probes, patterns, and prognoses. *J Assoc Genet Tech* 2002;28(2):40–46.
38. Wan TS, Ma SK, Au WY, Chan LC. Derivative chromosome 9 deletions in chronic myeloid leukemia: interpretation of atypical D-FISH pattern. *J Clin Path* 2003; 56:471–474.
39. Heng H, Tsui LC. Modes of DAPI banding and simultaneous *in situ* hybridization. *Chromosoma* 1993;102: 325–332.
40. Wolff DJ, Bagg A, Cooley L, Dewald G, Hirsch BA, Jacky PB, Rao KW, Rao PN, the Association for Molecular Pathology Clinical Practice Committee and the American College of Medical Genetics Laboratory Quality Assurance Committee. Guidance for fluorescence *in situ* hybridization testing in hematologic disorders. *J Mol Diag* 2007;9(2):1–24.
41. Wiktor AE, Van Dyke DL, Stupca PJ, Ketterling RP, Thorland EC, Shearer BM, Fink SR, Stockero KJ, Majoriwicz JR, Dewald GW. Preclinical validation of fluorescence *in situ* hybridization assays for clinical practice. *Genetics in Med* 2006;8(1):16–23.
42. American College of Medical Genetics. Standard and Guidelines for Clinical Genetic Laboratories. <http://www.acmg.net>.
43. American College of Medical Genetics. Technical and clinical assessment of fluorescence *in situ* hybridization: an ACMG/ASHG position statement. I. Technical considerations. *Genet Med* 2000;2(6):356–361.
44. College of American Pathologists Laboratory Accreditation Checklists. http://www.cap.org/apps/docs/laboratory_accreditation/checklists/checklistftp.html.
45. Schad CR, Dewald GW. Building a new clinical test for fluorescence *in situ* hybridization. *Applied Cytogenet* 1995;21:1–4.
46. Smith ACM, Magenis RE, Elsea SH. Overview of Smith-Magenis syndrome. *J Assoc Genet Tech* 2005;31(4): 163–167.
47. Stupca P, Meyer RG, Dewald GW. Using controls for molecular cytogenetic testing in clinical practice. *J Assoc Genet Tech* 2005;31:4–8.
48. Slovak M, Vedell V, Pagel K, Chang K, Smith D, Somlo G. Targeting plasma cells improves detection of cytogenetic aberrations in multiple myeloma: phenotype/genotype fluorescence *in situ* hybridization. *Cancer Genet Cell Genet* 2005;158(2):99–109.
49. Weber-Mathiesen K, Winkemann M, Müller-Hermelink A, Schlegelberger B, Grote W. Simultaneous fluorescence immunophenotyping and interphase cytogenetics: a contribution to the characterization of tumor cells. *J Histochem Cytochem* 1992;40(9):1427–1428.
50. Heng H, Squire J, Tsui LC. High resolution mapping of mammalian genes by *in situ* hybridization to free chromatin. *Proc Natl Acad Sci USA* 1992;89:9509–9513.
51. Heng H. High resolution FISH mapping using chromatin and DNA fibre. In: Beatty B, Sabine M, Squire J, eds. *FISH, A Practical Approach*. Oxford University Press: 2002; 77–92.
52. Verlinsky Y, Cohen J, Munné S, Gianaroli S, Simpson J, Ferraretti A, Kuliev A. Over a decade of experience with preimplantation genetic diagnosis: a multicenter report. *Fertil Steril* 2004;82:292–294.

53. The Practice Committee of the Society for Assisted Reproductive Technology and the Practice Committee of the American Society for Reproductive Medicine. Preimplantation genetic testing: a Practice Committee opinion. *Fertil Steril* 2008;90(3):S136–43.
54. Agerholm IE, Ziebe S, Williams B, Berg DG, Bruun Petersen G, Kolvraa S. Sequential FISH analysis using competitive displacement of labeled peptide nucleic acid probes for eight chromosomes in human blastomeres. *Hum Reprod* 2005;20(4):1072–1077.
55. Munné S. Predictability of preimplantation genetic diagnosis of aneuploidy and translocations on prospective attempts. *Reprod BioMed Online* 2004;9(6):645–651.
56. Wilton L. Preimplantation genetic diagnosis and chromosome analysis of blastomeres using comparative genomic hybridization. *Hum Reprod Update* 2005;11(1):33–41.
57. Gutierrez-Mateo C, Benet J, Starke M, Oliver-Bonet M, Munné S, Liehr T, Navarro J. Karyotyping of human oocytes by cenM-FISH, a new 24-colour centromere-specific technique. *Hum Reprod* 2005;20:3395–3401.
58. Munné S, Sandalinas M, Escudero T, Velilla E, Walmsley R, Sadawy S, Cohen J, Sable D. Improved implantation after preimplantation genetic diagnosis of aneuploidy. *Reprod Biomed Online* 2003;7:91–97.
59. Gleicher N, Weghofer A, Barad D. Preimplantation genetic screening: “established” and ready for prime time? *Fertil Steril* 2008;89(4):780–788.
60. Bielanska M, Seang LT, Asanla A. Chromosomal mosaicism throughout human preimplantation development in vitro: incidence, type, and relevance to embryo outcome. *Hum Reprod* 2002;17(2):413–419.
61. Chatzimeletiou K, Morrison EE, Prapas N, Prapas Y, Handyside AH. Spindle abnormalities in normally developing and arrested human preimplantation embryos in vitro identified by confocal laser scanning microscopy. *Hum Reprod* 2005;20(3):672–682.
62. Cooper M, Darilek S, Wun W, Angus S, Mensing D, Pursley A, Dunn R, Grunert G, Cheung S. A retrospective study of preimplantation embryos diagnosed with monosomy by fluorescence in situ hybridization (FISH). *Cytogenet Genome Res* 2006;114(3–4):359–366.
63. Coulom CB, Jeyendran RS, Fiddler M, Pergament E. Discordance among blastomeres renders preimplantation genetic diagnosis for aneuploidy ineffective. *J Assist Reprod and Genet* 2007;24(1):37–41.
64. Munné S. Analysis of chromosome segregation during preimplantation genetic diagnosis in both male and female translocation heterozygotes. *Cytogenet Genome Res* 2005;111(3–4):305–309.
65. Sampson JE, Ouhibi N, Lawce H, Patton PE, Battaglia DE, Burry KA, Olson, SB. The role for preimplantation genetic diagnosis in balanced translocation carriers. *Am J Ob Gyn* (2004);190:1707–1713.
66. Selvarajan S, Bay B-H, Choo A, Chuah K-L, Shim SH, Pan A, Huang XL, Tonk VS, Varma SK, Milunsky JM and Wyandt HE. FISH Variants with D15Z1. *J Assoc Genet Tech* 2003;29(4):146–151.
67. Evenson LJ, Drury KC, Kovalinskaia L, Williams RS. Rapid sequential FISH assays in human amniocytes and fibroblasts: a model for preimplantation genetic diagnosis. *J Assn Genet Tech* 2000; 26(3):96–98.
68. Drury KC, Kovalinskaia L, Clark P, Williams RS. Ultra-rapid (6 minute) FISH using microwave technology. *J Assist Reprod Genet* 1997;14:436–437 (Abstract 20).
69. Bahce M, Escudero T, Sandalinas M, Morrison L, Legator M, Munné S. Improvements of preimplantation diagnosis of aneuploidy by using microwave hybridization, cell recycling and monocolour labelling of probes. *Mol Hum Reprod* 6(9):849–854, 2000.
70. Dovalinskaia L, Li S, Al-Katanani Y, Williams RS, Drury KC. Rapid 2-round FISH aneuploidy screening for 9 chromosomes (X, Y, 13, 15, 16, 17, 18, 21, 22) using microwave technology. *Clin Embryol* 2006;9(1):13–21.
71. Ko E, Rademaker A, Martin R. Microwave decondensation and denaturation: a new methodology to maximize FISH data from donors with very low concentrations of sperm. *Cytogenet Cell Genet* 2001;95:143–145.
72. Thomas NS, Durkie M, Potts G, Sandford R, Van Zyl B, Youings S, Dennis N, Jacobs P. Parental and chromosomal origins of microdeletion and duplication syndromes involving 7q11.23, 15q11-q13 and 22q11. *Eur J Hum Genet* (2006); 14:831–837.

FISH troubleshooting		
Problem	Cause(s)	Solution(s)
1. Standard FISH		
No signal or weak signal	Target not present (e.g., SRY). Probe not added to buffer or poorly mixed. Probes overdiluted. Probe or target not denatured or incompletely denatured. Probe reannealed too long after denaturation and before adding to slide.	Always have a control probe for an internal control against hybridization failures. Repeat experiment from the beginning. Increase concentration of probe in buffer. Repeat experiment with proper denaturation of both probe and target at correct temperatures. Use probe immediately after separate denaturation. Repeat experiment from the beginning.
	Coverslip not removed before post wash, so probe is re-denatured and washed off cells.	Add coverslips slowly and incrementally to prevent bubbles; tap bubble out with tip of forceps; tap debris down with forceps to seat coverslip.
	Bubbles under coverslip or debris holding air under coverslip.	Repeat with fresh sample.
	Cells necrotic/dead.	Bouin's and certain other fixatives are too harsh—use 3:1 methanol acetic acid or formalin.
	Cells in wrong fixative.	Remove coverslips and re-mount slide with only one coverslip.
	Two coverslips added to counterstain, prevents visualization with oil immersion lens.	Repeat with new lot of probe; store probe in dark.
	Probe is too old or has been improperly stored.	Repeat protease step and hybridization or use longer time in protease starting with new, better slide.
	Too much protein on target for probe to penetrate.	Pepsin pretreatment may be helpful; try periphery of colonies for scoring.
	In situ harvested slide, protein over colonies inhibits probe penetration.	Post hybridization wash solution; check pH of formamide post wash; check accuracy of thermometers.
	Post hybridization wash is too stringent or improperly made.	Make fresh post-wash solution; check pH of formamide post wash; check accuracy of thermometers.
	Probe buffer is too stringent.	Be sure to use 50% formamide buffers with locus and telomere-specific probes, not 65% as with alpha satellite probes.
	Slide too old and improperly stored before hybridization.	Rehydrate old room temperature stored slides by running through 90, 80, 70% ETOH and then dH ₂ O before using for FISH. Store slides at -20°C under desiccation long-term for future FISH.
	If used, a formaldehyde step was too long.	Repeat, omitting formaldehyde step.
	Microscope bulb is too old or improperly centered.	Replace bulb at 200 hours and/or center it properly (especially important for subtelomere specific and very small probes).

Counterstain too concentrated (normal = 125 mg/ml; brighter for paints and paraffin-embedded cells = 1,000 mg/ml; lighter for M-FISH/SKY = 42 ng/ml).	Remove coverslip by soaking in 2× SSC. Destain slide by dehydrating through alcohols or in 3:1 fix for 1-2 min, dry, remount in more dilute counterstain.
Improper counterstain.	Red probes not visible with PI counterstain; Spectrum Blue not visible with DAPI counterstain.
Improper filters for probe being used.	Try another filter set or single band pass filter.
Probe has quenched due to exposure to excessive light conditions during storage.	Some probes fade faster than others. Store probe and hybridized slides in the dark. Use brief exposures to microscope light and use shutter to protect cell under lens when not scoping or capturing.
In a mix of centromere and telomere specific probes, the centromere signals are bright and the telomere signals are dim	Adjust concentration of the two probes with either less centromeric probe (one half the usual) or more telomeric probe per test, or both.
Cells are visible on low power, but cannot be focused on high power	Remove one coverslip and try again.
Poor chromosome morphology	Slide mounted upside down. Poor slide making method. Slide was improperly aged.
Slide was overheated during denaturation.	Decrease temperature of codenaturation hotplate or of formamide denaturation solution. Separate formamide denaturation is best for good morphology. Reduce denaturation time.
Slide was heated too long during denaturation.	Slide was digested in pepsin too long.
Signals spattered or very large and nonpunctate	Slide was not baked for 5–10 min at 90 °C before hybridization; slide was co-denatured too hot and/or too long.
Cells look bright on one side and dim on the other	Poor lamp alignment. Poor chromosome drying (differential drying).
Film or haze over nuclei (cytoplasm)	Poor slide-making technique.
Immersion oil has mixed with counterstain or incompatible second type of immersion oil.	When making slides check on phase; if drying differentially, change drying conditions to correct (see Chapter 2, 2.4.18 Slide-making for FISH studies). Remake slides using longer drying times and/or higher humidity; use pepsin to digest off cytoplasm; if pepsin does not work, try proteinase K digestion; if cytoplasm still a problem, use long and hot denaturation for slides and/or co-denaturation. Clean immersion lens and slide, press slide between 2 paper towels to remove excess mounting medium, and try again.

(Continued)

FISH troubleshooting (Continued)

Problem	Cause(s)	Solution(s)
Film all over slide	Microscope slides are too old or improperly cleaned.	Slides to be used for FISH need to be of good quality and properly cleaned—if poor slides are unavoidable, clean with either concentrated nitric acid for 1 hour and rinse thoroughly, or soak in 80% MeOH:20% conc. HCl overnight, 2 min H ₂ O rinse before use.
Natural film of cell metabolites and proteins.		Make sure cell suspension is not too concentrated; flood slide with fixative after putting cells on, even if cells have dried, to dilute the proteins and RNA, etc. Pretreat with RNase and/or protease to help clean metabolites off of cells.
Rapid counterstain fading	Old antifade.	Re-mount slide in new counterstain with good antifade.
Fluorescent background within nuclei/chromosomes	High light intensity on microscope. Alu-bands due to insufficient blocking DNA in probe mix.	Put neutral density filter in; close aperture diaphragm; de-focus lamp a little bit. Re-hybridize using increased Cot-1® DNA and/or human placental DNA to block repeats.
	Small regions of cross-hybridization due to low stringency conditions (see Figure 16.18).	Make sure correct probe buffer was used (60% formamide for alpha satellite DNA probes); wash slides in higher stringency conditions (either lower SSC concentration or 2–4 °C higher post wash temperature).
	If nuclei glow overall, wash solution was made improperly and/or at wrong salt concentrations.	Slide may be re-washed in correct post wash solution or start fresh with new slide.
	Cells were flow-sorted using a fluorescent dye.	Work with probe fluorophores of a different wavelength, if possible.
	Filter is too long bandpass.	Try a filter with smaller bandwidth or more specific bandpass to reduce background fluorescence.
Fluorescent background around periphery of nuclei/chromosomes	Slide has debris or bacteria that autofluoresce.	Flood slide with fixative during slide-making and after drying slide if it looks dirty on phase microscope. Check all FISH reagents for bacteria.
	Probe design not optimal.	If probe is too big (greater than 500bp), it can precipitate on slide: try another lot or brand of probe.
	Microorganisms present.	Check all of the FISH reagents for the presence of bacteria/fungi.
	Latex gloves contain talc which may contaminate slides.	Talc may autofluoresce and cause background; avoid use of latex gloves or powdered gloves.
	Post wash solutions were used too many times.	Replace post-wash solutions daily (SSC) or weekly (formamide).
	Cells were flow-sorted using a fluorescent dye.	Work with probe fluorophores of a different wavelength, if possible.
	Home-brew probe was not properly cleaned.	Re-label probe and/or repeat ethanol precipitation.
	Agglutination of detection agent, if used.	If the detection reagent has fluorescent precipitate in it, centrifuge it and use the supernatant.
Black or opaque debris on slides and over cells	Labeling ink/pencil has come off and spread over cells.	Use at least a #3–4 pencil or indelible pen to label slides.

Dirty solutions for slide preparation and FISH pretreatments.	Replace working solutions often if needed.
Probe is visible with different filter sets “Cross-talk” between different filters with certain fluorophores (e.g., between Spectrum Blue and Spectrum Aqua).	Sometimes unavoidable; ask microscope manufacturer or probe company for help with filter choices.
Wrong probe is pipetted onto slide but coverslip not yet added	Inattention to or misunderstanding of probes to be added to slides. Failure to use proper QC procedures.
Nonspecific signal (cross-hybridization) present on inappropriate chromosomes	Check to be sure there is not a cryptic rearrangement or variant using G-bands, other probes for the region, and numerous normal controls; if not, it is cross-hybridization artifact.
2. Paraffin-embedded Tissue FISH	
Tissue loss	Tissues were wet when floated onto slides and may not be completely dry, so may not adhere well. Pretreatment too harsh.
	Pretreatment too hot. Positively charged slides not used.
Cells appear “ghostly” with counterstain	Pretreatment too harsh. Insufficient time in pretreatment solution and/or protease.
Cells have persistent autofluorescence over tissues	Cells need more time in protease.
Cells exhibit mild autofluorescence and counterstain is very bright	
Loss of nuclear borders, difficult to tell which nuclei signals are in, dim counterstain	Overdigested in protease. Discard slide. Cut down time in protease or decrease concentration, or both.
No signal	Tissue overfixed in formalin before sectioning. Wrong fixative used.
	Formalin exposure should not exceed 24–48 hours. Eliminate Start with new slide and eliminate step for fixation in formalin at the end of FISH pretreatment protocol for overfixed tissues. FFPE tissue should be fixed in 10% neutral buffered formalin or paraformaldehyde Bouin’s, gluteraldehyde, or other fixatives may not work.

(Continued)

FISH troubleshooting (Continued)

Problem	Cause(s)	Solution(s)
Tissue too thin.	Less than 4 μm thick specimens may be oversensitive to enzyme pretreatment.	
Tissue was decalcified during sectioning.	Bone marrow is routinely decalcified and this procedure can degrade DNA/chromatin. Ask for re-section without decalcification, if possible.	
Tissue section not adequately digested so chromatin not accessible.	Check temperatures and concentrations for Pretreatment and Protease solutions; possibly use a stronger protease (250 mg/mL instead of 25 mg/mL or go to new enzyme type).	
Tissue appears "folded"	Positively charged slides not used. Tissue not sufficiently baked onto slide.	Re-section using positively charged slides with small + marks on the end. Bake overnight at 57–60 °C.
	Probe not hybridized long enough or at the correct temperature.	Hybridize at least overnight (14–16 hours), and ensure that temperature is 37 °C.
Cells too crowded to score	Tissue sections too thick or just too crowded.	Tissues sectioned thicker than 6 μm have too many overlapped nuclei and will make it difficult for probe to access DNA. Ask for 4–5 μm thick sections or score only areas where there are less cells (may not represent whole tissue section). Re-rinse slides in higher stringency post-hybridization wash (e.g., higher temperature and/or lower concentration of SSC) and check again.
Cross-hybridization of centromere enumeration probes	Stringency was not high enough, leading to nonspecific binding.	
3. PGD FISH		
Cannot find the single cell because it is not counterstained	Cell not pre-located on phase microscope.	If FISH scope has phase-contrast, note coordinates before hybridization; if FISH scope does not have phase, need marker slides or England finder slides for position of cell before hybridization.
	Cell was pre-located but still cannot be found.	Remove coverslip and counterstain with DAPI or lightly with PI. Find cell and capture all probe signals possible. Then remove coverslip, destain in fixative for 1–2 minutes, re-mount in antifade only, and capture the last signals.
Cell is covered with thick cytoplasmic debris	Rinse or digest off excess protein.	Flood slide with fixative and dry; re-examine for cytoplasm. If still present and problematic, pretreat with protease such as pepsin.
Probes not bright	Cytoplasmic background.	Needed pretreatment with fixative rinse or pepsin.
	Slides or probes denatured incorrectly (too short time and/or too cool of a temperature).	Remove coverslip, repeat hybridization with longer and/or hotter denaturation times/temperatures.
	Slide-making and/or cell is suboptimal.	Try to re-hybridize, heating longer and/or hotter during co-denaturation; if no better, report as uninformative test.

Fluorescent spots all over cell nucleus that fluoresce with all filter sets.	Pools of cell metabolites.	Try pepsin and formalin fixation and/or RNase treatment before re-hybridization.
Insufficient time to perform all hybridizations needed for 8–10 chromosomes	Reannealing times may be 4–16 hours for each probe set.	Try doing 2 sets of 5–6 probes each, the first set with the locus specific probes as well as centromere specific probes and preannealed for 1–4 hours; the second hybridization with centromere probes in the microwave oven with 5 minute hybridizations—(centromere probes work best in microwave).
Residual signal from first hybridization upon rehybridization	Probe was insufficiently destained.	Place slide in 72°C distilled water for 2 minutes to remove previous probe set.
Signal interpretation is difficult for one of the probes	Certain probe targets may be synthesizing, and may split; others may associate and appear as one signal.	Rehybridize with another probe for that locus or repeat the same probe to re-check ploidy.
4. Fiber FISH		
Dim signals	Insufficient probe.	Probe concentration is best at 2–3 times higher than with standard FISH.
	DNA not completely denatured.	Higher temperatures in formamide helps stretch and denature fibers. Then use ice-cold alcohol to preserve denatured state.
Heavy background signal	Improper blocking of repetitive sequences.	Cot-1® DNA should be double that used with standard FISH; incubate slide in BSA or skim milk solution to decrease nonspecific binding before detection with antibodies.
Fibers do not adhere to slide	Post wash not stringent enough. Improper fixation.	Use very stringent post wash. Bake slides briefly after preparation and before hybridization. Fix in alcohol series.

Contributed protocols

IMPORTANT: No protocol included in this manual should be used clinically unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements of all chemicals used in the laboratory.

General safety warnings

Consult SDS and laboratory protocol for health hazards, handling/contact risks, and environmental hazards, including discard directions for all chemicals. Gloves and lab coat should be worn for all FISH procedures, both to protect the technologist, and to prevent DNA from the skin being transferred into the test system. Many FISH reagents are biohazardous and require protective gloves. Examples are propidium iodide, DAPI, and formamide.

Formamide may be harmful by inhalation, ingestion or skin absorption; it causes skin irritation and may have reproductive effects. Do not breathe fumes from formamide; use under fume hood. In case of contact, flush skin or eyes, including inside eyelids, for 15 minutes and seek medical attention. Remove to fresh air if inhaled. If swallowed, call Poison Control and seek immediate medical attention.

Protocol 16.1 FISH (fluorescence in situ hybridization) methods

I. Principle

FISH methodologies can be used to answer specific questions about the cytogenetic status of patients, using metaphase or interphase cells under various conditions. The most commonly encountered tests are to detect microdeletions at a sub-microscopic level, to investigate the presence of gene fusions or amplification in neoplastic specimens, to determine the makeup of derivative or extra marker chromosomes, to look at engraftment from opposite sex donors in bone marrow specimens, to determine ploidy levels of certain chromosomes in uncultured cells from bone marrow or amniotic fluid (STAT cases only, as research only), and to correlate metaphase and interphase information when there is a question of cryptic or non-expressed mosaicism.

Chromosomes are denatured by exposure to hot formamide, and allowed to re-anneal to specific probes that have also been denatured; the non-specifically-bound probe is washed off with a solution designed to be "stringent" enough to leave the test probe but to remove the non-specific probe. Sequences of DNA in the probe will preferentially bind to like regions on the chromosomes (called the target) by the hybridization of the single strands of the denatured probe DNA to the single strands of DNA in the denatured chromosome. The sites at which the test probes anneal are visualized with fluorochromes. Probes are either directly labeled with a fluorochrome, or are labeled with haptens (biotin, digoxigenin, or phox) and detected by use of anti-hapten antibodies with fluorochromes attached. Hybridization is visualized with a fluorescence microscope with the proper excitation and barrier filters to detect the fluorochromes. Certain probes, such as whole-chromosome paints, must be pre-annealed with highly-repetitive DNA such as cot-1 DNA, to block out alu repeat sequences that might cross-hybridize to multiple non-specific sites.

II. Materials

Supplies

1. Water baths at 37°C and 75°C
2. Incubator at 37°C
3. Heat block with wells adjustable from 70–75 °C
4. Microfuge centrifuge
5. pH meter
6. Thermometers for 70–75 °C
7. Boxes with lids and tubing to rest slides on, and paper toweling for humidity, to incubate slides for hybridization.
8. Coplin jars, glass
9. Micropipettors, 20 µL, 200 µL, and 1000 µL
10. Rubber cement; frosting bottle for application
11. Coverslips, various sizes including 22 × 22 mm square glass and 12 mm round glass.

Reagents

1. Stock formamide: Gibco BRL Ultrapure redistilled formamide, #15515-026. Keep frozen until bottle is opened, then refrigerate. Shelf life 12 months at refrigerator temperature.
2. NP-40 (Nonidet P40): US Biological Corporation, Swampscott, MA, #N3500. Shelf life indefinite at room temperature.
3. 70% formamide:
 - a. 35 mL of formamide
 - b. 5 mL 10× SSC (pH 7.0)
 - c. 10 mL deionized water
 - d. pH to 7.0 with 1 M HCl
 - e. Store 70% formamide at 4°C. Shelf life 30 days.
4. Stock 20× SSC: (SSC stands for sodium chloride/sodium citrate)
 - a. 3.0 M NaCl
 - b. 0.3 M $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$

For 1 liter:

 - a. 175 g NaCl
 - b. 88 g $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$

Place both reagents in a liter volumetric flask. Bring volume to about 800 mL with deionized water. Adjust pH to 7.0 with 1 M HCl. Bring total volume to 1.0 L with deionized water.

Autoclave stock SSC and store at room temperature. Shelf-life 12 months.

OR: Abbott/Vysis 20× SSC powder with sodium chloride and sodium citrate, #32-804850: Add 264 g of powder into ~900 mL of distilled water. Stir with magnetic stirrer until dissolved. Bring volume up to 1000 mL and filter sterilize for storage at room temperature.
5. Working SSC solutions

Final concentration	20× SSC	Distilled Water	Total
0.25× SSC	6.25 mL	493.75 mL	500 mL
0.30× SSC	7.25 mL	492.75 mL	500 mL
0.4× SSC	10.00 mL	490.00 mL	500 mL
0.5× SSC	12.50 mL	487.50 mL	500 mL
1.0× SSC	25.00 mL	475.00 mL	500 mL
2.0× SSC	50.00 mL	450.00 mL	500 mL

Store working solutions at room temperature. Shelf-life 30 days.

6. 2× SSC/0.5% NP-40 solution:
 - a. 200 μL of NP-40
 - b. 4.0 mL of 20× SSC
 - c. 35.8 mL of distilled water

OR

 - a. 200 μL of NP-40
 - b. 40 mL of 2× SSC

Prepare fresh each time.
7. 80% ETOH
 - a. 800 mL of 95% ETOH
 - b. 150 mL of deionized water
8. 70% ETOH
 - a. 700 mL of 95% ETOH
 - b. 250 mL deionized water

95% ETOH: from Laboratory Stores, in flammable safety cans.
9. DAPI counterstain
 - a. Abbott/Vysis DAPI II, 125 ng/mL, #30-804931

Store at -20°C until expiration date. Use as is for mounting dual color FISH preparations.

- b. Abbott/Vysis DAPI I, 1000 ng/mL, # 32-804830
- c. Abbott/Vysis DAPI III, 42 ng/mL, #32-804932

Store in freezer until expiration date is reached.

10. Vectashield mounting medium for fluorescence: from Vector Laboratories, # H-1000.

Store at refrigerator temperature for up to 2 years.

11. Propidium iodide: Abbott/Vysis 07J06-001, 1000 µL

I. Slide preparation

Make slides according to environmental conditions, label with accession#, slide #, name, date, and type of probe(s) to be used. Slides must be used within 2 weeks for optimum results. Do not bake slides until ready to FISH. If slides must be kept longer than two weeks before hybridization, they should be dehydrated in a series of alcohols (two minutes in each, 70%, 80%, and 95% ethanol), dried, and stored desiccated at -20 °C.

Optional steps to harden chromosomes for better morphology:

Bake slides 5–10 minutes (NO LONGER) at 90 °C.

Incubate in 2× SSC at 37°C for 30 minutes. *Optional:* Pretreat slides with pepsin at this point to remove excess protein (e.g., for uncultured amniocytes (see Protocol 16.5 Interphase FISH for amniotic fluid specimen aneuploidy). Dehydrate slides: two minutes in each of 70%, 80%, and 95% ethanol). Dry slide at an angle, label down. They may be either air-dried or blown dry with an air jet. Slides may be hybridized using separate denaturation (slide is denatured in hot formamide and probe is denatured in a hot water bath) or codenaturation (ThermoBrite or HYBrite).

II. Probe preparation

Read manufacturer's instructions for each probe before use. The following are generalizations, and exceptions may arise.

Bring the probe up to room temperature and mix well before aliquoting for use, as the DNA is viscous and tends to go to the bottom of the tube. It is best to centrifuge probe tubes before each step of aliquoting, denaturing, prehybridizing, and adding it to the slide. This will ensure that all of the probe and its diluent are together and that the concentration and volume of the probe are correct. Usually, the total volume per slide needed is 10 µL of probe. Some probes are ready to use out of the vial, and others need to be diluted before use. For Abbott/Vysis probes, the usual dilution is 1 µL of probe, 2 µL of distilled water, and 7 µL of buffer. The Abbott/Vysis LSI buffer is used for paints and locus specific probes, and the CEP buffer is used for CEP probes. If two different probes are used simultaneously, add some of each into a microfuge tube with buffer if needed, subtracting 1 µL of distilled water for each additional probe added. Mix and centrifuge, and add the mixture to the slide. If probe instructions say "do not denature this probe," it has been denatured and stabilized at the manufacturer, and need not be denatured. However, it will not hurt it if it is denatured before use.

Usually, 10 µL of probe per slide is required (a 22×22 mm coverslip is used to cover this volume of probe). See Probe Selection and Hybridization flow chart below for probe and buffer volumes for standard probes. Some probes are designed to be used with 3-µL aliquots: for these, small 12 mm circle coverslips are used. Some probes are in a concentrated form, and these should be diluted according to the manufacturer's directions. If two different diluted probes are used simultaneously, add 5 µL of each into a microfuge tube, mix and centrifuge, and add the mixture to the denatured slide. **Note:** almost any probes can be mixed together. If mixing together a CEP and an LSI, use the LSI buffer (lower stringency) to be sure that the LSI probe works. Some cross hybridization of the CEP probe may occur.

III. Procedure

Denaturation steps

Separate denaturation the probe and slide

Separate denaturation is used for some hybridizations to obtain better DAPI band resolution and/or to accommodate paint methods. For denaturing the probe, the general rule is: all paints are denatured in a 70 °C water bath for 10 minutes, and all other probes are denatured at 75 °C for 5 minutes. All human paints must be pre-hybridized with non-labeled cot-1 DNA (usually included in the commercial probe vials) for 1-2 hours at 37°C in a floating tube rack in a water bath. Most others do not require prehybridization. **Note:** for metaphase cells requiring good DAPI bands, it is better not to use the HYBrite, but rather to separately denature the slide and the probe and then put them together to reanneal.

Put glass Coplin jar with 70% formamide into a water bath which is no hotter than 45 °C (or it will break). Turn water bath up to approximately 80 °C and bring the formamide inside the jar to 70–76°C (check temperature with a clean

thermometer). Sensitive materials such as fresh slides of bone marrow or amniotic fluid chromosomes will require lower temperatures, and resistant slides, such as older pellets of blood cultures or very cytoplasmic preparations will require hotter temperatures. Dip slide(s) into hot formamide for 2–6 minutes. The standard time is two minutes for most slides, with longer times used for very resistant preparations. If several slides are run in formamide, be sure that the temperature stays above 69 °C for the entire incubation time. If it falls below 69 °C, wait until it returns to 69 °C before starting timer.

Immediately plunge slides into cold ethanol baths to preserve chromosomes in the denatured state: two minutes each in each of the three Coplin jars with 75%, 85%, 95% ethanol in an ice bucket. Air dry slides (or use an air jet). Use denatured slides the same day (see below, “Hybridization”).

Codenaturation HYBrite Program Settings: The tissue type dictates what programs are best.

Program no.	Specimen or probe type	Denaturation temperature and time	Reannealing time and temperature
1	CEP for hi stringency, if needed	72 °C for 2 min	42 °C overnight
2	Paraffin tissues	75 °C for 10 min	37 °C overnight
3	Paraffin tissues	48 °C HOLD** for drying slides	–
4	Uncultured amnios, overnight hybe	75 °C for 3 min	37 °C overnight
5	PGD slides	73 °C for 5 min	37 °C for 4 hours to overnight
6	HER2/neu alternative for paraffins	80 °C for 5 min	37 °C overnight
7	Uncultured amnios, short hybe	75 °C for 3 min	37 °C for 4 hours
8	Any pre-hybridized slide	37 °C HOLD**	–
9	Most standard cytogenetic preps	72 °C for 2 min	37 °C overnight
10	All telomere panel	70 °C for 3 min	37 °C overnight

** No ramping up and down, just constant temperature

When slides have completed the HYBrite program, they are ready to be post-washed.

Currently, most probes are co-denatured on the Abbott/Abbott/Vysis HYBrite Hybridization machine. The exception is that painting probes are better if separately denatured since they need 20–30 minutes of prehybridization before adding to the slide. Add 3–10 µL of probe to each half-slide to be hybridized, coverslip with a 12-mm circle (3 µL) or a 22 mm² square coverslip (10 µL), and seal with rubber cement.

Sealing slides for FISH

Because there is such a small volume of fluid (3–10 µL), the heating up of the slide and probe during denaturation and reannealing can dry out the preparation and ruin the hybridization (see “Background Signal” in glossary). The coverslip may be sealed to prevent drying using either parafilm, for small coverslips, or rubber cement for larger ones. The parafilm will melt onto the slide during the HYBrite denaturation step and seal the coverslip down. This is the method of choice for preimplantation FISH on single nuclei.

For a whole slide, use 30 µL of probe and a 50 × 22 mm² coverslip. Check HYBrite for rubber cement balls on its surface that would interfere with slide contact. If the probe volume is small (e.g., 3 µL), put moistened strips of paper towel into the side troughs to prevent overdrying of slides. Place slides in HYBrite. Fill all of the empty spaces with a blank slide. Pick the HYBrite program according to the tissue type (see chart, later), and start program. Slides will heat up to the denaturation temperature for a few minutes and then the HYBrite will ramp down to 37–42 °C for reannealing for 15 minutes to overnight, depending upon the tissue type and probes.

Stepwise, the overall procedure is:

1. Add the (premixed) probe solution(s) to the slide in the region where the most cells are located.
2. Coverslip with a 22 × 22 mm coverslip, avoiding bubbles if possible.
3. Seal with rubber cement, using a syringe without needle to apply it.
4. Place slide on HYBrite hotplate.
5. Program HYBrite (usually #9). Close lid, press “yes” to start program. HYBrite will ramp up to 72 °C and hold for 2 minutes, and then ramp down to 37 °C to reanneal the probe with the target.
6. Hybridize overnight, or as specific protocol requires (sometimes as little as 15 minutes to 4 hours is sufficient).

Post wash

When slides have completed the HYBrite program, they are ready to be post-washed.

Sample/probe type	Post wash SSC	Time and temperature
Standard cytogenetics preparations with standard probes both LSI and CEP	0.4× SSC with 0.3% NP-40 detergent	2 minutes at $72 \pm 2^\circ\text{C}$ with agitation the first 3 seconds
Paraffin-embedded tissue	2× SSC with 0.3% NP-40	Presoak in 2× SSC 0.3% NP-40 at RT to remove coverslip; then, 2 minutes $72 \pm 2^\circ\text{C}$ with NO agitation
Paints	0.4 to 2× SSC with 0.3% NP-40, with brighter results using 2× SSC but usable results with 0.4× SSC	2 min at $72 \pm 2^\circ\text{C}$ with agitation the first 3 seconds
PGD probes	0.4× SSC with 0.3% NP-40	5 minutes with NO agitation

When the post-wash is completed, for all slide types EXCEPT paraffin-embedded tissues, place the slide in room temperature 2× SSC with 0.15% NP-40 for 5–30 seconds to cool off the slide and remove the stringency wash. Then dry the slide and mount with appropriate mounting medium (DAPI II or antifade with no stain if blue dyes are present in the probe mix). For paraffin embedded tissues, simply dry the slide and mount in DAPI I. For FITC probes/paints propidium iodide can be used to visualize small chromosome regions that are difficult to see with DAPI.

Post-wash step overview

Place Coplin jar(s) with appropriate post-wash solutions (see chart) into the water bath, and turn water bath up to approximately 80°C. Place clean thermometer in Coplin jar; when temperature reaches desired level (see chart), prepare one to four slides for washing. Keep direct-labeled probe preparations in the dark as much as possible to prevent bleaching of fluorochromes. After probes have hybridized 4 hours overnight, remove the rubber cement from the slides with forceps, taking care to leave the coverslip on the slide.

For paraffin-embedded tissue sections, to keep tissues from coming off of the slide, presoak coverslips off in 2× SSC with 0.3% NP-40 for a few minutes before adding slide to post-wash solution. For suspension culture type slides, tap slide gently on edge until the coverslip starts to slide off. Grasp the edge of the coverslip and remove by gently pulling it straight up, without any slipping motions. Immediately plunge into the post-wash solution (see chart, later). There is usually a 3-second agitation, with PGD probes being the exception (no agitation). When incubation time has completed, remove slide to the cool rinse (for Abbott/Vysis probes, 2× SSC with NP-40). The slide remains in the cool rinse (usually 2× SSC with 0.15% NP-40) for the required time, usually 30 seconds to 3 minutes (not critical).

Stepwise, the overall procedure is:

1. Add Coplin jar of 0.4× SSC with NP-40 to the water bath and turn temperature to 9 on the dial.
2. When inside of Coplin jar is $72 \pm 2^\circ\text{C}$, it is ready to use.
3. Remove slide from HYBrite and remove rubber cement carefully. Pop coverslip off slide (do not slide it off).
4. Dip slide for 9–10 dips in hot SSC, then incubate at $72 \pm 2^\circ\text{C}$ for 2 minutes.
5. Rinse slide in room temperature 2× SSC with NP-40 for about 30 seconds.
6. Put 18–20 µL of DAPI II counterstain on cell area of slide and coverslip with a 24×50 mm coverslip. Press between paper towels to make the counterstain layer as thin as possible. Slide is ready to scope, or can be put in the freezer until ready to scope.

Mounting in counterstain

Put 18–20 µL of **counterstain on cell area of slide and coverslip with a 24×50 mm coverslip. Press between paper towels to make the counterstain layer as thin as possible. Slide is ready to scope, or can be put in the freezer until ready to scope.

** DAPI II is used for the majority of slides. DAPI I is used for paraffin embedded tissue slides. DAPI III is used for M-FISH. Propidium iodide is a red counterstain to be used with FITC or green paints to better visualize the chromosome when very small rearrangements are in question.

Microscopy

The following filters are used to visualize the probe signals

Fluorophore	Counterstain	Probe filter excitation/emission	Filter location
FITC/Spectrum Green™	DAPI	490/515	Wheel
Rhodamine	DAPI	540/550	Wheel
Texas Red	DAPI	595/615	Wheel
Spectrum Orange™	DAPI	539-558/574-587	Front filter set
Cy3	DAPI	550-570	Wheel
Cy3.5	DAPI	581/596	Wheel
Cy5	DAPI	649/670	Wheel
Cy5.5	DAPI	675/694	Wheel
Spectrum Blue™	None (Vectashield antifade) or propidium iodide	40/450	Wheel
Spectrum Gold™	DAPI	530/555	Front filter set
Spectrum Red™	DAPI	592/612	Wheel
Far Red	Light DAPI	620/700	Front filter set
Spectrum Aqua™ (Diethylamino-coumarin/ DEAC)	DAPI	432/472	Front filter set
DAPI counterstain	—	539-574	Wheel
Propidium iodide counterstain	—	520/610	Wheel

Microscopy

Turn on fluorescent microscope bulb, making sure that the computer monitor is turned off during bulb firing.

When microscope bulb light turns on, monitor may be turned on. Log in to CytoVysis. Open or create a case. Go to “Probe” in Capture Mode. The “Excitation” button will start up the filter wheel. Find cells on low power using a DAPI filter. Go to high power oil immersion and switch to dual filter or triple BP filter to observe probe signals.

Scanning slide: Find the area of hybridization on low power and focus on the DAPI stain using the DAPI filter. Move to oil immersion high power and refocus. Change to the filter set that shows the probe colors on the slide. Methodically move up and down on the hybridized area to find the cells on oil immersion, and score all that are clear. Reject unclear patterns, cells that are too close together or overlapped, or cells in areas with a high degree of auto-fluorescence or debris.

One-color signal interpretation. Courtesy of Abbott Abbott/Vysis.

For fusion and break-apart probes, be conservative and do not overinterpret cells that are suboptimal. If an abnormal pattern not usually seen is present in multiple cells, it could be a true abnormal, and should be noted.

The currently available TEL/AML probe is of a design called ES for “extra signal.” Instead of showing both translocation partners as yellow signals like a double fusion probe, one partner has a yellow fusion and one has a vestigial red signal, usually small.

Double fusion patterns: Some double fusion patterns, notably the BCR/ABL1, can occur with concurrent deletions of *BCR* and/or *ABL1*. This results in several different patterns.

Break-apart signal patterns: Break-apart probes are useful when a breakpoint is liable to have multiple partners, so specific double fusion probes cannot be used for all of the different rearrangements.

When scoring interphase cells it is good to know nuclear morphology and watch for unusual or abnormal findings.

When not viewing slide, be sure to block the light on the microscope so the signal is not quenched.

Store slide in the dark and/or in a freezer until no longer needed.

QA/QC for FISH

Probes should be validated before use to determine how they behave both in the normal population and in affected individuals. Validations should be carried out for any parameter that could be tested for with a given probe; e.g., for trisomy, monosomy, deletions, duplications, etc.

Some probes require control probes. For probes to be used for duplication or deletion testing, a control needs to be located on the chromosome that the test region is located on (e.g., CEP 17 for p53). If the probe is for ploidy of a whole chromosome, it is better to use a control on another chromosome so that the ploidy of the cell can be determined. For example, in prenatal 13, 18, 21, X and Y ploidy FISH, the 13 and 21 are cohybridized on one slide, and the 18/X/Y are cohybridized on another. If there are 2 signals for 13 and 3 for 21 on the first slide, one can determine that the cell is probably trisomy 21 rather than triploid. If all probes show 3 signals it is most likely triploid, and if all probes show 4 signals it is likely tetraploid.

Some FISH assays require a control slide. All HER2/neu FISH slides are run with a nonamplified and two amplified controls, which are scored the same as the test slides. Any probe that is potentially nullisomic for the probe tested (e.g., Y chromosome probes, preimplantation blastomere probe sets) requires a normal control slide. All microarray FISH studies require a normal control slide. Any unexpected results on a FISH study may have retroactive normal FISH studies run.

Note that in the Analysis Mode in CytoVision there is a tool for moving signals on the picture. This should only be used for aneuploidy probes, and not for fusion or break-apart probes, as the latter depend on proximity for interpretation, so the laboratory Director needs to see the native positions of all of the probes.

Protocol 16.2 LSI, CEP, and paint probe protocol

Contributed by Vancouver General Hospital, Vancouver, BC

I. Principle

To provide a fluorescence in situ hybridization (FISH) procedure using Locus Specific Identifier (LSI), Centromeric Enumeration Probe (CEP) and whole chromosome paint probes on bone marrow, blood lymphocyte or amniotic fluid specimens for FISH analysis. Cytocell LSI, CEP and paint probes from Rainbow Scientific or Abbott/Vysis LSI, CEP or paint probes from Inter Medico are used for identification of genetic aberrations associated with genetic or hematopoietic disorders. These probes are designed to identify various chromosome aneuploidies, translocations, deletions, duplications and other rearrangements associated with specific genetic or hematopoietic disorders in interphase and metaphase cells (e.g., LSI BCR/ABL1 probe to identify translocation 9;22 for a CML patient).

II. Materials

Cytocell LSI, CEP or Paint FISH Probes	Rainbow Scientific
Abbott/Vysis LSI, CEP or Paint FISH Probes	Inter Medico
DAPI ES Counterstain, Cytocell	Rainbow Scientific #DES150L
DAPI II Counterstain, Abbott/Vysis	Inter Medico #32-804831
Vecta Shield Mounting Medium+DAPI	Vector Lab #H-1200
Vecta 411812200 Mounting Medium	Vector Lab #H-1000
Ethanol 99%, 4L	Brenntag Canada Inc. #EA100-4X4
Absolute Methanol, 4L	FISHER #A412-4
Glacial Acetic Acid, 2.5L	VWR #CAAX0073-59
20x Saline Sodium Citrate (SSC)	Gibco BRL #15557-044
Hybridization Solution B	Cytocell #HB500L
CEP Hybridization Buffer	Abbott/Vysis #32-804828
LSI/WCP Hybridization Buffer	AbbottVysis #32-804826
Triton X	Sigma #N-6507
dH ₂ O, autoclaved	Stores

Parafilm	Stores
Slides, Microscope 25 × 75 mm	James International Sales Ltd. #10202372
Glass Coverslips 18 × 18 mm	FISHER #12541A
Glass Coverslips 22 × 22 mm	VWR #CA 48367-106
Glass Coverslips 22 × 50 mm	VWR #CA 8393-059-1
Pipette Tips, 0.5–10 µL, autoclaved	FISHER #21-119-10
Pipette Tips, 20–200 µL, autoclaved	FISHER #21-197-8G
Eppendorf Tubes, 0.5 mL, autoclaved	Cole Parmer #P-02550-06
Tissue-Tek Rack/Containers	
Red Wax Pencil	Grand & Toy
Black Lab Marker	VWR #52877-310
Plastic squeeze Bottles, 250 mL	VWR #16651-573
Graduated Cylinders and Beakers	
Gloves	Core Lab
Kimwipes	Stores
Slide Holder, cardboard	
Beaker, plastic	
Styrofoam Tube Rack	
Float Rack (for Eppendorf tubes)	
Forceps	

Equipment

ThermoBrite StatSpin	ESBE Model #S500-12
Fume Hood, Labconco Basic 47	VWR Model #22473
Incubators, Multigas	SANYO Model #MCO1755M
Water bath (2)	FISHER #10L-M
Micropipettor, P200 Gilson Pipetman	MANDEL SCI. #GF-f123601
Micropipettor, P10 Gilson Pipetman	MANDEL SCI. #GF-F144802
Slide Warmer	FISHER #12-594
Vortex, Genie 2	FISHER #12-812
Timer	VWR #62344-586
Microcentrifuge	

Solutions

1× SSC:	100 mL 20× SSC 1900 mL dH ₂ O ■ Pour into large plastic 2-L labeled container and mix ■ Refrigerate at 4 °C
1× SSC/0.3% Triton X	200 mL 1× SSC
Working solution:	600 µL Triton X ■ Aliquot into Tissue-Tek container and mix

	■ Use same day
0.4x SSC	80mL 20x SSC
	1920mL dH ₂ O
	■ Pour into large plastic 2L labeled container and mix
	■ Refrigerate at 4°C
0.4x SSC/0.3% Triton X	200mL 0.4x SSC
Working solution	600µL Triton X
	■ Aliquot into Tissue-Tek container and mix
	■ Use same day
2x SSC	200mL 20x SSC
	1800mL dH ₂ O
	■ Pour into large plastic 2-L labeled container and mix
	■ Refrigerate at 4°C
2x SSC/0.1% Triton X	200mL 2x SSC
Working solution	300µL Triton X
	■ Aliquot into Tissue-Tek container and mix
	■ Use same day
3 : 1 fixative	10mL acetic acid
	30mL methanol
	■ Mix in a labeled plastic squeeze bottle

Probe solutions

Notes: *Turn off room lights for probe preparation!*

For the regularly used hematologic probes, six times the formulas below are prepared, labeled and stored in freezer in a box labeled "made up probes"

Cytcell probes	3µL probe
Unmade, (per slide)	7µL Hybridization Solution B
	Aliquot into Eppendorf tube
	Vortex and pulse spin suspension in microcentrifuge to mix before use
Abbott/Vysis probes	1 µL probe
Unmade (per slide)	2 µL dH ₂ O autoclaved
	7 µL LSI buffer
	Aliquot into Eppendorf tube
	Vortex and pulse spin suspension in microcentrifuge to mix before use

III. Procedure

Slide preparation

1. Soak slides in 99% ethanol to remove any debris. Wipe slides with Kimwipes and place in a plastic beaker of dH₂O. Rinse 2x with dH₂O.
2. Make slides with a 2–3x denser cell suspension than for regular analysis, using 1–2 drops of fixative overlay.

Probe addition and denaturation

1. Turn off lights to prevent probes from fading.
2. Place probes for use in 37°C incubator for approximately 5–10 min.

3. If not already made up, prepare probes according to solutions section.
4. Turn on ThermoBrite by hitting the “on” switch at the back of the machine (the temperature will go to 37°C).
5. For blood/bone marrow specimens with plentiful nuclei, add 3 µL of probe mixture to each slide and cover with a glass coverslip (18 × 18 mm). For other specimens, add 10 µL to each slide and cover with a glass coverslip (22 × 22 mm).
6. Soak the sponge with water on the inside of the ThermoBrite lid.
7. The cursor on the ThermoBrite LCD display will be sitting at “run program.” Press the enter key.
8. Select program #07 (called “mediumdn”) by pressing the up arrow until #07 appears on the display. Press the enter key.
9. The cursor will display “start.” Put slides into slots on the heating surface of the ThermoBrite.
10. Close ThermoBrite lid. Press the enter key. The ThermoBrite will denature the slides at 73°C for 10 minutes and then cool to 37°C.
11. Wraps slides in Parafilm.
12. Put slides in Tupperware container and incubate overnight in FISH incubator at 37°C.
13. Turn lights back on.

Post-hybridization washing and counterstaining

1. Turn on appropriate water bath to the required temperature of 73.5°C for post-hybridization washing.
2. Dispense 1× SSC working solution into a Tissue-Tek container for washing LSI and Paint probes and place in water bath.
3. Dispense 0.4× SSC working solution into a Tissue-Tek container if washing CEP probes and place in water bath.
4. Dispense 2× SSC working solution into an additional Tissue-Tek container and leave at room temperature.
5. Allow water bath to reach a steady 73.5°C.
6. Turn off room lights. Remove slides from the Tupperware container, remove parafilm and take off coverslips with forceps and discard into a sharps container. Place slides in a Tissue-Tek rack to wash in the appropriate SSC working solution at 73.5°C for 2 minutes, agitating at the 1 minute mark.
7. Remove rack containing slides from previous solution and place in the room temperature container of 2× SSC working solution, agitating for 10 seconds.
8. Air dry slides in the dark cupboard underneath bench.
9. Add 10 µL of DAPI ES or DAPI II to slides and coverslip (22 × 50 mm).
10. Place slides in the cardboard slide holder that protects probes from light.
11. Store in refrigerator to preserve fluorescence.

Protocol 16.3 FISH protocol for multiprobe® FISH panels

Contributed by Vancouver General Hospital, Vancouver, BC

I. Purpose

To provide a fluorescence in situ hybridization (FISH) procedure using Chromoprobe Multiprobe® System CLL Panel, Chromoprobe Multiprobe® – I System, Chromoprobe Multiprobe® – OctoChrome System on bone marrow, blood lymphocyte or amniotic fluid specimens for FISH analysis.

II. Principle

Cytocell Chromoprobe Multiprobe® panels from Rainbow Scientific are used for identification of genetic aberrations associated with genetic or hematopoietic disorders. These panels are designed to identify various chromosome aneuploidies, translocations, deletions, duplications and other rearrangements associated with specific genetic or hematopoietic disorders in interphase and metaphase cells.

III. Materials

In addition to those required for regular FISH protocol; see Protocol 16.2.

CDEN-HYB2 Hybridization Solution	Cedarlane Labs D002
Chromoprobe Multiprobe® System CLL Panel	Rainbow Scientific PMP 016
Chromoprobe Multiprobe® – I System	Rainbow Scientific PMP 004
Chromoprobe Multiprobe® – OctoChrome System	Rainbow Scientific PMP 803

IV. Procedure

Slide preparation

1. Soak template slides (with grid) in 99% ethanol to remove any debris. Wipe slides dry with Kimwipe.
2. Make slides with a 2–3× denser cell suspension than for regular analysis, drop suspension down each horizontal row, make sure that suspension covers each cell of the grid with enough metaphases if needed; if not you can drop more onto that area.
3. Drop a layer of fix on each half of the template slide.

Probe addition and denaturation

1. Place Multiprobe® device (grid side up) and hybridization solution for use on 37°C slide warmer for approximately 10 minutes.
2. Turn on ThermoBrite by hitting the “on” switch at the back of the machine (the temperature will go to 37°C).
3. Wet the sponge with dH₂O on the inside of the ThermoBrite lid.
4. Spot 2 µL of hybridization solution for the CLL and Octochrome, and 1 µL for the I system onto each square of the device.
5. Position the template slide onto the device, using the red dot as a guide.
6. Carefully turn the slide-device over and place on the ThermoBrite, so that the slide is on the bottom.
7. The cursor on the ThermoBrite LCD display will be sitting at “run program.” Press the enter key.
8. Select program #07 by hitting the up arrow until #07 appears on the display. Press the enter key.
9. The cursor will display “start. Close ThermoBrite lid. Press the enter key. The ThermoBrite will denature the slides at 73°C for 10 minutes.
10. Carefully remove slide-device from ThermoBrite, wrap in Parafilm and place in 37°C incubator for two nights.

Post-hybridization washing and counterstaining

See Protocol 16.2.

Protocol 16.4 Slide pretreatment with pepsin for FISH

Contributed by Clinical Genetics Laboratories at Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Purpose

For sub-optimal slides for FISH, pretreat with pepsin to reduce proteinaceous material on nuclei and increase hybridization efficiency: Very useful for uncultured amniocytes at <25 weeks gestation, CD138+ plasma cells, blood smears, buccal smears, or old cytoplasmic pellets.

Safety warnings

Formaldehyde may cause cancer with chronic exposure. Harmful if inhaled or absorbed through the skin, wear gloves and lab coat at all times. May be fatal or cause blindness if swallowed. Cannot be made nonpoisonous. Flush skin or eyes for 15 minutes in case of contact. Remove to fresh air if inhaled. If swallowed and victim is conscious, give milk, activated charcoal or water, get medical attention immediately.

II. Materials

1. 2× SSC
 - a. 100 mL of 20× SSC (see FISH recipes)
 - b. 900 mL deionized water
pH to 7.0.
2. 0.01 N HCl
 - a. 1 mL of 1 M HCl from the stock bottle in the fume hood
 - b. 99 mL of deionized water

3. 10% pepsin stock solution:
 - a. 100 mg of Sigma pepsin (#P7012): store at -20°C under desiccation.
 - b. 1 mL of deionized water
 Mix well, Aliquot into 20- μL portions in microfuge tubes. Store at -20°C .
4. Pepsin working solution
 - a. 20 μL of stock pepsin
 - b. 40 mL of 0.01 N HCl.
5. Phosphate buffered saline (PBS):
 For one liter:
 - a. NaCl 10.0 g
 - b. KCl 0.25 g
 - c. Na_2HPO_4 1.3 g
 - d. KH_2PO_4 0.25 g
6. Post-fixation solution: 1 mL of 37% formaldehyde
 - a. 0.18 g of MgCl_2
 - b. 39 mL PBS
 Store at 4°C and use within 1 month.
7. 70%, 80%, and 100% ethanol

III. Method

1. Place slides in 2 \times SSC for 30 minutes to 1 hour at 37°C .
2. Place slides in freshly made 37°C pepsin for 13 minutes.
3. Rinse slides in PBS at room temperature for 5 minutes.
4. Place slides in post-fixation solution for 5 minutes at room temperature.
5. Rinse slides in PBS for 5 minutes at room temperature. Dry slides.
6. Dehydrate slides in 70%, 80%, and 100% ethanol for 2 minutes each at room temperature
7. Proceed to hybridization.

Protocol 16.5 Interphase FISH for amniotic fluid specimen aneuploidy

Contributed by Clinical Genetics Laboratories at Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Purpose

To determine the ploidy of the chromosomes most commonly found in trisomic fetuses (e.g., 13, 18, 21, X, and Y). FISH may also be used in certain cases to detect microdeletions of the DiGeorge/Velocardiofacial syndromes region of 22q, especially in late gestation pregnancies with heart defects. This test is used as a preliminary result only, and is not intended to stand alone. It must be followed up by traditional chromosome studies, and it is recommended that no irreversible actions be taken prior to obtaining the full karyotype.

II. Principle

Amniotic fluid cells from whole fluid are centrifuged, treated with hypotonic solution, fixed, and slides made by usual methods according to environmental conditions. Slides are baked for a short time to harden the chromatin (less than 10 minutes or probes will not hybridize as well), treated in 2 \times SSC, pretreated with pepsin, and denatured with the Vysis Aneuvysion probes for 13, 18, 21, X, and Y, and occasionally the TUPLE 1 probe. Slides are reannealed (hybridized) for 4 hours to overnight, rinsed in 0.4 \times SSC with NP-40, and counterstained with DAPI.

Safety warnings

Formaldehyde may cause cancer with chronic exposure. It is harmful if inhaled or absorbed through the skin; wear gloves and lab coat at all times. *It may be fatal or cause blindness if swallowed* and cannot be made nonpoisonous. Flush skin or eyes for

15 minutes in case of contact. Remove to fresh air if inhaled. If swallowed and victim is conscious, give milk, activated charcoal or water; get medical attention immediately.

III. Materials

1. 0.075 M KCl (0.56% in water) or amniotic fluid culture hypotonic (see prenatal methods).
2. 3 : 1 methanol–acetic acid
3. 2× SSC: 100 mL of 20× SSC (see FISH recipes) plus 900 mL of deionized water. pH to 7.0.
4. 0.01 N HCl (1 mL of 1 M HCl from the stock solution plus 99 mL of deionized water).
5. 10% pepsin stock solution:
 - a. 100 mg of Sigma pepsin (#P7012): store Sigma powder at –20 °C under desiccation.
 - b. 1 mL of deionized water
 Mix well, aliquot into 20 µL portions in microfuge tubes. Store at –20 °C.
6. Pepsin working solution:
 - a. 20 µL of stock pepsin
 - b. 40 mL of 0.01 N HCl
7. Phosphate buffered saline (PBS): For 1 L:
 - a. NaCl 10.0 g
 - b. KCl 0.25 g
 - c. Na₂HPO₄ 1.3 g
 - d. KH₂PO₄ 0.25 g
 Add the above in 1000 mL of distilled water and mix well.
8. Post-fixation solution:
 - a. 1 mL of 37% formaldehyde
 - b. 0.18 g of MgCl₂
 - c. 39 mL of PBS
 Store at 4 °C and use within 1 month.
9. 70%, 80%, and 100% ethanol.
10. 0.4X SSC with 0.3% NP-40: 10.00 mL 20× SSC to 490.00 mL distilled water.
11. Vysis AneuVysion probes for 13 and 21, and 18/X/Y (#05J38-050, 50 assays).
12. Vysis DiGeorge Region Probe for TUPLE1/ARSA (#05J21-028).

IV. Method

1. Centrifuge cells out of whole amniotic fluid. Usually 2–4 mL proteinaceous will yield enough cells to do two slides, one for each probe set, depending upon gestational age (2 mL are enough for 25–30 weeks; for 15–16 weeks, 3.5–4 mL usually suffice). Floating cells may be recovered from cultures up to 6–8 days after set up if needed for interphase FISH studies, as well.
2. Resuspend cells in 2–3 mL of 0.075 M KCl or amnio hypotonic solution at 37 °C for 15 minutes. Add about ½ mL of fresh 3 : 1 methanol–acetic acid pre-fixative; mix and centrifuge cells; remove hypotonic with prefix. Mix and add 2–4 mL of fixative. Incubate cells in the freezer at –20 °C for at least 20 minutes before centrifuging and making slides. Make one slide per probe. Label with patient last name, GL#, culture, slide number, date, and probe.
3. Bake slides on aluminum tray for 5 minutes at 90–95 °C.
4. Add to 37 °C 2× SSC solution for 30 minutes (time not critical).
5. Place slides in freshly made 37 °C working solution pepsin for 13 minutes (reduce time if specimen is only slightly cytoplasmic as the pepsin will overdigest the cells).
6. Rinse slides in 1× PBS at room temperature for 5 minutes.
7. Place slides in post-fixation solution for 5 minutes at room temperature.
8. Rinse slides in 1× PBS for 5 minutes at room temperature.
9. Dehydrate slides in 70%, 80%, and 95–100% ethanol for 2 minutes each at room temperature. Dry slides either by air-drying or blowing with compressed air.
10. Pre-warm Vysis AneuVysion probes for 13/21 and 18/X/Y to room temperature.
11. Centrifuge probe vials for 1–3 seconds to collect probe in bottom of vial.

12. Using a micropipettor and fresh tip, remove 10 µL of probe for each mixture and place on slide in area of greatest cell concentration. Coverslip with a 22 × 22 mm coverslip, and seal with rubber cement.
13. Place on HYBrite. Run program #4 or #7. Both denature at 75 °C for 3 minutes, but program #7 will reanneal for 4 hours and program #4 will reanneal overnight. Either program is acceptable for the probes to hybridize well.

Post wash (after 4–18 hours)

1. When the program is finished, remove rubber cement and coverslip and place slides in 0.4× SSC with 0.3% NP-40 at 72 °C for 2 minutes with agitation for the first 30 seconds.
2. Place slides in room temperature 2× SSC with 0.1% NP-40 for 5 seconds to 2 minutes (time not critical – this step just cools slides down).
3. Mount with 18 µL of DAPI II and a 50 × 22 mm coverslip. Press excess DAPI out between paper towels. Store in dark until ready to scope, or in freezer if more than a few hours will pass before using slide.
4. Score 100 interphase cells, if possible, per probe set. Capture four images per probe set for normals, and six per probe set for abnormalities.
5. If more than 50% of cells exhibit an abnormal pattern, the result is abnormal for that probe.
6. Correlate findings with phenotype and chromosomes.

Protocol 16.6 FISH on direct preparations from abortus tissue

Adapted from a protocol contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

For details on the specimen preparation through the slide-making, see Protocol 4.24 making direct FISH preparations from abortus tissue.

I. Principle

A small sample of each POC (products of conception) specimen received for chromosome analysis is reserved for FISH testing, which is only performed on those specimens that have not grown sufficiently for chromosome analysis after 14 days of culture. By this method it can be determined if aneuploidy exists for chromosomes 13, 15, 16, 18, 21, 22, X, and Y in an otherwise unsuccessful analysis. These probes were chosen based on the incidence of these aneuploidies typically found in products of conception.

II. Materials

1. Pepsin Stock Solution: 1 gram lyophilized pepsin dissolved in 10 mL deionized tap water. Aliquot; store in 80 µL aliquots at -20 °C.
2. Pepsin Working Solution: 80 µL pepsin stock solution in 40 mL 0.01 N HCl. Make fresh daily and place in a Coplin jar in a 37 °C water bath.
3. Post Fixation Formaldehyde Solution: Combine 39 mL phosphate buffered saline, 40 µL magnesium chloride, and 1 mL 37% formaldehyde solution. Make fresh daily.
4. 20× SSC, pH 7.0: Dissolve 264 grams 20× SSC powder in 1 liter deionized water. Check pH; adjust to 7.0 if necessary with HCl. Store at room temperature.
5. 2× SSC, pH 7.0: Combine 900 mL deionized tap water and 100 mL 20× SSC solution. Check pH, change to 7.0 with HCl if necessary. Store at room temperature. Place a Coplin jar containing 50 µL of solution in a 37 °C water bath.
6. 70% Ethanol: Combine 700 mL ethanol with 300 mL deionized tap water. Mix and store at room temperature.
7. 80% Ethanol: Combine 800 mL ethanol with 200 mL deionized tap water. Mix and store at room temperature.

III. Method

FISH pretreatment

1. Place a Coplin jar containing 50 mL of 2× SSC in a 37 °C water bath along with another Coplin jar containing 40 mL of 0.01 N HCl. Take 40 µL of aliquoted pepsin from freezer and allow to thaw. Prepare a Coplin jar containing 50 mL PBS and another Coplin jar containing 40 mL of post-fixation formaldehyde at room temperature for later use.
2. Place slides prepared from uncultured amniocytes or products of conception into the prewarmed 2× SSC for 20 minutes at 37 °C. Just before 20 minutes expires, add pepsin to the other Coplin jar containing 0.01 N HCl.

3. Transfer slides to the Coplin jar containing prewarmed pepsin working solution for 13 minutes at 37°C. Agitate occasionally.
4. Transfer slides to a Coplin jar containing 50 mL of PBS at room temperature for 3 minutes.
5. Transfer slides to a Coplin jar containing 40 mL of post-fixation formaldehyde solution for 5 minutes at room temperature.
6. Transfer slides to a Coplin jar containing 50 mL fresh PBS at room temperature for 3 minutes.
7. Immerse slides in 70%, 80% and 100% ethanol series at room temperature for one minute each.
8. Allow slides to air dry and proceed with probe application.

FISH hybridization

9. Apply 10 µL of Aneuvysion X/Y/18 and 10 µL of 13/21 probe cocktails onto a double circle siliconized slide and cover each circle with a 22 mm coverslip. Seal with rubber cement. Denature and hybridize with SOP for your laboratory.

FISH post-wash

10. Place 50 mL of 0.4× SSC/ 0.3% NP-40 in a 73°C water bath and allow to reach temperature.
11. Remove slides from humidity chamber. Carefully remove rubber cement and coverslips.
12. Transfer to a solution of 0.4× SSC/ 0.3% NP-40 at 73°C and agitate for 2 minutes.
13. Transfer to a room temperature solution of 2× SSC/ 0.1% NP-40 and agitate for 1 minute.
14. Drain off excess liquid and apply 10 µL of DAPI or Vectashield and coverslip.
15. Store slides in freezer until ready to read.

FISH scoring signals

16. Analyze 50 cells for each probe. If these are normal, place 50 mL of Denaturation Solution in a 73°C water bath and allow to reach temperature. Mix α satellite 15, α satellite 16 and LSI 22 BCR Probes according to Vysis protocol.

FISH rehybridizing

17. Immerse slides in Denaturation Solution at 73°C for 6 minutes.
18. Denature probes for 4 minutes in a 73°C water bath.
19. Dehydrate slides in a series of ethanol washes (70%, 80%, 100%) for 1 minute each at room temperature.
20. Apply probes to circles on slides. Make sure slides are labeled with probes applied.
21. Cover each circle with a 22-mm coverslip and seal with rubber cement.
22. Place in a humidity chamber and incubate overnight at 37°C.

Protocol 16.7 FISH on cultured non-mitotic abortus tissue

Contributed by Debra Saxe, Emory University School of Medicine, Atlanta, GA

Continued from Protocol 4.24 Making direct FISH preparations from abortus tissue

I. Principle

The following FISH testing is performed on POC specimens that have not grown sufficiently for chromosome analysis after 14 days of culture. By performing FISH, the most commonly seen aneuploidy, for chromosomes 13, 15, 16, 18, 21, 22, X, and Y, can be detected on products of conception that otherwise would have failed. Uncultured amniocytes and products of conception often benefit from a pre-treatment step to minimize excess protein and cell cytoplasm in order to facilitate penetration to the cellular DNA. This protocol is applied to amniotic fluid specimens at above 25-week gestations, or for any amniotic fluid specimen that appears to have a “trashy” appearance when first spun, and also products of conception slides made from tissue.

II. Materials

1. Pepsin Stock Solution: Dissolve 1 g of lyophilized pepsin in 10 mL deionized tap water. Aliquot; store in 80-µL aliquots at -20°C.
2. Pepsin Working Solution: Add 80 µL pepsin stock solution to 40 mL 0.01 N HCl. Make fresh daily and place in a Coplin jar in a 37°C water bath.

3. Post-Fixation Formaldehyde Solution: Combine 39 mL phosphate buffered saline, 40 µL magnesium chloride, and 1 mL 37% formaldehyde solution. Make fresh daily. Discard as Environmental Hazardous Waste.
4. 20× SSC, pH 7.0: Dissolve 264 g of 20× SSC powder in 1 L of deionized water. Check pH, change to 7.0 if necessary with HCl. Store at room temperature.
5. 2× SSC, pH 7.0: Add 100 mL 20× SSC solution to 900 mL deionized tap water. Check pH, change to 7.0 with HCl if necessary. Store at room temperature. Place a Coplin jar containing 50 µL of solution in a 37°C water bath.
6. 70% Ethanol: Add 700 mL ethanol with 300 mL of deionized tap water. Mix and store at room temperature.
7. 80% Ethanol: Add 800 mL of ethanol to 200 mL deionized tap water. Mix and store with flammables at room temperature, using appropriate ventilation. Label as flammable.

III. Method

Follow Rapid Screen Protocol for uncultured amniotic fluid. Air dry.

A. FISH pretreatment

1. Pretreat slides with pepsin according to Amniotic Fluid Pretreatment Protocol. Place a Coplin jar containing 50 mL 2× SSC in a 37°C water bath along with another Coplin jar containing 40 mL of 0.01 N HCl. Take 40 µL of aliquoted pepsin from freezer and allow to thaw. Prepare a Coplin jar containing 50 mL PBS and another Coplin jar containing 40 mL of post-fixation formaldehyde at room temperature for later use.
2. Place slides prepared from uncultured amniocytes or products of conception into the prewarmed 2× SSC for 20 minutes at 37°C. Just before 20 minutes expires, add pepsin to the other Coplin jar containing 0.01 N HCl.
3. Transfer slides to the Coplin jar containing prewarmed pepsin working solution for 13 minutes at 37°C. Agitate occasionally.
4. Transfer slides to a Coplin jar containing 50 mL of PBS at room temperature for 3 minutes.
5. Transfer slides to a Coplin jar containing 40 mL of post-fixation formaldehyde solution for 5 minutes at room temperature.
6. Transfer slides to a Coplin jar containing 50 mL of fresh PBS at room temperature for 3 minutes.
7. Immerse slides in 70%, 80%, and 100% ethanol series at room temperature for one minute each.
8. Allow slides to air dry and proceed with probe application.

B. FISH hybridization

1. Apply 10 µL of Aneuvysion X/Y/18 and 10 µL of 13/21 probe cocktails onto a double circle siliconized slide and cover each circle with a 22 mm coverslip. Seal with rubber cement. Denature and hybridize with SOP for your laboratory.

C. FISH post wash

1. Place 50 mL of 0.4× SSC/ 0.3% NP-40 in a 73°C water bath and allow to reach temperature.
2. Remove slides from humidity chamber. Carefully remove rubber cement and coverslips.
3. Transfer to a solution of 0.4× SSC/ 0.3% NP-40 at 73°C and agitate for 2 minutes.
4. Transfer to a room temperature solution of 2× SSC/ 0.1% NP-40 and agitate for 1 minute.
5. Drain off excess liquid and apply 10 µL of DAPI or Vectashield and coverslip.
6. Store slides in freezer until ready to read.

D. FISH scoring signals

1. Analyze 50 cells for each probe. If these are normal, place 50 mL denaturation solution in a 73°C water bath and allow to reach temperature. Mix α satellite 15, α satellite 16 and LSI 22 BCR probes according to Vysis protocol.

E. FISH rehybridizing

1. Immerse slides in Denaturation Solution at 73°C for 6 minutes.
2. Denature probes for 4 minutes in a 73°C water bath.

3. Dehydrate slides in a series of ethanol washes (70%, 80%, 100%) for 1 minute each at room temperature.
4. Apply probes to circles on slides. Make sure slides are labeled with probes applied.
5. Cover each circle with a 22-mm coverslip and seal with rubber cement.
6. Place in a humidity chamber and incubate overnight at 37°C.

Protocol 16.8 FISH on smears

Contributed by Vancouver General Hospital, Vancouver, B.C.

I. Purpose

To provide a fluorescence in situ hybridization (FISH) procedure using Locus Specific Identifier (LSI), Centromeric Enumeration Probe (CEP) and whole chromosome paint probes on bone marrow and peripheral blood for FISH analysis on smears.

II. Principle

1. Cytocell LSI, CEP and paint probes from Rainbow Scientific or Abbott LSI, CEP or paint probes from Inter Medico are used for identification of genetic aberrations associated with genetic or hematopoietic disorders.
2. These probes are designed to identify various chromosome aneuploidies, translocations, deletions, duplications and other rearrangements associated with specific genetic or hematopoietic disorders in interphase cells (e.g. LSI BCR/ABL probe to identify translocation t(9;22) for a CML patient).

III. Materials

Methanol	Fischer A412-4
Cytocell LSI, CEP or Paint FISH Probes	Rainbow Scientific
Abbott LSI, CEP or Paint FISH Probes	Inter Medico
DAPI ES Counterstain, Cytocell	Rainbow Scientific #DES150L
DAPI II Counterstain, Abbott	Inter Medico #32-804831
Vecta Shield Mounting Medium + DAPI	Vector Lab #H-1200
Vecta 411812200 Mounting Medium	Vector Lab #H-1000
Ethanol 100%, 4L	Brenntag Canada Inc. #EA100-4X4
Absolute Methanol, 4L	FISHER #A412-4
Glacial Acetic Acid, 2.5L	VWR #CAAX0073-59
20× Saline Sodium Citrate (SSC)	Gibco BRL #15557-044
Hybridization Solution B	Cytocell #HB500L
CEP Hybridization Buffer	Vysis #32-804828
LSI/WCP Hybridization Buffer	Vysis #32-804826
Triton X	Sigma #N-6507
dH ₂ O, autoclaved	Stores
Parafilm	Stores
Slides, Microscope 25 × 75 mm	James International Sales Ltd #10202372
Glass Coverslips 18 × 18 mm	FISHER #12541A
Glass Coverslips 22 × 22 mm	VWR #CA 48367-106
Glass Coverslips 22 × 50 mm	VWR #CA 8393-059-1
Pipette Tips, 0.5–10 µL, autoclaved	FISHER #21-119-10
Pipette Tips, 20–200 µL, autoclaved	FISHER #21-197-8G
Eppendorf Tubes, 0.5 mL, autoclaved	Cole Parmer #P-02550-06
Hybridization Chamber	Rainbow Scientific

Tissue-Tek Rack/Containers	
Red Wax Pencil	Grand & Toy
Black Lab Marker	VWR #52877-310
Graduated Cylinders and Beakers	
Gloves	Core Lab
Kimwipes	Stores
Slide Holder, cardboard	
Beaker, plastic	
Styrofoam Tube Rack	
Float Rack (for Eppendorf tubes)	
Forceps	
Coplin Jar, glass	VWR #25457-006
Sharps container	Stores
Stackable Hiboys	

Equipment

ThermoBrite StatSpin	ESBE Model #S500-12
Fume Hood, Labconco Basic 47	VWR Model #22473
Incubators, Multigas	SANYO Model #MCO1755M
Water bath (2)	FISHER #10L-M
Micropipettor, P200 Gilson Pipetman	MANDEL SCI. #GF23601
Slide Warmer	FISHER #12-594
Vortex, Genie 2	FISHER #12-812
Timer	VWR #62344-586
Microcentrifuge	
Biohazard Hood, NuAire	ESBE Lab Supplies Model #NU425-400

Solutions

1× SSC	200 mL 20× SSC
	3800 mL dH ₂ O
	Pour into stackable Hiboy container and mix
1× SSC/0.3% Triton X	200 mL 1× SSC
Working sol'n	600 µL Triton X
	Aliquot into Tissue-Tek container and mix
	Use same day
0.4× SSC	80 mL 20× SSC
	1920 mL dH ₂ O
	Pour into large plastic 2-L container and mix
	Refrigerate at 4 °C
0.4× SSC/0.3% Triton X	200 mL 0.4X SSC
Working sol'n	600 µL Triton X
	Aliquot into Tissue-Tek container and mix

	Use same day
2× SSC	400 mL 20× SSC
	3600 mL dH ₂ O
	Pour into stackable Hiboy container and mix
2× SSC/0.1% Triton X	200 mL 2× SSC
Working Sol'n	300 μL Triton X
	Aliquot into Tissue-Tek container and mix
	Use same day
Fixative	3 Methanol : 1 Acetic Acid

Probe solutions

Note: *turn off room lights for probe preparation!*

Cytocell probes	3 μL probe
Unmade (per slide)	7 μL hybridization sol'n B
	Aliquot into Eppendorf tube vortex and pulse spin suspension in microcentrifuge to mix before use
Abbott probes	1 μL probe
Unmade (per slide)	2 μL dH ₂ O autoclaved
	7 μL LSI buffer
	Aliquot into Eppendorf tube vortex and pulse spin suspension in microcentrifuge to mix before use

IV. Procedure

Making a smear from a peripheral blood specimen

1. Spin down blood tube in centrifuge for 10 min at 1100 RPM.
2. Soak slides in 99% ethanol to remove any debris. Wipe slides with Kimwipes and place in a plastic beaker of dH₂O. Rinse 2x with dH₂O and dry with Kimwipes.
3. **In a biohazard hood, using gloves, gown and sterile technique:** Use a cotton plugged Pasteur pipette to add one drop buffy coat to a slide.
4. Using a second slide placed on top of the drop of buffy coat, smear the drop to create a thin layer on the slide.
5. Air dry slide.
6. Check quality of smear under microscope in the slide making room.

Carry out procedure in fume hood

1. Make up enough fixative to fill a glass Coplin jar.
2. Place the bone marrow or peripheral blood smear into the fixative and set timer for 5 min.
3. Air dry slide, and check quality of preparation under microscope. Mark area to place probe with a wax pencil.
4. Proceed with regular FISH (see Protocol 16.2).

Protocol 16.9 FISH on very small samples

Contributed by Clinical Genetics Laboratories at Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

If a sample is so small that processing it with hypotonic and fixation steps with centrifugation would lose cells and compromise the FISH assay, the cells must be put directly on a microscope slide and fixed *in situ*. Because the cells have not been properly

swollen in hypotonic, their cytoplasmic proteins may be thick enough to prevent proper probe penetration. Therefore, a pepsin or other protease pretreatment is recommended. This is only suitable for interphase FISH studies.

II. Materials

1. Centrifuge
2. Glass slides
3. Pipettes and bulbs
4. 3 : 1 methanol–acetic acid fixative
5. Coplin jars
6. 2× SSC
7. 10% pepsin stock solution:
 - a. 100 mg of Sigma pepsin (#P7012)
 - b. 1 mL of deionized water
 Mix well. Aliquot into 20- μ L portions in microfuge tubes. Store at –20°C.
8. Pepsin working solution: Add 20 μ L of stock pepsin to 40 mL of 0.01 N HCl.
9. Phosphate Buffered Saline (PBS): For one liter:
 - a. NaCl 10.0 g
 - b. KCl 0.25 g
 - c. Na₂HPO₄ 1.3 g
 - d. KH₂PO₄ 0.25 g
 Add the above in 1000 mL of distilled water and mix well.
10. Post-fixation solution:
 - a. 1 mL of 37% formaldehyde
 - b. 0.18 g of MgCl₂
 - c. 39 mL PBS
 Store at 4°C and use within 1 month.
11. 70%, 80%, and 100% ethanol.
12. 0.4× SSC with 0.3% NP-40 (or other post wash)

III. Method

1. Obtain sample with small number of cells such as an amniotic fluid or fibroblast culture with poor attachment, cerebrospinal fluid, flow-sorted cells, etc.
2. Centrifuge the sample at 1000 RPM for 8–10 minutes.
3. Carefully remove the supernatant to just above the pellet.
4. Very gently resuspend the cells using a micropipettor (set at 10 μ L).
5. Flood a glass slide with fixative in a thin layer. Add the cell suspension to the part of the slide where the hybridization will occur. If aqueous, the solution will bead up on the fixative layer.
6. Slowly, using small drops of fresh 3 : 1 methanol–acetic acid fixative, drop fixative around the perimeter of the cell suspension on the slide. As the fluid from the cell beads up, drop a small drop of fixative on top of it. The beaded up fluid will spread out. Drop a few more drops of fixative to rid the slide of any remaining aqueous fluid. It is O.K. to drain the droplets off after 3–4 drops of fixative have been dropped on top of the cell suspension. Concentrate on keeping the cells in a 22 × 22 mm area, if possible. If there are insufficient cells on the slide, and there is remaining cell suspension, continue repeating step 6, and layer cells onto the hybridization area. When sufficient cells are present on the slide, dry the slide and put it in a Coplin jar of fresh fixative for 20–30 minutes. Re-dry the slide.
7. Bake slide for 5 minutes at 90–95°C.
8. Go to the 2× SSC/pepsin steps (pepsin treatment is for 13 minutes at 37°C), as per uncultured amniocytes.
9. Rinse slides in 1× PBS for 5 minutes.
10. Post fix slides in formaldehyde solution for 5 minutes.
11. Rinse slides in 1× PBS for 5 minutes.

12. Dehydrate in 70/80/100% ethanol series for 2 minutes each.
13. Proceed to hybridization.
14. Post wash cells according to manufacturer's instructions for the probes used.

Protocol 16.10 Paraffin-embedded tissue FISH method

Contributed by Clinical Genetics Laboratories at Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Purpose

Interphase FISH has many uses in the diagnosis and prognosis of disease, both acquired and constitutional. Occasionally the only tissue available to do the FISH study is paraffin embedded tissue from the pathologist. The slides must be de-paraffinized and pretreated to optimize the FISH assay.

II. Materials

1. Coplin jars
2. Forceps
3. Water baths, 37 and 80 °C
4. Propar
5. 100% Ethanol
6. Slide warmer, 45–50 °C
7. HYBrite
8. 0.2 N HCl: 1 mL of concentrated HCl/50 mL H₂O
9. 2× SSC, pH 7.0
10. 1 M NaSCN (sodium thiocyanate): 4.05 g/50 mL H₂O
11. Pepsin, 0.5 mg/mL (Sigma #P7012): 25 mg/50 mL of 0.01 N HCl (1 mL concentrated HCl/1000 mL water): store at –20 °C under desiccation.
12. 10% buffered formalin
 - a. 10 mL formaldehyde
 - b. 90 mL H₂O
 - c. 0.4 g NaH₂PO₄
 - d. 0.65 g Na₂HPO₄
 Store at room temperature. Good at least 1 year.
13. Probes, buffers
14. Abbott Molecular Laboratories DAPI I or Vector Laboratories DAPI

III. Methods

1. Obtain 4–5-μm-thick paraffin sections on a positively-charged slide (has + signs on it).
2. Bake slides for 1 hour at 60 °C. Cool.
3. De-oil in 3 × 10 minutes of Propar.
4. 100% ethanol 2 × 5 minutes.
5. Dry slides on 45–50 °C warming plate 2–5 minutes.
6. Pretreat:
 - a. 0.2 N HCl for 20 minutes at RT
 - b. H₂O 3 minutes
 - c. 2 × SSC 3 min
 - d. Pretreatment solution (sodium thiocyanate) 30 min @80 °C
 - e. H₂O 1 min RT
 - f. 2× SSC 2 × 5 min RT
 - g. Blot slides
 - h. Add slides to pepsin solution 10 min @37 °C

- i. 2× SSC 2 × 5 min RT
 - j. Dry slide 45–50°C 2–5 min
 - k. Immerse in 10% buffered formalin 10 min (optional; skip this step if tissue has previously failed hybridizations).
 - l. 2× SSC 2 × 5 min followed by a dip in distilled water
 - m. Dry on 45–50°C slide warmer 2–5 minutes or at room temperature
7. Go to probe protocol as per individual probe locus and brand.
Slides are usually denatured on HYBrite at 75°C for 10 minutes followed by hybridization overnight at 37°C.

Post wash

1. Remove coverslip by immersing slides in 2× SSC with 0.3% NP-40 (150 µL/50 mL) – ROOM TEMPERATURE!
 2. Drain off excess fluid and place slide in 2× SSC with 0.3% NP-40 at 72°C for 2 minutes.
 3. Dry slide in the dark.
 4. Counterstain with DAPI I (1000 ng/mL)** and coverslip.
- **Vector DAPI may be diluted 2 parts to one part of their antifade solution.

Protocol 16.11 VP2000 automated slide processor method for FFPE FISH

Contributed by Clinical Genetics Laboratories at Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

The VP2000 instrument is designed to automate and standardize processing of slide preparations for fluorescence in situ hybridization (FISH). Applications include deparaffinization of thin sections, and FISH pretreatment and dehydration of paraffin sections as well as other specimen types. Preprogrammed protocols are provided for use with Vysis FISH assays. In addition, the instrument is custom programmable for user-specific applications.

The VP2000 is a computer-driven robotic mechanism with 15 reagent basins (12 at ambient temperature and three heated), one water bath, and a heated air drying station. Up to 50 slides are loaded in a slide basket attached to a robotic arm. Following specific protocols loaded onto the computer software in a Word format, slides are transported through various treatment steps that can include slide deparaffinization, specimen pretreatment (2× SSC), protease deproteinization, fixation, water rinses, and ethanol dehydration series. Slides are then ready for manual or automated FISH procedures using FISH reagents of various vendors.

Specimen

Fixed slide preparations of interphase or metaphases cells as appropriate for any FISH protocol. These may include paraffin-embedded thin section slides (must be positively charged slides), standard methanol/acetic acid fixed cytogenetic slide preparations, or other slide preparations such as cytocentrifuge slides, and directly-applied fresh cells which have been fixed after dropping cells on.

II. Reagents and supplies

Reagents

1. PathVysis pretreatment Vysis #30-801250 reagent, 500 mL
2. PathVysis Protease I Vysis #30-801255 buffer solution, 500 mL
3. Abbott Molecular Labs Protease, #02J08-032 (250 mg) or 06J93-001(750 mg)
4. Formalin, 10% buffered S/P #C4320-101
5. Propar Clearant, Anatech Catalog #511
6. Ethanol, 100%, 95%, 70% (100% From Lab Stores or Fisher Scientific)
7. Sodium citrate J.T. Baker #3646-05
8. Sodium chloride VWR #EM-SX0420-1
9. Phosphate buffered saline, Sigma #1003 1 L tablets
10. Magnesium chloride J.T. Baker #2444-01
11. Hydrochloric acid, 1.0 N Sigma #920-1
12. H₂O, distilled

Supplies and equipment

- Abbott Molecular Laboratories VP2000 Slide Processing unit attached to hose for venting to plenum, with PC computer with software.

Safety warnings

10% buffered formalin contains 4% formaldehyde, a toxin and known cancer agent. Handle with standard personal protective equipment. Dispense in a fume hood and use in the VP2000 in conjunction with the exhaust fan unit. Propar is an aliphatic hydrocarbon and may be toxic on contact or with inhalation. Dispense in a fume hood and use with the exhaust fan in the VP2000.

III. Procedure

- Remove any basins from the refrigerator that will be needed and allow 45 minutes to return to room temperature. Fill (**or top up**) basins with appropriate reagents as per specific protocol (see appendix). The basins require 500 mL of reagent equivalent to a 5.0 cm depth. The maximum fill line is 600 mL. Basins can be removed for easy filling and cleaning. Keep the basin covers in place when instrument is not in use or if run is not to be started immediately.
- If necessary, turn on the power switch located at the left side of the instrument.
- Power the PC and monitor. At the login window enter the username [**Vysis**] and the password [**Password**]. Click **OK**.
- If new protease is to be used, add appropriate protease powders to basins as needed (250 mg or 750 mg per 500 mL of 0.02 M HCl). Pipette until dissolved.

Loading slide basket

- Insert tabs on end of basket into slots on the robotic arm.
- Insert slides vertically into the slide basket guide slots. Load up to 50 slides with the label end up.
- Gently press basket down to seat.

Running a protocol

- Press the **Run Screen** button on the left side of the screen to display the Run Screen window.
- Click the **Protocol** button (it may say “no protocol selected”) to select a protocol. A File Open window will display. Select icon of the desired protocol from the list and click **Open**.
- The Heated Basin Temperature window will now display. Click the **Set to Protocol Requirements** button. If the protocol requires heated reagents, color-coded borders appear around the heated basin icons of the Run Screen window as follows:
 Gray: heater off
 Blue: temperature low
 Red: temperature high
 Green: temperature within specified range
- When heated basins have reached specified temperatures, click the **Start** button. Program will run and upon completion will position the slide basket in the home position over the drying station.
- Remove slides from basket and hybridize as appropriate. Wipe down the surface area of the processor. Remove the Protease and pretreatment basins, cover them and store at 4°C.
- Replace basin covers, turn off processor and shut down the PC.

Protocol parameters

Parameters of commonly used VP2000 protocols are documented in a Spreadsheet called VP2000 OHSU Protocols.

Operation notes

- Heated basins
 - Maximum temperatures are
80 °C, Basins 1 and 2
40 °C, Basin 3

- b. If a protocol has been started before the basin(s) have come up to temperature, the Start Run window will appear, indicating that a basin is not ready. Override the window by clicking the **Start Now** button if the basin has time to reach temperature before it is needed. Otherwise click the **Auto Start** button to have the protocol start when all basins reach specified temperature. The **Auto Start** button is the safer choice.
2. Adjusting protocol start
Click on the **Next Step** or **Previous Step** buttons of the Run Program window to start the protocol at a different step.
3. Protocol run window information
As the protocol runs, the display window has color-coded areas displaying the following information
 - a. Upper Area: Displays latest status message, e.g., “Running.” Messages including warnings and errors, and the area is color-coded green, yellow or red to alert user of problems.
 - b. Center Area: Provides specific information on the run, providing an ongoing progress check. Information includes step number, status of the reagent basin used during the step, time elapsed and time remaining.
 - c. Lower Area: Displays the contents of each basin as per the selected protocol.
4. Pausing a run
Click the **Pause** button in the upper right section of the Run window. Pause the run to shorten the timing of a step using the Time Set control, repeat the current step, or move forwards or backwards among the steps. Click the **Continue** button to resume the run
5. Aborting a run
Click the **Abort** button in the upper right section of the Run window. Click **Yes** in the confirmation window that appears. The Basket arm will return to the home position. There is no recovery from an aborted run.
6. Viewing the event log
Click the **Event Log** button on the left margin of the Run window. The log provides the date, day and time of each occurrence, the protocol and step being executed, a description of the event, and the name of the current user. The last 25 runs are maintained automatically on a “first in, first out” basis.

Instrument operating guide

Editing a program

1. Press the **Protocol Editor** button on the left side of the main operation window to display the Protocol Editor window.
2. Click **Open** to display the Open window.
3. Select the icon of the protocol to be edited in the Open window.
4. Edit protocol using the Creating a Custom Protocol instructions of the VP2000 Processor user guide, pages 36–39.

Creating a custom protocol

NOTE: Creating a new protocol may be more easily accomplished by opening an existing protocol, saving under a new name (**Save As** button), editing and resaving.

1. Press the **Protocol Editor** button on the left side of the main operation window to display the Protocol Editor window.
2. Click **New** to display a blank Protocol Editor window.
3. Create new protocol using the Creating a Custom Protocol instructions of the VP2000 Processor user guide, pages 36–39.

Periodic maintenance procedures

Procedure	Interval
Wipe/clean instrument surfaces	Each usage
Clean water bath	Weekly
Oil blower fan	Six months
Annual preventive maintenance	Annually

Reagent expirations

Alcohols: 1 week

SSCs: 3 days

Pretreatment solution: 1 day

Protease: 2 days if stored at 4 °C; discard if left at room temperature longer than 8 hours.

Propar: 30 days

HCl: 30 days

References

VP2000 Processor User Guide, (1999)

PathVysion HER2 DNA Probe Kit package insert (2000)

Protocol 16.12 Plasma cell targeted FISH

Contributed by Vancouver General Hospital, Vancouver, BC

I. Principle

Purpose

To provide a protocol for targeted FISH on plasma cells (PC).

Cytocell and Vysis LSI probes are used for identification of genetic aberrations associated with multiple myeloma. These probes are designed to identify deletion 13q, t(4;14) and deletion 17p. Refer to Protocol 16.2 for method details.

II. Materials

Acetone 100%	Fisher #A949-1
AMCA anti-lambda antibody	Vector #CI-3070
AMCA anti-kappa antibody	Vector #CI-3060
AMCA anti-goat antibody	Vector #CI-5000
Carnation skim milk powder	
Phosphate buffered saline 10× (PBS)	Gibco #70011
Triton X	Sigma #N-6507
Vectashield without DAPI	Vector #H-1000

Solutions

1× PBD (Phosphate buffered detergent)	100 mL 10× PBS 1900 mL dH ₂ O Pour into a large 2-L plastic container Add 2 mL triton X and mix
PBDM (phosphate-buffered detergent with milk)	500 mL PBD 5 g Carnation skim milk powder Shake vigorously and incubate overnight in a 37 °C water bath Decant supernatant and spin at 3000 RPM for 30 min Repeat Freeze supernatant in 10-mL aliquots.
AMCA anti-lambda or anti- Kappa antibody working solutions	50 µL AMCA anti-lambda or kappa antibody 450 µL PBDM Mix and store at 4 °C
AMCA anti-lambda and	50 µL AMCA anti-lambda antibody

Anti-kappa antibody	50 µL AMCA anti-kappa antibody
Working solution	400 µL PBDM
	Mix and store at 4 °C
AMCA anti-rabbit working solution	28 µL AMCA anti-rabbit antibody 472 µL PBDM
	Mix and store at 4 °C

III. Procedure

Procedure for Lambda/Kappa stain on bone marrow smears

1. Fix smears in 100% acetone at 4 °C for 1–15 minutes depending on condition of smears.
2. Apply first antibody layer (AMCA anti lambda, anti kappa or a mixture of both anti lambda- and anti-kappa antibodies), cover with plastic coverslip and incubate smears in a humid chamber at 37 °C for 20 minutes.
3. Remove coverslip and wash smears 3× 1 minute each in room temperature PBD.
4. Apply second antibody layer (AMCA anti-rabbit), cover with plastic coverslip and incubate smears in humid chamber at 37 °C for 20 minutes.
5. Wash smears three times in RT PBD.
6. Dry on 37 °C hotplate.
7. Apply Vectashield without DAPI and coverslip.
8. View smears under fluorescent microscope to assess numbers and morphology of PCs.
9. Mark good areas with black permanent marker on edge of slide.
10. Remove coverslip and wash 3× in RT PBD.
11. Dry on 37 °C hotplate.
12. Proceed with standard FISH procedure (see Protocol 16.2) EXCEPT:
 - Add probe to good area marked out earlier
 - Before post-hybridization wash, soak coverslips off in RT 1× SSC for approximately 3 minutes.
 - After post-hybridization wash, dry on 37 °C hotplate for 5 min.
 - Use Vectashield without DAPI for mounting.

Protocol 16.13 Plasma cell separation for interphase FISH using easy SEP magnet method

Contributed by Oregon Health & Science Center, Knight Diagnostic Cytogenetics Laboratory, Portland, Oregon

Safety warnings

The Big Easy EasySep Magnet® produces a strong magnetic field. Keep away from pacemakers, magnets, computer disks, watches, and other objects that respond to magnetic fields. Magnetic nanoparticles are dextran-coated iron-oxide particles and are not known to be hazardous. CD138 Positive Selection Cocktail contains no known hazardous components.

EasySep® RBC Lysis Buffer is irritating to the eyes. Protective gloves, laboratory coat, and safety glasses are required. In case of contact, immediately flush eyes or skin with copious amounts of water for 15 minutes while removing contaminated clothing and shoes. If inhaled remove to fresh air. If swallowed, wash out mouth with water provided the person is conscious. In all cases of exposure get medical care immediately in the Emergency Room. Clean up spills with absorbent material and put in container for disposal. Read the SDS.

I. Principle

The purpose of this procedure is to separate plasma cells from blood or bone marrow for FISH. Many plasma cell (CD138+) dyscrasias are represented at such a low percentage in bone marrow or blood that interphase FISH abnormality rates approach the normal cutoff values and are difficult to interpret. Separation of these cells from other white blood cells before doing the interphase FISH panel yields a much higher abnormality rate than non-separated samples. The assay will be used to concentrate the plasma cells in order to perform meaningful interphase FISH.

Following are some important definitions regarding plasma cells:

Plasma cells, also called plasma B cells, plasmocytes, and effector B cells, are white blood cells that secrete large volumes of antibodies. They are transported by the blood plasma and the lymphatic system. Like all blood cells, plasma cells ultimately originate in the bone marrow; however, these cells leave the bone marrow as B cells, before terminal differentiation into plasma cells, normally in lymph nodes.

Plasma cell dyscrasias are a diverse group of neoplastic diseases involving proliferation of a single clone of cells producing a serum M component (a monoclonal immunoglobulin or immunoglobulin fragment) and usually having a plasma cell morphology; it includes multiple myeloma and heavy chain diseases.

Multiple myeloma, also known as plasma cell myeloma or Kahler's disease (after Otto Kahler) is a cancer of plasma cells. In multiple myeloma, collections of abnormal plasma cells accumulate in the bone marrow, where they interfere with the production of normal blood cells. Most cases of myeloma also feature the production of a paraprotein—an abnormal antibody which can cause kidney problems. Bone lesions and hypercalcemia (high calcium levels) are also often encountered.

CD138: "CD" stands for "cluster of differentiation." CD molecules are cell surface molecules (antigens) used for immunophenotyping cells. They are usually receptors or ligands important to the cell type. The CD138 antigen is expressed in normal and malignant plasma cells but not in mature B cells. Plasma cell dyscrasias usually carry this cell surface marker; however, it may be lost in some tumors and magnetic separation is not useful for these cases.

II. Materials

Specimen

1. Specimen description

An 0.2–1.0 mL (up to 4.5 mL) EDTA or sodium heparin anti-coagulated sample of blood or bone marrow is used per separation run. Plasmacytoma tumors may also be used, either from short-term cultures or collagenased cell suspensions in medium. Anticoagulated blood/marrow cells in RPMI or minced tumor cell suspension in RPMI are also appropriate. The specimen is kept at ambient temperature.

2. Specimen acceptability

Acceptable specimens are 0.2–1.0 mL (up to 4.5 mL) EDTA or sodium heparin anti-coagulated samples of blood or bone marrow or plasmacytoma tumors in medium or collagenase at ambient temperature. Criteria for specimen acceptability and rejection follow the procedures described in Accessioning Standard Operating Procedures.

Unacceptable specimens are formalin-fixed samples, frozen samples, or any sample exceeding 7 days since collection.

Note: This test requires the expression of CD138+ cell surface antigens that are labile when left for long periods of time. Therefore, it is best to process the sample as soon as possible.

3. Actions if the specimen becomes unavailable or unusable

Specimens are evaluated for storage conditions, labeling, integrity, packaging, sample type, age (if applicable), and overall appearance. The technologist will notify Customer Service to contact the client group if the sample cannot be processed; otherwise, the technologist will process the samples. If the sample results indicate the sample results are unreliable or do not match the submitted order, then the technologist will notify Customer Service to contact the client group and report the laboratory is unable to produce accurate results.

Reagents

1. EasySep® Human Whole Blood CD138 Positive Selection Kit, StemCell Technologies, #18387, for labeling 60 mL whole blood.

Reagents may be purchased separately or in a kit. See below for individual requirements of each component.

Separate components

- a. Monoclonal antibodies purified from hybridoma culture supernatant, bound in bispecific tetrameric antibody complexes that are directed against CD138 and dextran. EasySep® Human Whole Blood CD138 Positive Selection Cocktail 3×, 1.0 mL, StemCell Technologies #18387. Stable at 4°C for 2 years. Do not freeze this product. Contents sterile in unopened tube. Shipped at room temperature (15–25 °C), but should be refrigerated upon receipt.

- b. RoboSep® Buffer, StemCell Technologies #20104

Store at 4°C. May be substituted with phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) and 1 mM EDTA (Ca^{2+} and Mg^{2+} free).

- c. EasySep® Whole Blood Magnetic Nanoparticles, 3× 1.0mL (magnetic dextran iron nanoparticles), StemCell Technologies #18180.
Store at 4°C.
- d. EasySep® RBC Lysis Buffer 10× Concentrate, StemCell Technologies #20110 for 10 mL; #20120 for 100 mL.
Stable at 20°C (room temperature) for 2 years. Contents sterile in unopened tube. This product may be shipped at room temperature (15–25°C), and should be refrigerated upon receipt.
- e. Modified Carnoy's methanol–acetic acid fixative
Prepare before each use. Stable at room temperature for 4–8 hours.
- f. Sanimaster disinfectant

Equipment

1. Centrifuge, Sorvall Legend XT
2. Polypropylene centrifuge tubes, 15 mL, Corning #430052
3. Sterile pipettes, 1 mL, Falcon® #35-7521
4. Sterile pipettes, 10 mL, Falcon® #35-7551
5. Pipette Aid, Drummond 100
6. Tips, 0.5 mL, Fisher #1368362A
7. Tips, 1.25 mL, Fisher #1368362B
8. Tips, 2.5 mL, Fisher #1368362C
9. The Big Easy™ EasySep® Magnet, StemCell #18001
Caution: Strong magnetic field. Keep away from pacemakers, magnets, computer disks, watches, and other objects that respond to magnetic fields.
10. Round bottom snap cap tubes, 14 mL, Falcon® #352057

III. Method

1. Remove the RoboSep® buffer, EasySep® 1× Lysis Buffer (1:10 dilution: concentrate is diluted at least 1 hour before use 1:9 with distilled water), and the EasySep® CD138 Positive Selection Kit from the refrigerator to warm to room temperature.
2. Prepare fresh 3 : 1 methanol–acetic acid fixative.
3. Transfer 0.2–1.0 mL of bone marrow, whole blood, or minced tissue, depending on the volume received in the laboratory, to a labeled 15 mL conical centrifuge tube. If the bone marrow is in transport medium, pellet the cells and remove most of the supernatant before beginning the separation method.
4. Add RoboSep® buffer to sample until final volume reaches 10–14 mL.
5. Centrifuge sample for 10 minutes at 1400 RPM to remove any soluble factors, fat cells, and platelets that can interfere with subsequent separation.
6. Remove supernatant with a disposable transfer pipette. Be careful not to disturb the cell pellet. Discard excess in waste container containing Sanimaster disinfectant.
7. Gently resuspend the buffy coat/cell pellet with 1 mL RoboSep® buffer and transfer sample/buffer suspension to a labeled 14 mL round-bottom tube.
8. Add 1 mL 1× RBC lysis buffer to sample to obtain a ratio of 1:1 cell suspension to lysis buffer.
9. Gently mix sample suspension well with a disposable transfer pipette, and proceed to CD138 Positive Cell Selection step immediately.

CD138-positive cell selection method

1. Mix CD138 Selection Cocktail vial by inversion. Do not vortex.
2. Add 50 µL of CD138 Positive Selection Cocktail to specimen/RBC lysis buffer mixture.
3. Gently mix by pipetting up and down with a disposable transfer pipette and incubate at room temperature for 15 minutes.
4. After the 15 minute incubation, mix (by inverting vial) and add 50 µL of EasySep® Magnetic Nanoparticles. Add the Nanoparticles directly into the round bottom tube containing the CD138 Selection Cocktail/Specimen/RBC Lysis buffer mixture and gently pipette up and down to mix. Incubate 10 minutes.

5. After the 10 minute incubation add RoboSep® buffer to specimen mixture until final volume reaches 5 mL. Gently mix with a disposable transfer pipette and place the round bottom tube in EasySep® Magnet. Incubate for 5 minutes at room temperature.
6. After the 5 minute incubation, pick up the magnet (do not remove tube), hold the tube in place by pressing with index finger to push the tube against the side of the magnet and in one continuous motion, invert the magnet and tube, pouring off the supernatant into a waste container. The magnetically labeled cells will remain bound inside the tube, held by the magnetic field of the magnet.
7. Hold the magnet and tube in the inverted position for 2–3 seconds; return the tube to the upright position. Note: Do not shake or blot off any drops that may remain hanging from the mouth of the tube. This completes one round of magnetic separation.
8. Remove the tube from the magnet. The CD138+ cells/Magnetic Nanoparticle mixture will appear as an orange film around the side of the tube the same height as the magnet walls.
9. Resuspend cells with RoboSep® buffer until final volume reaches 5 mL with a disposable transfer pipette. While adding the buffer to the specimen, rinse the remaining cells off the walls of the tube.
10. Repeat steps 5–9 for a total of 3 rounds of magnetic separation/washing.
11. Pour off the final round of supernatant and remove the round bottom tube from the magnet.
12. Add approximately 300 µL of RoboSep® buffer using a disposable transfer pipette to the highly purified cells. Gently pipet up and down rinsing the CD138+ purified cells/buffer mixture along the inside of the tube walls to wash all the cells into the bottom of the tube.
13. Slowly add 1 mL fixative drop by drop, with agitation of cells between each drop.
14. Add fixative until final volume reaches 5 mL.
15. Transfer contents to a 15 mL conical tube properly labeled with the patient identification.
16. Store tube in –20 °C freezer until ready for slide making.

Slide-making

1. Centrifuge specimen for 10 minutes at 1000 RPM. Remove the supernatant to within ¼–1 mL of pellet.
2. Add fixative to resuspend pellet, as needed.
3. Using a micropipettor, apply 5–10 µL of cell suspension to slide. Allow to dry and evaluate on a phase microscope for cell density. Apply more cell suspension to the slide, if needed.
4. Bake slide for 5 minutes at 90 °C. Proceed to FISH method with pepsin pretreatment as for amniocyte interphase FISH.

Protocol 16.14 Preimplantation genetic testing (PGD) for aneuploidy

Contributed by Clinical Genetics Laboratories at Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Purpose

Interphase cells biopsied from 8–10 cell-stage embryos may be biopsied for single-cell FISH analysis using probes specific for chromosomes with increased risk for aneuploidy (e.g., 13, 15, 16, 17, 18, 21, 22, X, and Y) or unbalanced translocations (customized probe sets). Embryos with aneuploidy for these chromosomes, unbalanced translocations, or polyploidy, are not used for transfer, thus increasing a couple's chance of producing a normal offspring.

II. Principle

Interphase cells, which have been fixed onto glass slides by the embryologist, are denatured and hybridized with specific probes that have been labeled with different colors of fluorochrome. After annealing the probes to the target sequences, excess signal is washed off and the slide is mounted in antifade for examination on a fluorescence microscope with multi-color capability and an imaging system to capture all the signals. Additional hybridizations may be done on the same cells to investigate the status of parental translocations or for additional aneuploidy probe sets.

Safety warnings

Probes contain formamide, which is a harmful substance. Denaturation temperatures may be very hot, and care should be taken not to touch the hotplate with bare skin.

Formamide may be harmful by inhalation, ingestion or skin absorption. Causes skin irritation. May have reproductive effects. Flush skin or eyes for 15 minutes in case of contact. Remove to fresh air if inhaled. Seek medical attention if swallowed. Wear gloves and lab coat at all times when handling.

III. Materials

Specimen

Single blastomeres are received on slides from the Andrology/Embryology Laboratory or from outside referrals after 24 hours notice. Unacceptable specimens are those in which a nucleus is not visible or is fragmented, damaged, or covered with debris. However, these specimens are not refused.

Reagents and supplies

Abbott Multivision PB probes, Catalog #30-111085 (13, 16, 18, 21, 22) 60 µL prediluted
 Abbott Multivision 4 color custom probes (15, 17, X and Y), #30-111086, 60 µL prediluted.
 HYBrite hotplate (Abbott)
 37°C incubator
 12-mm circular coverslips
 Parafilm
 Glass Coplin jars
 Micropipettor (20 µL capacity)
 Tips for micropipette
 Timer
 Microfuge
 Water bath with 80°C capability
 Forceps
 Thermometer
 NP-40
 0.4× SSC with 0.3% NP-40
 2× SSC with 0.3% NP-40
 Antifade II solution (Abbott #32-804030 or Vector Vectashield #H-1000)
 Control male slides from peripheral blood sample
 Distilled water, or, 70% formamide in 2× SSC pH 7.0 for removing first probe set from slides, 72°C.

IV. Method

Either probe mixture may be used first; however, if the sex of the embryos is needed, start with the 15, 17, X, and Y probe mixture. Any abnormal embryos in the first hybridization need not be rehybridized with the second set unless the physician requests it. For patients being tested for translocation probes, see Translocation PGD probe method.

A. Abbott probe set for 15, 17, X, and Y:

1. Check all slides under phase microscope to find nuclei, and make a marker slide of each preparation.
2. Turn HYBrite on and program it to denature (melt) at 73°C for 5 minutes and hybridize (HYB temp) at 37°C for 4 hours to overnight. Moisten the paper towel strips in the troughs of the HYBrite.
3. Fill any slots in the HYBrite that will not be used with blank slides.
4. Pre-warm PGT probes to room temperature for 5–10 minutes.
5. Centrifuge probe bottles in the microfuge for 1–3 seconds to collect the probe in the bottom of the bottle.
6. Remove 3 µL of probe and deposit it on the circle where the blastomere nucleus is located. Carefully add a coverslip, avoiding bubbles.
7. Cut a piece of Parafilm and lay it over the coverslip to prevent the probe from drying out.
8. Put the slide into the HYBrite machine. When all slides have been added, close the cover and push the “Yes” button on the HYBrite to start the program.
9. While the hybridization takes place, or the next morning if overnight hybridization is used, fill one Coplin jar with 0.4× SSC/0.3% NP-40 (and mix well), and a second jar for destaining with distilled water **or** 70% formamide in 2× SSC.

- Place both jars in the water bath at about 40–50°C, and then turn the water bath up to 8 within 45–60 minutes of the end of the hybridization period. (If Coplin jar is added to very hot water it will break).
10. Fill a second jar with 2× SSC with 0.15% NP-40, mix well, and leave at room temperature.
 11. When the HYBrite program has finished, remove the slides from the HYBrite and remove the Parafilm carefully. Gently but persistently shake the slide to dislodge the coverslip. When it has slid off to one side, it can be popped off using forceps with an upward motion.
 12. Immediately add the slide to the hot 0.4× SSC with 0.3% NP-40. DO NOT AGITATE! Check temperature to be sure it stays at 73 +/−1 °C. Incubate for 5 minutes. Do only a few slides at a time to control the temperature.
 13. Place slides in the room temperature 2× SSC with 0.15% NP-40 for 1 minute, then place them in a drawer to drain dry in the dark.
 14. Mount slides with antifade (18 µL/slide) and coverslip with a 24×50 mm coverslip. Press out any excess antifade. If any additional probes will be used sequentially, place a Coplin jar of distilled water to warm up in the 72°C water bath for removal of the first probe set after scoping the slides (start them in the 42°C water bath to prevent breaking the jar).
 15. Observe on the Zeiss or Nikon microscope with the Cytovision imaging computer.
 16. Finding the nucleus is very time consuming and may require up to 10–20 minutes. Start by focusing on the ground glass label of the marker slide. Then center the dot on the marker slide on the microscope under DAPI filtered epi-illumination. Then place the FISH slide onto the stage without moving it. Look on low power for the scribed circle around the cell. If it is visible, focus it, and then move to high power and change to the dual bandpass filter set. Scan the area with the circle inscribed around the cell, being sure that the plane of focus is correct. The best filter to use to scan is the FITC/Rhodamine filter, as the correct plane of focus can be found by identifying fluorescent debris floating in the mounting medium. If the nucleus cannot be found, use the marker slide to locate it more precisely. Once the cell is located, capture it in the following order: “gray” counterstain (antibody to proteins labeled aqua), followed by aqua, blue, and the last 3 colors, green, red, and gold, in any order. Write the coordinates and number of signals on a count sheet.

B. Additional probes for ploidy of 13, 16, 18, 21 and 22:

1. Clean oil off of slides with alcohol wipe and soak off coverslip in 2× SSC with 0.15% NP-40 for a few minutes.
2. Soak slide in a second 2× SSC for 5 minutes.
3. Place slide into 72 °C distilled water or 70% formamide/2× SSC for 2 minutes.
4. Dehydrate through 70, 80, and 95% ethanols for 2 minutes each. Dry slide.
5. Mix probes with index finger and centrifuge for a few seconds.
6. Place 3 µL of probe mixture onto each scribed area of each slide and coverslip with a 12 mm circle coverslip. Parafilm the coverslip.
7. Turn HYBrite on and program it to denature (melt) at 73°C for 5 minutes and hybridize (HYB temp) at 37°C for 4–18 hours. Moisten the paper towel strips in the troughs of the HYBrite. Fill any slots in the HYBrite that will not be used with blank slides.
8. Put the slide into the HYBrite machine. When all slides have been added, close the cover and push the “Yes” button on the HYBrite to start the program.
9. Hybridize slides 4 hours to overnight.
10. Rinse slides as in A, steps 12 and 13. Mount slides in antifade without any counterstain.
11. Observe as in A.
12. Capture the cell as before, and record the results on the Analysis Sheet.
13. If additional probes are to be used, repeat section B using the new probe sets.

Immediately FAX results to referring physician/counselor.

Color key for probes:

PB set

- LSI 13 = Spectrum Red
- CEP 16 = Spectrum Aqua
- CEP 18 = Spectrum Blue
- LSI 22 = Spectrum Gold
- LSI 21 = Spectrum Green

5-color custom set

CEP 15 = Spectrum Orange
 CEP 17 = Spectrum Aqua
 CEP X = Spectrum Green
 CEP Y = Spectrum Blue

Protocol 16.15 Preimplantation genetic testing (PGD) FISH for translocations

Contributed by Clinical Genetics Laboratories at Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Purpose

Couples with balanced translocations are at risk for unbalanced offspring. Using arm and centromere-specific probes, interphase cells from preimplantation blastomeres can be tested for the balanced versus unbalanced state. Interphase cells biopsied from 8–10 cell-stage embryos may be biopsied for single-cell FISH analysis using probes specific for the translocation products as well as sequential hybridization to test for aneuploidy (e.g., 13, 18, 21, 16, 22, X, and Y). The 15, 16, and 22 ploidy is not performed unless there is adequate time, and usually there is not, because there is a 36 hr. time frame for results. Embryos with the unbalanced state, or with aneuploidy for the other chromosomes, or any evidence for polyploidy, are not used for transfer, increasing a couple's chance of producing a normal offspring. In interphase, there is no way to determine whether the balanced state represents a balanced translocation or absence of the translocation with normal homologues.

II. Principle

For non-acrocentric chromosome translocations, probes for one of the centromeres and both of the sub-telomere specific regions of the involved arms should be pre-tested on parental chromosomes to be sure they are specific and do not cross hybridize with any other chromosome(s). Laboratory policy requires both parents to have all probes tested on their chromosomes due to variability in centromeric and subtelomeric specific probe signal patterns within the normal population. For Robertsonian translocations, only the sub-telomere specific probes for the involved arms are needed for the testing. The probes should be chosen with different colors so they may be distinguished from each other. This may require mixing probes from different manufacturers, since some probes are limited in color choices. After the parental translocation has been tested with the probe set, some donated blastomeres should then be tested to be sure that the probes work in the proper tissue type. Metaphase (parental) and interphase cells (parental and blastomere) which have been fixed onto glass slides are denatured and hybridized with specific probes that have been labeled with different colors of fluorochrome. After annealing the probes to the target sequences, excess signal is washed off and the slide is mounted in DAPI/antifade for examination on a fluorescence microscope with multi-color capability and an imaging system to capture all the signals.

Safety warnings

Probes contain formamide, which is a harmful substance. Formamide may be harmful by inhalation, ingestion or skin absorption. Causes skin irritation. May have reproductive effects. Flush skin or eyes for 15 minutes in case of contact. Remove to fresh air if inhaled. Seek medical attention if swallowed. Wear gloves and lab coat at all times when handling.

Denaturation temperatures may be very hot, and care should be taken not to touch the hotplate with bare skin.

Specimen

For probe development, sodium heparinized blood is cultured as for constitutional blood chromosomes. For probe use after validation, single blastomere cells are received on slides from the Andrology/Embryology Laboratory after at least 24 hours notice

Unacceptable specimens are those in which a nucleus is not visible or is fragmented, damaged, or covered with debris. However, these specimens are not refused.

III. Materials

Supplies

1. HYBrite hotplate (Abbott)
2. 37°C incubator
3. 12-mm circular coverslips
4. 22 × 22 mm coverslips
5. 24 × 50 mm coverslips
6. Parafilm
7. Abbott, Cytocell, Q-Biogene, and/or other probes to be used for the translocation with hybridization buffers
8. Abbott PGT probes for subsequent aneuploidy study
9. Glass Coplin jars
10. Micropipettor (20 µL capacity)
11. Tips for micropipette
12. Timer
13. Microfuge
14. Rubber Cement
15. Water bath with 80°C capability
16. Forceps
17. Thermometer
18. NP-40
19. 0.4x SSC
20. 2x SSC with 0.1% NP-40
21. DAPI II (Abbott)
22. Antifade II solution (Abbott #32-804030)
23. Control male slides from peripheral blood sample

IV. Method

A. Parental blood probe studies

1. Make slides for FISH as usual. Bake slide 5 minutes at 90–95°C.
2. Place slides in 2x SSC at 37°C for 30 minutes, and dehydrate slides through 2 minute changes of 70, 80, and 95% ethanol. Dry slides.
3. Centrifuge probe bottles in the microfuge for 1–3 seconds to collect the probe in the bottom of the bottle.
4. Mix probes: Use manufacturer's recommended volume for each probe, e.g., 1 µL for Abbott, 3 µL for Cytocell, and bring to 10 µL with buffer.
5. Coverslip with a 22 × 22 mm glass coverslip, seal with rubber cement, and run on the HYBrite, program #9 (72°C for 2 minutes, 37°C overnight).
6. The next morning, fill a jar with 0.4x SSC/0.3% NP-40, and mix well. Place in the water bath at about 40–50°C, and then turn the water bath up to 9. (If Coplin jar is added to very hot water it will break). Fill a second jar with 2x SSC/0.3% NP-40, mix well, and leave at room temperature.
7. Remove rubber cement and pop off coverslip. Add slide to 0.4x SSC with NP-40 with agitation for the first 3 seconds, and allow to rinse for 2 minutes.
8. Place slides in 2x SSC with NP-40 at room temperature for 30 seconds or so.
9. Mount slide with DAPI II and a 24 × 50 mm coverslip. Press out excess mounting medium.
10. Observe at least 10 metaphase cells for signal specificity and sensitivity. Observe at least 50 interphase cells for signal intensities and background.
11. If probes are acceptable, FISH study is complete. (Note: if OHSU laboratory has never performed chromosomes on the patient, prepare at least one good G-banded metaphase to demonstrate the translocation).
12. If probes show too much background or cross hybridize, other manufacturer's probes may be tried.

B. Blastomere testing

1. Check all blastomere preparation slides under phase microscope to find nuclei, and make a marker slide of each preparation. Prepare control slide with metaphases.
2. Turn HYBrite on and program it to denature (melt) at 73°C for 5 minutes and hybridize (HYB temp) at 37°C for overnight. Moisten the paper towel strips in the troughs of the HYBrite.
3. Fill any slots in the HYBrite that will not be used with blank slides.
4. Pre-warm translocation probes, and probe buffers to room temperature for 5–10 minutes.
5. Centrifuge probe bottles in the microfuge for 1–3 seconds to collect the probe in the bottom of the bottle.
6. Mix probes: Use manufacturer's recommended volume for each probe, e.g., 1 µL for Abbott, 3 µL for Cytocell, and bring to 10 µL with buffer. This is enough for one parental blood study or 3 embryos.
7. Remove 3 µL of probe mix and deposit it on the circle where the blastomere nucleus is located. Carefully add a 12 mm circular coverslip, avoiding bubbles.
8. Cut a piece of Parafilm and lay it over the coverslip to prevent the probe from drying out.
9. Put the slide into the HYBrite machine. When all slides have been added, close the cover and push the "Yes" button on the HYBrite to start the program.
10. Allow hybridization to take place overnight for the translocation probe set.
11. The next morning, fill a jar with 0.4× SSC/0.3% NP-40, and mix well. Place in the water bath at about 40–50°C, and then turn the water bath up to 9. (If Coplin jar is added to very hot water, it will break).
12. Fill a second jar with 2× SSC/0.3% NP-40, mix well, and leave at room temperature.
13. Fill a third jar with distilled water for use after microscopy, to destain the slide for the next probe set to be hybridized. Place in a 72°C water bath.
14. When the HYBrite program has finished, remove the slides from the HYBrite.
15. Mount slides with DAPI II (18 µL/slides) and coverslip with a 24 × 50 mm coverslip. Press out any excess mounting medium.
16. Observe on the Zeiss microscope with the Cytovision imaging computer.
17. Once the cell is located, capture it and label each capture with the embryo designation (e.g., E-1/B-1 for embryo 1 blastomere 1). Write the coordinates and number of signals on a count sheet.
18. When all captures are completed and pictures printed out, remove oil from coverslip with alcohol and remove coverslip by soaking slide in 2× SSC with 0.15% NP-40 until the coverslip can easily be slipped off. Immediately place slide into 72°C distilled water for 2 minutes to remove probe signal, if rehybridization is necessary.
19. Prepare the report which should describe whether each embryo is euploid or aneuploid, and if it is balanced or unbalanced for the translocation probe set. Include a note that the balanced embryos may carry the translocation.

Protocol 16.16 Post-FISH BrdU antibody detection

Adapted from a protocol contributed by Amy Hanlon-Newell, Knight Diagnostics-Oregon Health & Science University Research, Cytogenetics Laboratory, Portland, OR

I. Method

1. Remove slides from post-wash RT (room temperature) SSC.
2. Drain off slides.
3. Preblock slides in 2× SSC/BSA solution (0.05 g BSA in 10 mL 2× SSC + 0.15% NP-40) at RT for 10 minutes in dark.
4. Drain slides.
5. Combine 30 µL FITC-BrdU with 270 µL 2× SSC/BSA.
6. Apply 60 µL of the antibody/SSC/BSA mixture (from step 5) to each slide.
7. Cover with plastic coverslip.
8. Incubate 30 minutes in humid chamber at 37°C.
9. Remove plastic coverslip.
10. Rinse slides three times, 2 minutes each, in 2× SSC + 0.15% NP-40 solution.
11. Mount glass coverslip with 18 µL DAPI II.
12. Read.

Protocol 16.17 Same-day HER2 IQ-FISH pharmDx™ for breast tissue

Anonymous Contributor

I. Principle

Nuclei from formalin-fixed, paraffin-embedded (FFPE) breast tumor tissue are examined for the presence of *HER2* (*ERBB2*) gene amplification. Processing steps include deparaffinization, rehydration, pre-treatment, proteolytic digestion, denaturation, hybridization (60–90 minutes), stringent post wash, and DAPI counterstaining [1]. Two independent readers count the number of *HER2* (red) and 17-centromere (green) signals observed in 20 invasive tumor nuclei (see Note 1, Scoring Criteria for exclusion rules). If more than one tumor population is present, twenty nuclei are examined from each different invasive tumor population that is present in the breast tissue.

ASCO/CAP 2013 (2) guidelines define *HER2* FISH reportable categories as follows:

Negative: *HER2/CEN-17 ratio < 2.0 and average HER2 signals/total cells < 4.0*.

Equivocal: *HER2/CEN-17 ratio < 2.0 and average HER2 signals ≥ 4.0 and < 6.0*. If a second test could not be ordered, e.g., due to technical problems, the result is reported as Indeterminate.

Amplified: *HER2/CEN-17 ratio ≥ 2.0 OR average HER2 signals per nucleus ≥ 6.0*.

Note: H&E slides must be reviewed with a pathologist, who will identify the infiltrating tumor area(s) to be examined. In situ tumor should not be scored. The pathologist can also mark normal cells that can be used as an internal control.

Scoring criteria must follow ASCO/CAP 2013 guidelines (unless superseded by more current guidelines). Because certain conditions fall under reporting restrictions, alert the director if amplified cells were only observed within in situ tumor cells, or if amplification was observed only in 10% or less of the tumor.

Two “blind” readers should read each case; the pathologist or director can be the second reader. When results are equivocal in the first 20 invasive tumor nuclei, the first reader will examine 20 additional nuclei. If still equivocal, the second reader will examine 20 additional nuclei. If still equivocal, a reflex test (same specimen using the alternative test) or new test (new specimen using same or alternative test) should be done.

Specimen requirements

All surgically excised or biopsied breast tissue must be promptly placed in a sufficient volume (at least 10 : 1 formalin to specimen ratio) of 10% neutral-buffered formalin fixative, and fixed for at least 6 hours, but no more than 72 hours prior to tissue processing. The exception to the above minimum fixation interval requirement is core needle breast biopsies, which require a minimum of 1 hour fixation. The final report includes these details and warns when a specimen has not met ASCO/CAP 2013 guidelines.

Tissue specimens should be cut into sections of 4–6 µm and mounted on Dako silanized slides #S3003 (recommended) or poly-L-lysine-coated slides. A minimum of two serial sections should be cut, one stained with hematoxylin and eosin (H&E stain) for tumor evaluation, and one for *HER2* gene amplification detection. Specimens should be analyzed within 4–6 months of sectioning when stored at room temperature (20–25 °C).

Slide review

H&E-stained slides are reviewed with a pathologist, who marks both the tumor cells being targeted and normal cells that can serve as an internal control. The pathologist will also determine if there are additional invasive tumor populations requiring examination. The technologist will etch the borders of the targeted tumor areas on the back of the silanized slide to be processed. Each *HER2* run concurrently processes a control that has amplified and normal tissue; using a new kit, new lot or a different shipment date of the same lot must also include an equivocal control. The pathologist reviews hybridized slides and confirms signal results.

Shipment warning

HER2 IQFISH pharmDx™ (2) should not be exposed to high temperatures during transport; therefore, dry ice should still be present upon receipt. Some kit components may arrive unfrozen; this will not affect the kit. Negative and amplified controls must be run with every hybridization session. New lots and old lots with different shipment dates must add an equivocal control in order to verify that the probe works as expected.

Precautions

1. For in vitro diagnostic use only.
2. For professional users.
3. Specimens (before and after fixation) and all materials exposed to them should be handled as if capable of transmitting infection and should be disposed of with proper precautions. Never pipet reagents by mouth and avoid contact the skin and mucous. If reagents come in contact with sensitive areas, wash with copious amounts of water.
4. Minimize microbial contamination of reagents to avoid erroneous results.
5. Incubation times and temperatures, or methods other than those specified, may give erroneous results.
6. Tissue fixation methods and thickness of specimen other than those specified may affect tissue morphology and/or signal intensity.
7. Avoid evaporation of *HER2/CEN-17 IQISH Probe Mix* during hybridization by ensuring sufficient humidity in the hybridization chamber.
8. Reagents have been optimally diluted. Further dilution may result in loss of performance.
9. Wear appropriate personal protective equipment to avoid contact with eyes and skin. Refer to the Safety Data Sheet (SDS) for additional information.
10. Only clean staining jars should be used for the pepsin immersion method (step 2, method C).

II. Materials (for breast tissue assays)

A. Materials provided in the Dako kit [1]

Kit materials are sufficient for 20 tests if using the following quantity per slide:

1. Target area = 22 × 22 mm
2. Pepsin = 250 µL (5–8 drops) per slide (vial 2A),
3. Probe mixture = 10 µL per slide (vial 3*)
4. DAPI mounting mixture = 15 µL per slide (vial 5*).

*Note: Solutions in vial 3 and vial 5 are viscous and may require quick micro-centrifugation to collect the reagent.

Important: Further dilution of the reagents or alteration of incubation temperatures may give erroneous or discordant results. Differences in tissue processing and technical procedures in the user's laboratory may also invalidate the assay results.

1. **Stock Vial 1: Pre-Treatment Solution (20×)**, 150 mL, concentrated 20× MES (2-[N-morpholino]ethanesulphonic acid) buffer. Store in the dark at 2–8 °C. Tolerates frozen storage. Freezing and thawing up to 10 times does not affect performance. When thawing, crystals may occur, but they will dissolve at room temperature. Ensure that no crystals are present before preparing reagent. At product concentrations, these substances do not require hazard labeling; however, used and unused reagents should be collected for regulated disposal and should not be discarded down the sink. See **Preparing diluted buffer solutions** later for dilution instructions.

2. **Vial 2A: Pepsin**, 4 × 6.0 mL, pH 2.0. Contains stabilizer and an antimicrobial agent. Pepsin may be affected adversely if exposed to heat; therefore, storage and application should be at 2–8 °C. Do not leave at room temperature. Pepsin also contains 5–10% propan-2-ol, but at product concentrations does not require hazard labeling. Because it contains pepsin A, it may cause an allergic reaction. Consult with Chemical Hygiene Officer for disposal requirements.

Vial 2B: Pepsin diluent (10×), 24 mL, pH 2.0. Contains an antimicrobial agent. Store in the dark at 2–8 °C. Tolerates frozen storage, and can tolerate freezing and thawing up to 10 times without affecting performance. Discard all used/unused solution for regulated disposal. Do not discard down the sink. For a five-slide capacity Coplin jar, prepare 60 mL pepsin solution:

- a. Add 48 mL of room temperature (20–25 °C) distilled or deionized water to the container.
- b. Add 6 mL of cold (2–8 °C) Pepsin Diluent (10×) (Vial 2B) to the container.
- c. Add 6 mL of cold (2–8 °C) Pepsin (Vial 2A) to the container.
- d. Put lid on the container and equilibrate the pepsin solution to 37 (±2) °C in a water bath.
- e. Equilibrated pepsin solution should be used within 5 hours.

Precautions: Pepsin diluent (10×) contains 60% propan-2-ol and should be properly labeled:

- a. Highly flammable.
- b. Irritant, including to eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
- c. Vapors may cause drowsiness and dizziness.

- d. Keep container in a well-ventilated place.
 - e. Keep away from sources of ignition – No smoking.
3. **Vial 3: HER2/CEN-17 IQ-ISH Probe Mix**, 0.2 mL, ready-to-use. Contains a mix of Texas Red-labeled *HER2* DNA probes and fluorescein-labeled CEN-17 PNA probes in IQ-ISH hybridization buffer. Store at -18 °C in the dark. The *HER2/CEN-17* Probe Mix may be affected adversely if exposed to heat or excessive light levels; therefore, do not leave this component at room temperature and in strong light, such as direct sunlight. **Caution:** Because *HER2/CEN-17* IQ-ISH Probe Mix separates into two phases while stored at -18 °C, equilibrate the probe mix to room temperature (20–25 °C) for a maximum of 30 minutes (protect from strong light), followed by thoroughly mixing the vial for 15 seconds at 2500 RPM using a vortex mixer. Discard empty container in biohazard box.
4. **Stock Vial 4 – Stringent Wash Buffer (20×)**, 150 mL, concentrated 20× SSC (saline-sodium citrate) buffer with detergent (Tween-20). Store in the dark at 2–8 °C. Tolerates frozen storage. Freezing and thawing the reagent up to 10 times does not affect performance. One Coplin jar of diluted Stringent Wash Buffer (see below) is equilibrated to room temperature, and a second jar is brought to 63 (±2) °C prior to use. For disposal, check with Laboratory Chemical Hygiene Officer. See **Preparing diluted buffer solutions** below for dilution instructions.
5. **Vial 5 – Fluorescence Mounting Medium** with 500 µg/L DAPI (4',6-diamidine-2-phenylindole), 0.4 mL, ready-to-use. Store in the dark at 2–8 °C. Tolerates frozen storage. Freezing and thawing the reagent up to 10 times does not affect performance. Medium may be applied at any temperature from 2–25 °C, but may be affected adversely if exposed to heat; therefore, do not leave at room temperature. Collect all used/unused solution for regulated disposal. Do not discard down the sink.
6. **Stock Vial 6 – Wash Buffer (20×)**, 500 mL, concentrated 20× Tris/HCl buffer. Store in the dark at 2–8 °C. Tolerates frozen storage. Freezing and thawing the reagent up to 10 times does not affect performance. Contains 1- $<$ 20% trometamol, but at product concentrations, these substances do not require hazard labeling. See **Preparing diluted buffer solutions** for dilution instructions.
7. **Coverslip Sealant**, 1 tube, ready-to-use. Store in the dark at 2–8 °C, but may be applied at any temperature from 2–25 °C. Tolerates frozen storage. Freezing and thawing the reagent for each analysis does not affect performance. Prior to discarding original tube, leave uncapped in the fume hood until all remaining material has solidified. Discard tube in regular trash. **Precautions:** Coverslip sealant contains 60-100% naphtha (petroleum), hydrotreated light, and is labeled
 - a. Extremely flammable.
 - b. Dangerous for the environment.
 - c. Toxic to aquatic organisms may cause long-term adverse effects in the aquatic environment.
 - d. Keep container in a well-ventilated place.
 - e. Keep away from sources of ignition – No smoking.
 - f. This material and its container must be disposed of in a safe way.
 - g. Avoid release to the environment. Use appropriate container to avoid environmental contamination. Refer to Safety Data Sheet (SDS) for additional information.

B. Preparing diluted buffer* solutions (1×)

*For Stock Vial #1 (pretreatment), #4 (stringent wash), and #6 (wash buffer):

1. Remove Stock Vial* from freezer one day prior to preparation and thaw in the refrigerator. Dilute 50 mL from Stock Vial* with 950 mL DDI water in a 1000 mL volumetric flask. Fill properly labeled bottle and store in refrigerator. Mark the date that the Stock Vial* is opened. Refreeze Stock Vial* in flam freezer. Unused diluted wash buffers (1×), stringent wash buffer (1×) and pretreatment solution (1×) may be stored at 2–8 °C for 1 month. Discard any 1× buffer that looks cloudy in appearance. On the day of use, all 1× buffer solutions should be poured an hour before use so that they can equilibrate to room temperature (20–25 °C). They are discarded after use.

C. Other laboratory

1. Distilled or deionized water
2. Ethanol series, 70%, 85%, and 96%. Store covered jars at room temperature under fume hood or at 2–8 °C, and use for a maximum of 200 slides or one month from date of pour-off from stock (stock alcohols expire 3 months from date of prep).

Discard solutions if cloudy in appearance. Collect all 70% and 96% from deparaffination series for chemical disposal. During periods of high humidity, replace alcohols and deparaffination reagents every two weeks.

3. Xylene or xylene substitutes, e.g., Hemo-De Clearing agent (#HD-150, from Mercedes #S-CIHD150B). Use under fume hood. Store at room temperature (20–25 °C) away from flame, and use for up to 200 slides or 1 month. Expiration: See bottle. Collect for chemical waste disposal.

D. Laboratory supplies and equipment

1. Absorbent KimWipes
2. Adjustable Eppendorf™ pipettors and tips
3. Calibrated partial immersion thermometer (range 37–100 °C)
4. Calibrated surface thermometer (range 37–100 °C)
5. Coverslips, 22 × 22 mm (or larger when tissue exceeds this size)
6. Forceps
7. Fume hood
8. Funnel for Hemo-De transfer
9. Dako Hybridizer (code S2450 or S2451) or heating block with moisture strips for denaturation at 66 °C (± 1) and hybridization at 45 °C (± 2); a humid oven/chamber can be substituted for hybridization.
10. Microcentrifuge
11. Slides, Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides (see **Specimen requirements** earlier)
12. 21 acetal staining jars, Simport #M900 – EasyDip™ slide staining system, slide-staining rack #M905-12DGY, and aluminum holder
13. Plastic slide staining dish, lid, and rack that is resistant to pretreatment solvent at microwavable temperatures +100 °C. IHCWorld #IW-2511,2512
14. Glass Coplin jar and cover
15. Timer (capable of 2–90 minute intervals)
16. Vortex mixer
17. Water bath(s) with lid, (capable of maintaining 37 (± 2) °C, 63 (± 2) °C and 95–99 °C). The latter can be substituted with a microwave oven with sensing capability for the pre-treatment step. If using a microwave, use microwavable plastic staining dish with lid, capable of sustaining 100 °C for 10 minutes.
18. Insulated oven mitts
19. Slide etching pen, LabScientific #ETCH-PEN
20. Non-fluorescing oil
21. Cardboard slide folders with hinged cover that holds 20 slides
22. A microscope area with minimal light
23. Fluorescent microscopy, including:
 - a. Leica CytoVision FISH Analysis Software, with Z-stack capturing capability
 - b. Epifluorescence microscope
 - c. Mercury lamp (100 watt); log for hours. A 50 watt lamp is not recommended.
 - d. Objectives include:

Fluorescence dry 10× or fluorescence oil immersion 16× objective;
100× fluorescence oil immersion objective. 40× and/or 60× fluorescence oil immersion objectives are optional.
 - e. Filters

DAPI filter, e.g., Chroma filter # 31000.
Texas Red/FITC double filter, e.g., Chroma filter # 51006. Triple filters are not recommended.
Texas Red and FITC single filters can be used for confirmation.

Fluorochrome	Excitation Wavelength	Emission Wavelength
FITC	495 nm	520 nm
Texas Red	596 nm	615 nm

III. Procedure (breast)

This procedure provides a quick reference for running the test. For a more thorough description of the procedure, see Dako's online protocol (1).

A. FISH processing

JAR	Duration	°C	(+/-)	Reagent	Notes
STEP 1: Prepare Daily Solutions					
All buffers (see Materials above) and coverslip sealant are brought to room temperature (about 1 hour). Prepared stock reagents are stored at 4 °C for 1 month.					
If using water bath pretreatment method, place pretreatment solution and jar in water bath before increasing temperature. Verify water level in water bath is ≥ solution level in Coplin jar. Keep jar(s) covered in order to stabilize temperature and avoid evaporation. Temperature in jar must maintain at 95–99 °C for at least 30 minutes before use. Expires after use. <i>Regulated disposal.</i>					
If processing >5 slides, prepare Pepsin Immersion solution. Can be used for 5 hours at 37 °C. <i>Regulated disposal.</i>					
STEP 2: Deparaffinize and Dehydrate					
1	5 min	RT		Hemo-de #1	Tap off excess. QC: Change all Hemo-de and ETOH washes after 200 slides or one month from pouring. In high humidity, change deparaffinization reagents every 2 weeks. <i>Regulated disposal.</i>
2	5 min	RT		Hemo-de #2	Tap off excess. While waiting, saturate white humidity strips in Dako hybridizer. Change when strips are not adequately absorbing.
3	2 min	RT		96% ETOH	Tap off excess.
4	2 min	RT		96% ETOH	Tap off excess.
5	2 min	RT		70% ETOH	Tap off excess.
6	2 min	RT		70% ETOH	Tap off excess.
7	2 min	RT		Wash Buffer	Can remain in this solution for up to one hour. Tap off excess. Discard solution in sink with copious water.
STEP 3: Pretreatment					
8	10 min	95–99	(±1)	Pretreatment	If using microwave, make sure turntable is clean. Place pretreatment container in center of microwave, leaving the lid slightly askew. Press sensor cook, bring dial to appropriate program to reach 99 °C (e.g., #13), and press start. Set timer to 10 minutes. When the sensor detects that the solution has reached its desired temperature, it will beep. Immediately start timer (10 minutes) and stop microwave at 10 minutes. If using water bath method, immerse slides, keeping thermometer within the jar until the pretreatment solution reaches the 95–99 °C range, at which time you start timer (10 minutes). Record temp on hybridization record.
8	15 min			Pretreatment	Solution will be HOT when done! Using insulated gloves, carefully remove hot jar from water bath or microwave, and leave uncovered. Wipe down microwave surface with 70% ETOH.
9	3 min			Wash Buffer	Tap off excess.
10	3 min			Wash Buffer	Tap off excess.

STEP 4: Pepsin Treatment					
11	13–15 min or 20 min	RT or 37°C (\pm 2 °)	Pepsin or Pepsin Immersion	With a clean KimWipe per slide, carefully remove excess buffer around tissue. If processing <5 slides, lay slides flat and apply 5–8 drops cold pepsin to target area for 13 min (15 min if tissue was in formalin for an extended time). While waiting, remove probe from flam freezer and place in drawer (do not exceed 30 min for the probe to be left at RT). If processing 5 or more slides, immerse in pepsin solution at 37°C for 20 minutes. Record temp on hybridization record.	
12	3 min	Post-Pepsin Wash Buffer		Tap off excess.	
13	3 min	Post-Pepsin Wash Buffer		Wet humidifying strips in hybridizer.	
14	2 min	70% ETOH		Tap off excess.	
15	2 min	85% ETOH		Tap off excess.	
16	2 min	96% ETOH		Tap off excess.	
STEP 5: Probe Hybridization					
	5 min	Air Dry		Vortex probe for 15 seconds and microcentrifuge before use. When slides are dry, apply 10 μ L probe*; cover with 22 x 22 mm coverslip and seal with sealant. *If target area is larger than 22 x 22 mm, increase probe amt and coverslip size accordingly.	
	10 min	66	(\pm 1)	Dako Hybridizer	Place slides in slots, making sure that they are resting flat on the surface. Using a surface slide-thermometer, record denaturation and hybridization temperatures in hybridization record. Run HER2-IQ program.
	60–120 min	45	(\pm 2)		While slides are hybridizing, bring Stringent Buffer Post wash gradually to temp (63 °C) in water bath. Once stabilized, record temperature in hybridization record. Make sure water level \geq solution level.
STEP 6: Post-wash Treatment					
17		Room Temperature Stringent Post wash		When hybridization is complete, end program and wait for program to end. Working with one slide at a time, remove sealant with forceps and place in room temperature Stringent Wash until all coverslips have been removed (if coverslip does not come off easily, place in buffer to loosen glass).	
18	10 min	63	(\pm 2)	Hot Stringent Post wash	When all coverslips have been removed, place slides in hot Stringent Wash Buffer for 10 minutes. Do not leave unattended. While waiting: (a) wipe down hybridizer with alcohol and remove any sealant residue. (b) Remove DAPI from flam freezer and keep n dark, e.g., closed drawer (limit to 30 min at RT).
19	3 min	Wash Buffer		Tap off excess.	
20	3 min	Wash Buffer		Tap off excess.	
21	2 min	70% ETOH		Tap off excess.	
22	2 min	85% ETOH		Tap off excess.	

23	2 min	96% ETOH	After last alcohol rinse, air dry in dark at ~45° angle, label down. Vortex and microcentrifuge before using. Cover hybridized area with 15 µL DAPI* and use a coverslip that covers all targeted areas. *If target area is larger than 22 x 22 mm, increase DAPI and coverslip size accordingly. Place in cardboard slide holder, close and leave at room temperature for 15 min or until ready to score. Return DAPI to flam freezer. Slides should be read within 7 days after mounting.
Interpretation Criteria – Breast			
1. Repeat the test if controls are not as expected.			
2. Reject if non-uniform signals exceeds 25%.			
3. Reject if autofluorescence is high.			
4. Reject if nuclear resolution is poor.			
5. Reject if >10% signal cover cytoplasm.			

B. Troubleshooting – breast [1]

Problem	Probable cause
1. No signals or weak signals	1a. Ensure that kit was properly shipped, and vials are stored as expected and in the dark. 1b. Check microscope filters, mercury lamp, lens or immersion oil. 1c. Pretreatment temperature and time were adequate. 1d. Ensure sufficient humidity in the hybridization chamber.
2. No green signals	2a. Incorrect Stringent wash conditions, or coverslips not removed before stringent wash.
3. No red signals	3a. Pre-treatment conditions incorrect.
4. Areas without signal	4a. Probe volume too small. 4b. Air bubbles caught during denaturation/hybridization step
5. Excessive background staining	5a. Ensure that only formalin fixed, paraffin-embedded tissue sections are used. 5b. Paraffin was incompletely removed. 5c. Stringent wash temperature was too low or not 63 (±2) °C.
6. Poor tissue morphology	6a. Incorrect pre-treatment conditions may result in unclear or cloudy appearance. 6b. Incorrect Pepsin treatment. For example, too long Pepsin treatment or very thin section thickness may cause ghost cells or donut. Ensure that the section thickness is 4-6 µm.
7. High level of green autofluorescence on slide, including areas without FFPE tissue	7. Glass slides may be too old. Ensure that the coated glass slides have not passed expiration date.

If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, contact Dako Technical Services for further assistance.

IV. Notes

1. Signal counting guide [1]

- Do not count overlapping nuclei, because not all areas of nuclei are visible.
- Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one signal.

- Do not count over- or underdigested nuclei, or nuclei that are missing signals in its center (donut-shaped nuclei).
- Generally speaking, do not score nuclei with signals of only one color. If, however, their occurrence is so frequent that it raises suspicion, discuss situation with laboratory director.
- Because paraffin-embedded breast tissue has been sliced for slide preparation, some nuclei may show signal absence or size differentiation as a result of being sliced.

V. References

1. Dako HER2 IQFISH pharmDx™, Code K5731, 9th edition. <http://www.dako.com/download.pdf?objectid=122737002>.
2. Wolff AC, Hammond MEH, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JMS, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G, Hayes DF. Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update. *J Clin Oncol* November 1, 2013; 31(31): 3997–4014.

CHAPTER 17

Multicolor FISH (SKY and M-FISH) and CGH

Turid Knutsen

(retired), Section of Cancer Genomics, Genetics Branch, Center for Cancer Research National Cancer Institute, National Institute of Health, Bethesda, MD, USA

17.1 Introduction

Molecular cytogenetics, the marriage of classical cytogenetics and recombinant DNA technologies, led to the development of fluorescence *in situ* hybridization (FISH) technologies more than two decades ago. Introduced in the late 1970s and first applied to metaphase chromosomes in 1986 [1], FISH has added important, dynamic components to diagnostic and research cytogenetics, allowing characterization of the origin of virtually all chromosome abnormalities, many of which were unidentifiable using banding alone.

This chapter will focus on two complementary FISH techniques, each of which provides a genome-wide picture of cytogenetic abnormalities in a single experiment. The first of these techniques, multicolor FISH (mFISH), introduced in 1996 as spectral karyotyping (SKY) [2] and multiplex-FISH (M-FISH) [3], permits the simultaneous visualization of each chromosome pair in a different color. The second technique, chromosomal comparative genomic hybridization (CGH) [4], developed in 1992, generates a map of changes in the DNA copy number. Array CGH (aCGH), a more recent development, is addressed in chapter 18; aCGH is rapidly replacing chromosomal CGH in many laboratories. These techniques have been made possible by the availability of spectrally discrete fluorochromes, the use of combinatorial probe labeling schemes, and technical advances in fluorescence microscopy, including the development of suitable optical filters and sensitive digital imaging devices.

Each of these techniques (SKY, M-FISH, and CGH) has its advantages and limitations (Table 17.1), but when taken together and combined with standard banding techniques they present a thorough picture of genomic alterations. The strength of multicolor FISH lies in its ability to define translocations, marker chromosomes, and complex rearrangements, and to reveal cryptic change; it cannot, however, detect intrachromosomal rearrangements, such as duplications, very small deletions, or small paracentric inversions. The advantage of CGH is that it requires only DNA and permits the study of archival material; it detects global chromosomal imbalances and is particularly useful for the study of solid tumors, which often do not yield adequate chromosomal preparations. Disadvantages of CGH include its inability to detect balanced, whole-genome copy number (ploidy) changes, balanced rearrangements, and imbalances present in less than 50% of the sample. See later for a more thorough discussion of this subject.

17.1.1 Online databases

There are a number of online databases that display data from these molecular cytogenetic FISH techniques. A few of them display multicolor FISH data: the NCI and NCBI's SKY/M-FISH & CGH Database (<http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi>) [5]; the Chromosome Rearrangements in Carcinomas database (<http://www.path.cam.ac.uk/~pawefish/>); and the Cell Line NCI60 Drug Discovery Panel (<http://home.ncifcrf.gov/CCR/60SKY/new/demo1.asp>).

Other online databases display CGH data: the Laboratory of Cytomolecular Genetics (CMG) (<http://www.helsinki.fi/cmg/>); the CGH Data Base (http://www.cghtmd.jp/cghdatabase/index_e.html); and Progenetix (www.progenetix.net/) [6].

Table 17.1 Comparison of G-banding analysis, FISH, SKY/M-FISH, and CGH

Study material	Banding	FISH	SKY/M-FISH	CGH
Metaphase spread	+	+	+	-
Interphase nuclei	-	+	-	-
DNA	-	-	-	+
DETECTS				
Copy number changes (ploidy)	+	+	+	-
Copy number changes (individual chromos)	+	+	+	+
Translocation – balanced	+	+	+	-
Translocation – unbalanced	+	+	+	+
Amplification (dmin, hsr)	+	+	+	+ ¹
Additions (>10 Mb)	+	+	+	+
Deletions (>10 Mb)	+	+	+	+
Deletions (<2–3 Mb)	+/-	+	-	+/-
Deletion of specific genes	-	+	-	+/-
Inversions ²	+	+	-	-
Insertions ²	+	+	+	-
Heterogeneity	+	+	+	-

¹ Does not distinguish dmin from hsr.² Size dependent.

Adapted from:

Bayani J and Squire JA, *Cancer Invest* 2002; 20:373–386 (Ref. 31)Speicher MR and Carter NP, *Nature Rev/Genet* 2005; 6:782–792 (Ref. 62)

The first two of these CGH databases display cases from a single laboratory, except the Progenetix database, which contains CGH profiles of over 32,000 cases from over 2500 references in the literature.

17.2 Multicolor FISH (SKY/M-FISH)

17.2.1 Introduction

Multicolor FISH has enhanced our understanding of the chromosomal basis of many genetic diseases, especially cancer. Before its development, many cryptic and complex translocations, insertions, and amplifications went undetected when only chromosomal banding techniques were employed. Multicolor FISH has been applied to metaphase preparations from a variety of tissues and diseases, and to species other than human, particularly the mouse. Table 17.2 lists some of the clinical and research applications of multicolor FISH (see also reviews in [7–9]).

17.2.2 Other multicolor FISH techniques

In addition to SKY and M-FISH there are several other multicolor FISH techniques; these are based on the use of different probe types, derived from microdissection of specific chromosome regions or based on specific loci. These techniques can be divided into two groups: those that reveal a multicolor banding pattern on the whole genome, and those that color specific chromosomal subregions such as centromeres or telomeres (reviewed by Liehr et al. [10]).

Included in the first group are RxFISH, COBRA, and MCB. RxFISH (also termed Harlequin FISH, zoo-FISH, or cross-species color banding) [11] is based on cross-species banding. Flow-sorted chromosomes from two closely related species of gibbons are labeled with three fluorochromes and hybridized to human chromosomes. Since there is a high degree of sequence homology between gibbons and humans, the probes paint the entire human karyotype, and the extensive structural rearrangements that have occurred during evolution produce a specific colored banding pattern [12]. The resolution is 100–200 bands

Table 17.2 Clinical and research applications of SKY/M-FISH

Clinical cytogenetics
Constitutional disorders
Prenatal diagnosis
Preimplantation genetic diagnosis
Cancer cytogenetics
Diagnosis
Prognosis
Progression
Etiology
Cancer research
Tumor studies
Tissue culture studies
Drug studies
Animal models of human disease
Knockout mutants
Oncogene overexpression
Transgenic mice
Radiation effects
Drug effects
Chemical effects
Karyotype instability
Comparative cytogenetics (evolution)

per haploid human karyotype. COBRA (COmbined BInary RAting labeling) [13], a variation of SKY/M-FISH, is based on the simultaneous use of combinatorial labeling and ratio labeling, so that the 24 colors required for painting human chromosomes can be reduced to only four fluorophores. COBRA also permits gene and viral integration site mapping [14]. High-resolution multicolor-banding (MCB) [15] employs differentially labeled overlapping microdissected libraries, and permits the differentiation of chromosome-specific areas at the band level.

Included in the second group of multicolor FISH techniques are arm FISH, mBAND FISH, M-TEL FISH, and cenM-FISH (CM-FISH). Arm FISH, a chromosome arm-specific multicolor FISH technique using a 42-color M-FISH technique, permits the detection of aberrations at the resolution of chromosome arms [16]. mBAND FISH comprises region-specific probes. The partial chromosome paints gradually decrease in intensity from the center toward the end of the chromosome. The overlap of the intensity profile of adjacent probes results in color-ratio variations along the chromosome. M-TEL FISH is a 24-color technique that detects subtelomere deletion and interchromosomal exchanges in the subtelomere regions [17]. CM-FISH is a combination of probes for all human centromeric satellite regions and is particularly useful for the characterization of small supernumerary marker chromosomes with no or little euchromatin [18,19]. A comprehensive database covering multicolor FISH techniques and their applications can be found at <http://ssmc-ti.com/Start.html>. RxFISH is presented in greater detail in chapter 16, but the other techniques are not widely employed in the clinical setting, and will therefore not be discussed in further detail in this chapter.

17.2.3 Theory

Multicolor FISH (SKY and M-FISH) is based on the simultaneous hybridization of specific painting probes for each chromosome in the genome of interest (e.g., 24 in humans), labeled with different fluorochromes or fluorochrome combinations (fluors). The number of useful Boolean combinations of N fluors is $2^N - 1$; thus there are 31 combinations for five fluors. Combinatorial labeling makes it possible to discriminate many more targets than the number of spectrally resolvable fluorophores. Five pure spectrally distinct dyes are used either singly or in combination to create a chromosome cocktail of probes, each with a unique spectral signature for each chromosome (Figure 17.1a). The only difference between

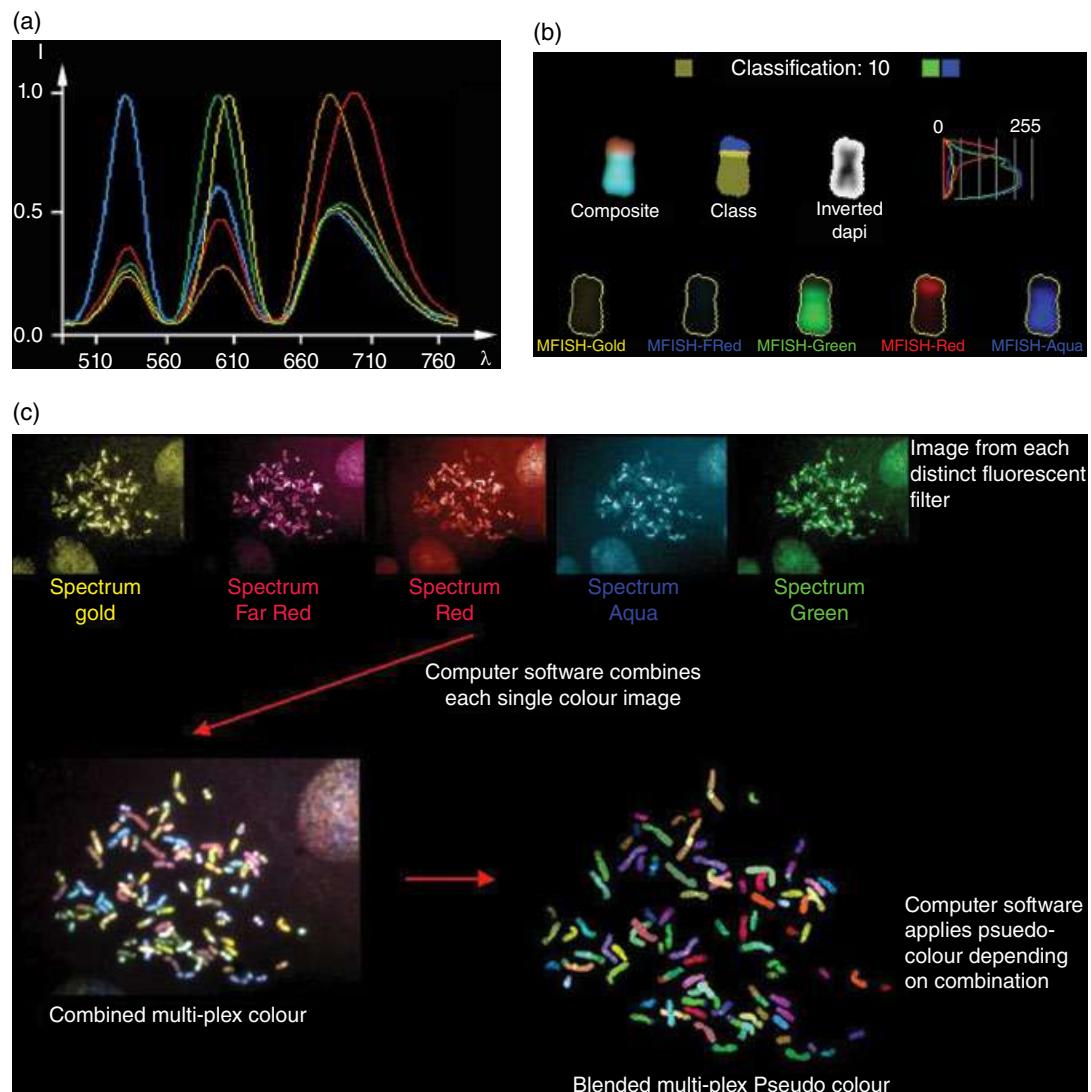


Figure 17.1 (a) Measured spectra of five different dyes used for combinatorial labeling of all 24 human chromosomes: blue, rhodamine 110; green, Spectrum Orange; yellow, Texas Red; orange, Cy 5; and red, Cy 5.5. Schröck 1999. Reproduced with permission of Springer. (b) Composite view of t(3;10) demonstrating a composite fluorochrome image, a pseudo-colored M-FISH classification image, an inverted DAPI image, a profile plot of each fluorochrome along the length of the chromosome, and each individual fluorochrome image. Reproduced with the kind permission of Michael Behme. (c) M-FISH analysis of blood from a case of epilepsy and mental retardation, demonstrating a balanced t(12;13) and insertion from chromosome 11 to chromosome 12: 46,XX,der(12)ins(12;11)(p11.2;p11.2p15)t(12;13)(q22;q32),der(13)t(12;13)(q22;q32). The translocations were confirmed by WCP. (d) Principle of spectral imaging and demonstration of the potential of separating overlapping fluorochromes with spectra-based classification. Simultaneous hybridization of three different chromosome painting probes that were labeled with Cy 3 (chromosome 7), Texas Red (chromosome 13), and a combination of Cy 3 and Texas Red (chromosome 4) is depicted. The emitted light is visualized through a triple bandpass filter, sent through an interferometer, and imaged with a CCD camera. The interferogram that is generated for each pixel is analyzed by Fourier transformation, a process that makes it possible to define the spectrum of the light. The measured spectra can then be converted to display colors or to classification colors. When display colors are used, chromosomes with similar spectra have similar colors. However, the subtle differences in the spectra of the Cy3 and Texas Red-labeled chromosome painting probes are sufficient to enable the discrimination of the chromosomes after spectral classification. OPD, optical path difference. Schröck 1996. Reproduced with permission of AAAS. See insert for color representation of this figure.

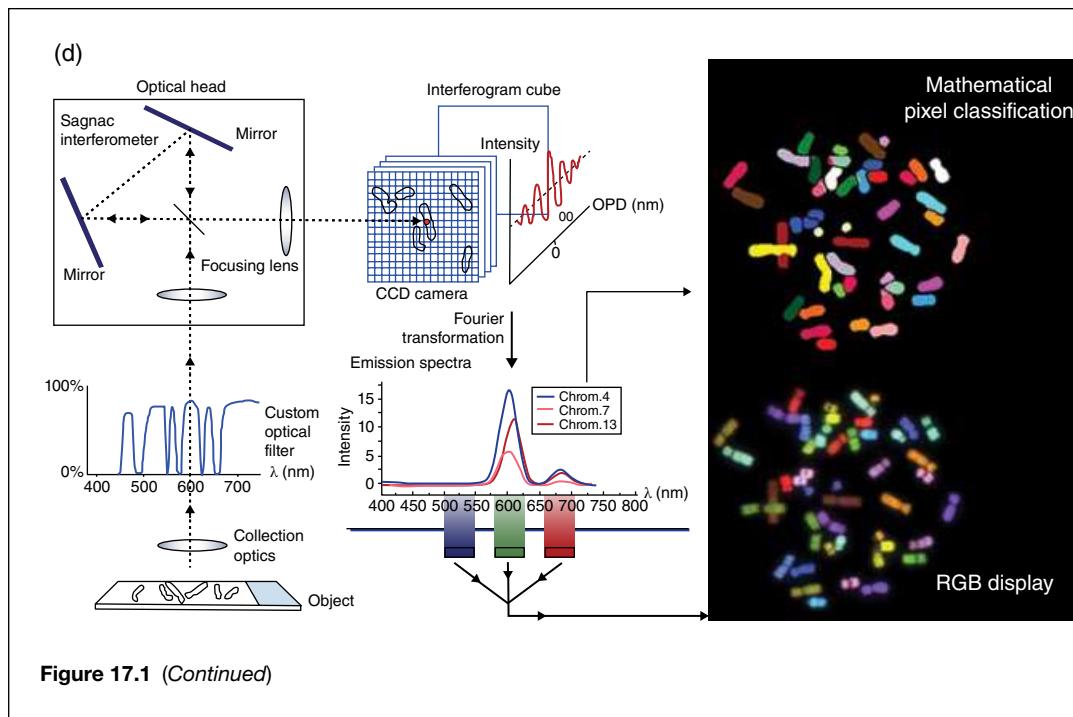


Figure 17.1 (Continued)

SKY and M-FISH lies in their image acquisition and analysis: SKY is based on the spectral signature of each fluorochrome in the probe cocktail, while M-FISH is conducted using filter sets specific for each fluorochrome. In M-FISH, images are captured for each of the five spectrally distinguishable fluorochromes separately and sequentially, then merged into a composite using a computer program (Figures 17.1b,c). For SKY, image acquisition is conducted using a combination of epifluorescence microscopy, charged-coupled device (CCD) imaging, and Fourier spectroscopy, permitting the measurement of the entire emission spectrum with a single exposure at all image points (Figure 17.1d). See Image acquisition for a more detailed discussion. Companies producing SKY or M-FISH analysis software provide detailed instructions and tutorials for image acquisition and analysis.

Figure 17.1c shows the results of M-FISH in a case of epilepsy and mental retardation; the classification karyogram demonstrates a balanced translocation and an insertion. Figure 17.2 demonstrates the results of a SKY hybridization performed on a secondary leukemia cell line showing the inverted DAPI image; the red, green, and blue (RGB) display image; the classification image; and the inverted DAPI/classification karyogram. SKY was able to identify the chromosomal origin of complex markers and inversions, one of which was very small; CGH on the same cell line mirrored the karyotype abnormalities showing interstitial deletions and chromosomal gain and amplifications (Figure 17.2e).

17.2.4 Applications of multicolor FISH

Constitutional disorders and prenatal diagnosis

The correct identification of constitutional chromosome abnormalities is crucial for the purposes of diagnosis, risk assessment, and genetic counseling. Multicolor FISH has been particularly useful in characterizing de novo balanced and unbalanced translocations, complex translocations, and ring chromosomes [20].

In prenatal studies, the identification of small markers known as “extra structurally abnormal chromosomes” (ESAC) or “small supernumerary marker chromosomes” (sSMC) – found in 0.076% of prenatal specimens and 0.043% of newborns [21] – presents a major diagnostic and counseling dilemma: some of these markers, such as those derived from pericentromeric regions of chromosome 10, appear to have no deleterious effect, while others, for example, those derived from proximal chromosome 22, are associated with specific abnormal phenotypes and a high incidence of mental retardation. At least half of the markers are de novo, and the risk of congenital abnormalities is about 13% [22]. Using FISH and excluding markers derived from chromosome 15, Crolla et al. [23] reviewed 49 cases in which a marker had been detected pre- or post-natally and estimated that the risk for abnormal phenotypes is approximately 7% for acrocentric markers and 28% for nonacrocentric

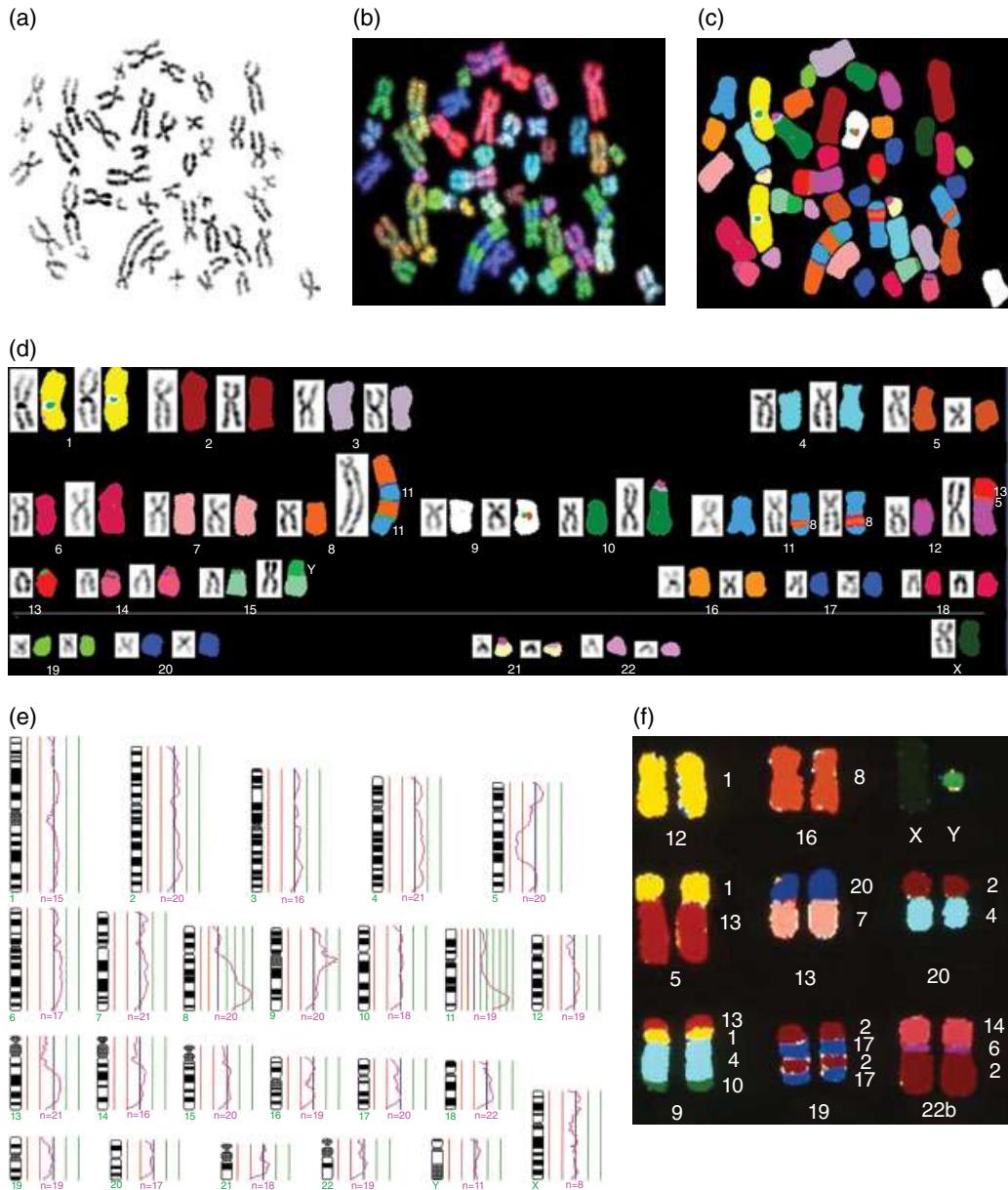


Figure 17.2 SKY and CGH analysis of secondary leukemia cell line SAML-2. (a) Inverted DAPI image. (b) RGB display image. (c) Spectral classification image. (d) DAPI and SKY karyogram showing 45,X,der(Y;15)(q10;q10),del(5)(q13q31), der(8)(8pter->8qter::11q21->11qter::8q13->8qter::11q21->11qter::11q?23->q11q?24),dup(10)(p?15p10),+11, der(11)(11pter->11q21::8q22.1->8q24.1::11q22->11qter)x2,der(12)t(12;13)(p13.3;q14)ins(12;5)(p13.3;q31q31),-13. (e) CGH profile; the three vertical lines to the right of each chromosome idiomogram represent different fluorescence ratio values between the tumor and the control DNA; the values were set at 0.75, 1.00, and 1.25 left to right, respectively. In this cell line, the CGH profile closely mirrors the karyotypic findings, demonstrating gain at 8q and 11q, and losses at 5q and 13q; amplified areas of 8q and 11q are those containing the *MYC* and *MLL* oncogenes (FISH confirmed the amplifications). (d,e) Knutson 2003. Reproduced with permission of John Wiley and Sons. (f) Classification-colored images of representative examples of gibbon chromosomes. The upper row presents examples of gibbon autosomes and the gibbon sex chromosomes that remain unchanged in the human karyotype. The center row shows gibbon chromosomes 5, 13, and 20 with whole-arm translocations from the human counterparts. Gibbon chromosomes 9, 19, and 22b (lower row) reveal multiple aberrations, producing a banding pattern. The numbers on the right side of the gibbon chromosomes indicate the corresponding human chromosome material. Schröck 1996. Reproduced with permission of AAAS. See insert for color representation of this figure.

markers. sSMC cases reported in the literature have been collected into an online database (<http://ssmc-ti.com/Start.htm>) by Thomas Liehr. According to this database, 74% of sSMC have no phenotypic effect, sSMC can be derived from any chromosome, mosaicism and structural variation (e.g., rings and isoduplications) are frequent, and multiple sSMCs of different origins can occur in the same patient. See Haddad et al. [24] and Brecevic et al. [25] for reviews of cases in which multicolor FISH studies have successfully determined the origin of such markers. The Brecevic article includes a study of sSMC in immortalized cell lines.

Preimplantation genetic diagnosis (PGD)

Multicolor FISH is a sensitive screening method for the in vitro fertilization (IVF) process: human oocytes that fail to be fertilized after IVF show a high rate of chromosomal abnormalities [26], and abnormalities are also very common in preimplantation human embryos. Oocytes, blastomeres, human placental cytotrophoblasts, and polar bodies have all been successfully studied using multicolor FISH, with the goal of implanting only cytogenetically normal embryos [27–29]. Interphase multicolor FISH applications in prenatal and preimplantation diagnostics are reviewed in Stumm et al. [28].

Meiosis

Multicolor FISH, cenM-FISH, and M-TEL FISH have all been applied to meiosis in both males and females, permitting the study of the meiotic process for each human chromosome, and making possible the detection of synaptic anomalies, the investigation of chromosome recombination and segregation, and the identification of de novo structural meiotic rearrangements. These techniques are employed in the study of chromosome behavior in normal individuals, in individuals with constitutional abnormalities, in infertility, in assessments of the effects of radiation and chemotherapy, and in determination of the differences between male and female meiosis. A review is presented in Oliver-Bonet et al. [30].

Cancer

The most common application of multicolor FISH is in the study of cancer, where it has been used to analyze hematologic malignancies (leukemias and lymphomas) [31,32], solid tumors, and tissue culture lines established from human malignancies [7–9,33,34]. An excellent and thorough review of the published applications of SKY in genetic diagnosis and research was published by Schröck et al. [9]. They describe the numerous new chromosomal translocations, cryptic changes, and other aberrations that have been revealed by SKY in prenatal and constitutional studies, leukemias, lymphomas, and solid tumors, as well as findings in a variety of mouse models. They also discuss other research applications such as studies of cell lines and of radiation-induced chromosome instability.

In cancer, multicolor FISH can result in the discovery of new recurrent translocations that can lead to new subgroup disease classifications and improvements in therapy. The cloning of breakpoints involved in cancer-specific translocations has led to the discovery of new oncogenes, which are involved in cell growth and regulation, and tumor suppressor genes, which are involved in signal transduction pathways from the cell membrane to the sites of gene transcription. Studies of multiple myeloma have shown the power of this technique in defining complex aberrations and in identifying new recurring translocations [35] and several novel recurring breakage sites [36]. Interestingly, multicolor FISH studies of acute myeloid leukemia (AML) patients with normal G-banded karyotypes, which account for 40% of newly diagnosed cases, have revealed few additional abnormalities, leading to the speculation that mutations and homozygosity may be the underlying biological cause in this subgroup of AML [37].

Solid tumors generally exhibit more chromosomal abnormalities than do hematologic malignancies. They are also more difficult to analyze, since many of them display a low mitotic index, poor growth rate, and poor chromosome morphology, which in the past often made it impossible to obtain any useful information as to chromosomal abnormalities using banding alone. Multicolor FISH has revealed the identity of numerous marker chromosomes, hidden translocations, and unsuspected insertions in carcinomas, sarcomas, and brain tumors [7–9,37], and has enabled the identification of the chromosomal origin of homogeneously staining regions (hsr) and double minutes (dmin), which are chromosomal manifestations of oncogene amplification [38].

Multicolor FISH is particularly useful in identifying the additional chromosomal abnormalities that are gained as malignancies progress through the premalignant → malignant → metastatic stages of disease. Since these changes increase in complexity, due to the development of multiple subclones and derivative markers, it is more difficult to distinguish the important changes from those that just represent genetic “noise.”

Animal models

Multicolor FISH is also a useful tool in the study of animal models of human disease. The mouse is a particularly important model because it can readily be genetically manipulated, permitting direct correlation between genotype and phenotype, and between human and mouse genetic pathways [12,39]. The sequencing of the mouse genome and the development of orthologous maps between mouse and human genes has also greatly facilitated the correlation between cytogenetic findings in mouse models and human disease. Mouse models can be used to study tumor-initiating aberrations, the effects of tumor suppressor gene inactivation (in mouse knockout mutants), oncogenic overexpression, and genetic instability. There are particular advantages to the use of mouse models: (a) tumors can be studied at an earlier stage of carcinogenesis, making it possible to detect tumor-initiating events in specific tumor types, and to follow the sequential activation of oncogenes, and (b) the effects of drugs, chemicals, and radiation can be studied *in vivo* [33]. Prior to the development of multicolor FISH, these studies were hampered not only by the difficulty in obtaining well-banded chromosomes from mouse tumors, but also by the morphology of the mouse chromosomes themselves, since all are acrocentric and similar in size. Numerous studies on a variety of mouse model systems (Table 17.2) have demonstrated that these difficulties can be overcome by the use of multicolor FISH [40]. A few examples of such studies are listed below:

1. Confirmation of the T(12,15) translocation in chemically induced plasmacytomas in BALB/c mice [41].
2. Studies of the genomic effects of the *cMyc* oncogene, the *p53* tumor suppressor gene, and *Brcal*, a breast cancer susceptibility gene, in mouse mammary tumors of transgenic mice [42,43].
3. Demonstration of karyotypic instability in mouse pre-B lymphoma cell line [44].

SKY probes are also available for rat chromosomes and have been applied to rat models [45].

Comparative cytogenetics

In comparative cytogenetics, SKY has revealed rearrangements that have occurred during the course of chromosome evolution, thereby demonstrating the evolutionary divergence of closely related species. These studies can be helpful in determining the genetic relationship among subspecies and in providing information about polymorphic variation [46]. In Figure 17.2f, SKY demonstrates the rearrangements that have occurred between the human and gibbon (*H. concolor*) chromosomes [2]. Such studies have also been performed with the chimpanzee, baboon, gorilla, orangutan, and muntjac (reviewed in [7,47]).

3D nuclei FISH

Multicolor FISH has been applied to three-dimensionally preserved cells (3D FISH) to study the spatial organization of the genome, providing information about the organization of chromosome territories and subchromosomal domains, and about patterns of chromatin density within a chromosome territory [48,49]. These studies have shown that chromosomes in the interphase nucleus occupy mutually exclusive territories, with each arm forming a separate domain, and that dmin and hsr do not overlap normal chromosome territories but are located in the interchromosomal domain (ICD) spaces, facilitating access of amplified genes to transcription and splicing complexes that lie in the ICD [49]. It has been found that gene-poor chromosomes (e.g., human chromosome 18) occupy a more peripheral position, while gene-rich chromosomes (e.g., human chromosome 19) are located in a more central position in the nucleus [50]. The position of individual chromosome territories may be cell type or cell cycle dependent. For a review of the technical aspects of 3D FISH, see Solovei et al. [48] and Walter et al. [51].

17.2.5 Methodology

Information for the following sections on slide pretreatment, hybridization, detection, and imaging was obtained from the protocols listed at the end of this chapter. These protocols were provided by individual laboratories at the National Cancer Institute (Protocol 17.1, 17.7), Mayo Clinic (Protocol 17.5), Ontario Cancer Institute (Protocol 17.2), Oregon Health and Science University-Knight Diagnostic Laboratories (Protocol 17.4), and by commercial vendors (Abbott Molecular Vysis, Des Plaines, IL (Protocol 17.8); Leica Biosystems, Buffalo Grove, IL; Applied Spectral Imaging, Vista, CA and Migdal Haemek, Israel (Protocol 17.3); Leica Microsystems, Buffalo Grove, IL; and MetaSystems, Altlussheim, Germany (Protocol 17.6), as well as others [8,9,33,46,47,52–65]. A basic outline of the steps involved in SKY, M-FISH, and CGH is presented in Table 17.3.

Table 17.3 Outline of SKY/M-FISH and CGH procedures

SKY/M-FISH direct-labeled probe	SKY/M-FISH indirect-labeled probe	CGH
Prepare metaphase spread	Prepare metaphase spread	Prepare normal metaphase spread
↓	↓	↓
Age slide	Age slide	Age slide
↓	↓	↓
Prepare probe	Prepare probe	Extract DNA
↓	↓	↓
Pretreat slide	Pretreat Slide	Nick translate DNA
↓	↓	↓
Denature and pre-anneal probe	Denature and pre-anneal probe	Precipitate DNA
↓	↓	↓
Denature slide	Denature slide	Denature and pre-anneal DNA probe
↓	↓	↓
Hybridize	Hybridize	Denature slide
↓	↓	↓
Wash	Wash	Hybridize
↓	↓	↓
Counterstain and mount	Blocking agent	Wash
↓	↓	↓
Capture images	Detection	Blocking agent
↓	↓	↓
Analyze images	Wash	Detection
	↓	↓
	Counterstain and mount	Wash
	↓	↓
	Capture images	Counterstain and mount
	↓	↓
	Analyze images	Capture images
		↓
		Analyze images

Specimens and slide preparation

Multicolor FISH can be performed on metaphase preparations from a variety of tissues, including blood, bone marrow, effusions, solid tumors, fibroblasts, amniotic fluid, chorionic villi, and others. Slide quality is one of the most important factors in successful hybridization; ideally, the best results are obtained on slides with an adequate number of mitoses and good chromosome spreading, with few or no overlapping chromosomes.

When preparing cytogenetic slides it is important to use phase contrast microscopy to check the quality of the cells. Dark, refractile cells hybridize poorly, and the best results are obtained from metaphases and nuclei that appear moderately gray or dull gray without visible cytoplasm surrounding the chromosomes. Chromosomes that appear shiny and hollow, indicative of high humidity and prolonged drying time during slide preparation, and those that appear light gray (too low a humidity) can result in poor hybridization patterns. Poor chromosome morphology can result in poor resolution of the (inverted) DAPI bands, making them difficult to interpret, and can also cause “bleeding” of colors at chromosome translocation junctions.

Slide aging and storage

In order to harden the chromosomal DNA matrix so that it can survive the harsh denaturation process, it is necessary to age the slides. This can be done naturally over time, generally at room temperature or by storage at 37°C. If time is of the essence, it can be accomplished artificially by heat (e.g., at 90°C for 10 minutes) or in a hot standard saline citrate solution (2× SSC). Some protocols state that artificial aging is not necessary for slides that are more than 2 weeks old. Generally, the best results are obtained with slides that are prepared one day to two weeks prior to hybridization: fresh slides produce the highest hybridization efficiencies with the brightest signals [66]. In general, as chromosome preparations age beyond 2 weeks, they become difficult to denature because the DNA eventually degrades.

Storage at -20°C in a moisture-free environment, either in 100% ethanol or with a dessicant, is recommended for slides that will not be used within two weeks; long term storage of dehydrated slides at -70°C to -80°C in the presence of a dessicant will preserve the preparations for several months [55]. Cell pellets (from chromosome harvests) can be stored in fixative at -20°C and slides dropped 1–3 days before hybridization. Alternatively, slides can be stored at 37°C in a dry incubator for up to one year and still provide adequate hybridizations; the key is to prevent exposure to moisture, which rapidly degrades the DNA. (Personal note: I have had successful SKY hybridization with bright signals on 3-year-old mouse chromosome preparations stored in a dry oven at 37°C; however, the DNA was somewhat degraded such that the chromosome banding morphology was of less than optimal quality).

SKY/M-FISH probes

SKY and M-FISH kits containing a combination of probes directly and indirectly labeled with five pure spectrally distinct dyes are used to create the unique probe cocktail (Figure 17.1a). Table 17.4 lists the common fluors and haptens currently used in SKY and M-FISH, along with their peak excitation and emission spectra. (A hapten is a small substituent on a protein that can elicit an antibody response.) With directly labeled probes, the reporter molecule, a fluorescent dye, is conjugated directly to the nucleic acid probe; this typically involves chemically binding a fluorescent tag into a nucleoside triphosphate, and subsequently using enzymes, such as polymerases, to replace the probe's nucleosides with labeled ones [67]. Directly labeled probes do not require blocking or detection after hybridization. Some fluors do not work well when directly coupled to DNA – probably because they affect binding of probe to target – but work very well when coupled to secondary reagents such as antibodies [68], so an indirect process is necessary. With indirect labeling, the intermediate molecule is coupled to the fluorescent dye in the detection step using an antigen-antibody reaction; the most popular intermediate molecules are two haptens: biotin, which binds to avidin or streptavidin with high affinity, and digoxigenin. Examples include fluorescein-avidin and anti-digoxigenin-rhodamine. One advantage of indirectly labeled probes is that the detection steps can be repeated to strengthen the signal, although this can also increase background fluorescence. Generally though, whole chromosome paints, such as those used for SKY and M-FISH, can be detected without amplification. New fluors are constantly being developed, and those that are photostable and produce bright signals are incorporated into labeling schemes.

Probe sets are combinations of whole chromosome paint (WCP) probes, one probe for each chromosome in the genome of interest, for example, 24 different WCP probes for the human genome. See Table 17.5 for various probe fluorophore combinations for each chromosome according to source. While these labeling schemes use five fluorochromes, more can be used, on the condition that there are measurable differences in the emission spectrum of each fluorochrome. WCP can be produced using somatic cell hybrids, by microdissection of specific chromosomes, or by flow sorting of the specific chromosomes. Flow cytometry has emerged as the method of choice for producing SKY/M-FISH probes. This method separates the individual chromosomes on the basis of size and relative nucleotide content [69,70]. Each new batch of flow-sorted chromosomes should be tested using 24 single-paint FISH experiments to assure the specificity of the paint. The flow-sorted chromosomes are amplified by two rounds of degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), an enzymatic process for amplifying selectively discrete segments of DNA using specific primers [71]. The degenerative primers used in the PCR reaction produce randomly amplified regions representative of the entire chromosome [72]. The advantage of flow-sorted chromosome paint probes is that subsequent DNA amplifications can easily be expanded [46].

Painting probes contain both the unique and the repetitive DNA sequences specific to each chromosome. To prevent cross-hybridization of the repetitive probe sequences with repetitive sequences of other chromosomes, the mixture is incubated with human genomic DNA (e.g., COT-1), which is rich in highly repetitive sequences. The COT-1 DNA binds to the repetitive probe sequences, so that only single-copy DNA probe sequences are available for hybridization to the target DNA.

Table 17.4 Common fluors and haptens currently used in SKY and M-FISH

Fluors and Haptens	Peak excitation ¹	Peak emission ¹
Fluor – direct label		
Cy 5	649	670
DEAC (diethylamino-coumarin)	426	480
Fluorescein (FITC)	502	530
Rhodamine	550	570
Spectrum Aqua	433	480
Spectrum FRed	655	675
Spectrum Gold	530	555
Spectrum Green	497	524
Spectrum Orange	559	588
Spectrum Red	592	612
Texas Red	595	649
Fluor – indirect detection		
Alexa Fluor 680	679	702
Cy 5.5	675	694
Hapten		
Biotin		
Digoxigenin		
DAPI	367	452

¹ Vary slightly by manufacturer.

The blocking capacity of COT-1 DNA can vary by batch, so each new batch should be tested. To minimize such testing, it is advisable to order large batches at a time.

The great majority of laboratories performing SKY or M-FISH purchase the labeled probe sets from commercial vendors such as Applied Spectral Imaging (SKY), Abbott Molecular-Vysis (M-FISH), or MetaSystems (M-FISH). However, the laboratory of Thomas Ried at the National Cancer Institute, NIH, makes its own SKY probe kits using procedures included in this chapter. Below are brief summaries of the four protocols used to produce the kit.

1. Amplification of flow sorted DNA using DOP-PCR

Each individual flow-sorted chromosome is amplified using DOP-PCR. After completion of the PCR, an agarose gel is run to determine the efficiency of the amplification reaction. Sterile techniques are crucial because any contaminant will be amplified along with the sample.

2. Secondary DOP-PCR

A second round of amplification is performed to increase the quantity of starting material for further labeling PCR reactions.

3. DOP-PCR labeling

This step is the direct incorporation of fluorochrome- and hapten-conjugated deoxyuridine 5'-triphosphate (dUTP) to the amplified flow-sorted chromosomes. It consists of a total of 57 individual PCR labeling reactions (human kit).

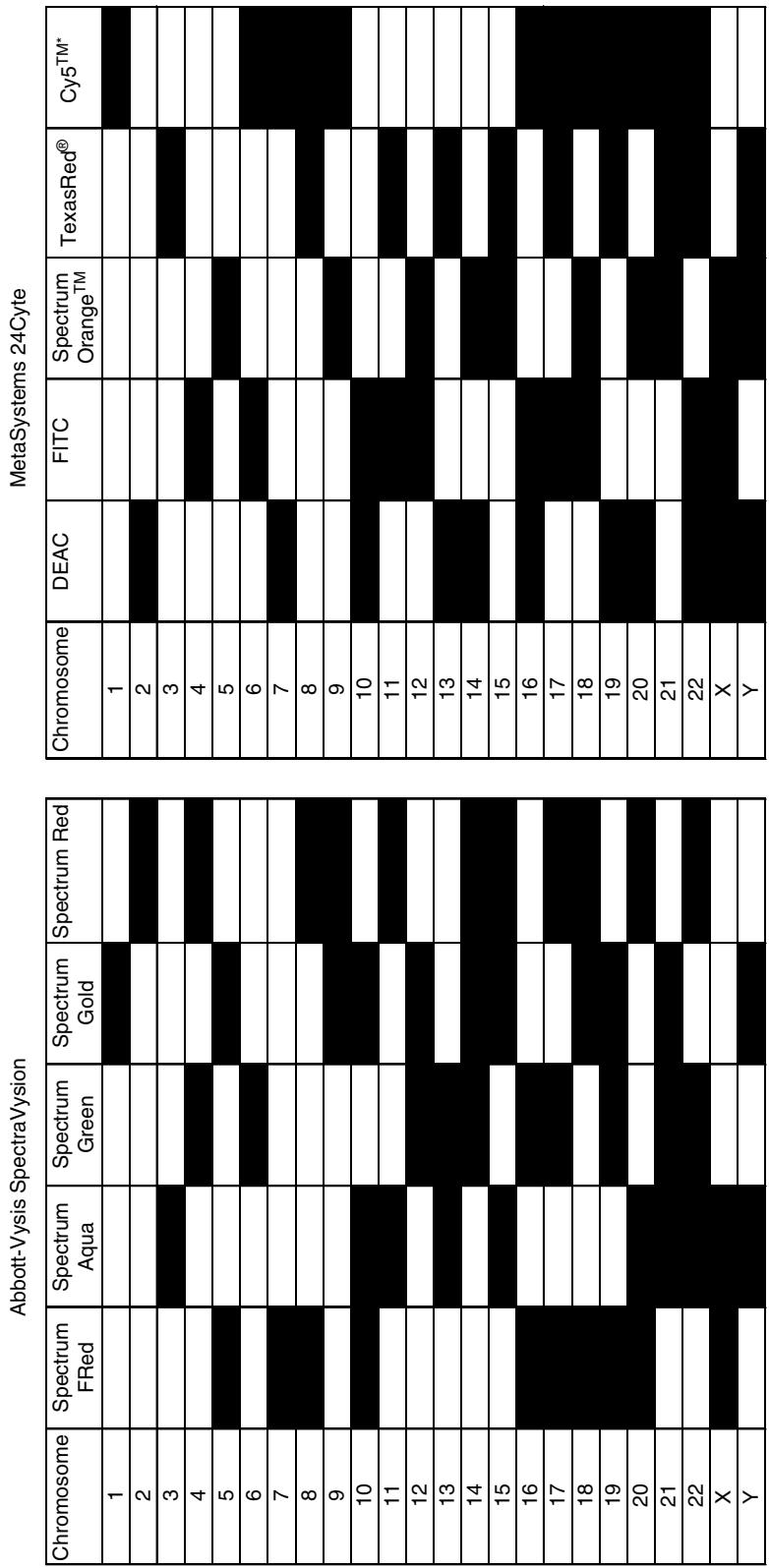
4. Precipitation of SKY kits and quality control (QC)

The precipitation step purifies and concentrates the probe by removing everything (primers, proteins, etc.) except the DNA. All labeled PCR products are pooled into one mixture and ethanol-precipitated with sodium acetate in the presence of COT-1 DNA and salmon sperm DNA, and then re-suspended in the hybridization solution (formamide, 2× SSC, and 10% dextran sulfate). Sodium acetate is a salt that binds to DNA and pulls it out of solution. Blocking DNA (COT-1) is

Table 17.5 Probe combinatorial labeling schemes for SKY/M-FISH

		Ried Laboratory, NCI						Applied Spectral Imaging (ASI)					
Chromosome	Spectrum Orange™	Texas Red®	Biotin	Spectrum Green	Digoxygenin	Chromosome	Rhodamine	Texas Red	Cy5	FITC	Cy5.5		
1						1							
2						2							
3						3							
4						4							
5						5							
6						6							
7						7							
8						8							
9						9							
10						10							
11						11							
12						12							
13						13							
14						14							
15						15							
16						16							
17						17							
18						18							
19						19							
20						20							
21						21							
22						22							
X						X							
Y						Y							

Biotin detected by streptavidin-Cy5, and digoxigenin by mouse anti-Dig and Alexa Fluor 680 goat anti-mouse IgG



All fluorophores are directly labeled (no detection required)

*Biotin is the reporter molecule and is detected by streptavidin-Cy5

used to reduce nonspecific hybridization of the probe to nontarget DNA. Carrier DNA (salmon sperm) is DNA from a distantly related species that is used to reduce background by associating with nonbiological sites such as the microscope slide. Dextran sulfate (a “crowder”) facilitates the hybridization process by decreasing the free space in the hybridization reaction; it is more hydrophilic than DNA, and because it is strongly hydrated in aqueous solution, the DNA has less access to water and hybridizes at a faster rate [53]. The probe, which at this point is ready for denaturation, is aliquoted into Eppendorf™ tubes to contain only as much material as is needed for each hybridization. One labeling PCR reaction yields approximately 22 kits, and for quality control purposes, one kit is hybridized to normal metaphases and the incorporation efficiency evaluated using the SKYVIEW imaging software. Probe kits are generally stored at –20°C and warmed to 37°C prior to denaturation. Because the probes are sensitive to enzymes that degrade DNA, gloves should always be worn when handling them.

Slide pretreatment

Slide pretreatment is outlined in Table 17.6. Usually, the first step, after aging, is equilibration in 2× SSC at room temperature. Each treatment step is followed by wash steps (either 2× SSC or PBS). It is recommended that slides be evaluated before, during, and after pretreatment to ensure successful hybridization.

Occasionally, slides are “clean” enough (i.e., very little cytoplasmic material) not to require pretreatment; the majority, however, benefit from enzymatic treatment. Cytoplasmic and nuclei debris may interfere with chromosomal denaturation, and thus impair hybridization, because of nonspecific adherence of probes to the proteins contained therein. Removal of the cytoplasm by enzyme digestion facilitates better probe accessibility to the chromosomal DNA and reduces background fluorescence. Several different enzymes can be used, including trypsin and proteinase K, but pepsin is the mildest of these proteases, and is the most widely used. The optimal pepsin concentration is dependent upon the age of the specimen, the amount of cytoplasm surrounding the chromosomes, and the strength of the pepsin. Each batch of pepsin is different; it is therefore necessary to empirically determine the optimum pepsin concentration for each sample. Digestion should be monitored (see the Ried Lab pretreatment in Protocol 17.1). Overtreatment impairs chromosome morphology and causes loss of structure, while undertreatment results in lower hybridization efficiencies and cytoplasmic autofluorescence [57]. The slide should be discarded if the chromosomes appear hollow and overdigested. Some protocols also recommend treatment with RNase to eliminate interfering remnants of cellular RNA. The MetaSystems protocol uses RNase only for mouse post-hybridization washes. RNase should be applied when using propidium iodide (PI) as a counterstain, since PI stains both DNA and RNA (DAPI stains only DNA).

Previously G-banded slides can be successfully hybridized using multicolor FISH techniques. The pepsin pretreatment is not necessary, as the chromosomes have already been partially digested with trypsin. In fact, G-banding enhances the DAPI banding and often results in brighter hybridization signals. Following the removal of immersion oil, the slides are destained, rinsed in water and PBS, and finally dehydrated in an ethanol series. For details, see the Ried Lab SKY (Protocol 17.1) and Mayo Clinic M-FISH procedures in Protocol 17.5, and Table 1 in “General guide for processing cytogenetic preparations for G-banding analysis followed by SKY or M-FISH” in Bayani and Squire [60]. G-banded slides can be hybridized immediately following preparation.

Table 17.6 Slide Pretreatment for SKY/M-FISH and CGH

Step	Action
Aging (Natural: time; Artificial: heat, hot 2× SSC)	Hardens DNA
2× SSC	Equilibration
RNase	Removes exogenous RNA
2× SSC	Wash to remove excess RNase
Enzyme (pepsin, proteinase K, weak HCl)	Digests protein and removes cytoplasm
Formaldehyde	Fixation of chromosomes
PBS Wash	Prevents formation of salt crystals while drying
PBS/MgCl ₂	Equilibration
Ethanol series	Dehydration
Air dry	

Slide denaturation

DNA is a double-stranded molecule. The whole *in situ* hybridization process is predicated on the separation of the two strands of both the probe DNA and the target DNA on the slide into a single-stranded state. When the two are combined in the hybridization process, the probe binds to complementary (target) DNA sequences to re-form the double-stranded molecule. DNA dissociates (separates) at 90–100 °C. This temperature, however, destroys cellular DNA; therefore, formamide, an organic solvent, is used to lower the melting temperature of DNA. Incubation of the cellular DNA on the slide in the presence of 70% formamide lowers the melting point of DNA to 70 °C without detrimental effects to cellular morphology. Once denaturation is achieved, the slide is immediately placed in 70% ethanol, either ice cold or at room temperature, followed by an increasing alcohol series to maintain the DNA in its denatured state and to remove any residual moisture.

Virtually all procedures use 70% formamide in 2× SSC in the denaturation process, but other chemicals such as 0.07 N sodium hydroxide (see the MetaSystems procedure, Protocol 17.6) can also be employed. The temperature of the 70% formamide treatment varies from 70 °C to 80 °C, according to the individual protocol, and the slides are either immersed in a formamide solution or treated with formamide (under a coverslip) while on a slide warmer set at 75 °C to 80 °C. The formamide used for denaturation must be of high quality, must be deionized, and must have a pH of about 7.0. Oxidation of deionized formamide causes DNA depurination, so proper preparation and storage are essential. Care should be taken in handling formamide since this chemical is caustic, toxic, teratogenic, and is a possible carcinogen. Since it is volatile at higher temperatures, one should also avoid breathing formamide fumes.

The timing of the slide denaturation process is critical and varies from 1 to 3 minutes, depending on the age of the slides, the species, and cell type: older slides generally require a longer incubation period, while mouse preparations may need a reduced time and temperature. Previously banded slides also require a shorter incubation time (10–30 seconds). Overdenaturation can reduce intensity due to loss of DNA and spreading of the chromosomal DNA over a larger area, so that the chromosomes appear “fluffy,” resulting in poor hybridization. Underdenaturation reduces the amount of single-stranded chromosomal DNA available for hybridization to the labeled probes, resulting in reduced hybridization efficiency with patchy hybridization.

Probe denaturation and hybridization

The probe is denatured at 72–80 °C for 5–7 minutes, either in a water bath or a Thermomixer, and then placed briefly on ice to prevent overdenaturation. Some procedures call for a pre-annealing step at 37 °C for 30–60 minutes; this allows the COT-1 sequences to anneal, that is, the repeat elements in the probe become double-stranded and are therefore prevented from hybridizing to the target DNA.

Once both the probe and the target DNA on the slide have been denatured, the probe can be applied to the slide, usually about 10 µL of probe per 18 × 18 mm or 22 × 22 mm coverslip and the edges sealed with rubber cement to prevent drying. The slide is placed in a light-proof, air-tight hybridization chamber, together with a moistened paper towel, and incubated at 37 °C for 1–4 days. The volume of probe and the size of the coverslip are dependent upon the mitotic index of the specimen and the quality of the metaphases.

Fluorophores are photo-bleached by exposure to light; therefore, all steps involved in handling the probes and the hybridized slide, including the incubation period and washes, should be performed in minimal light.

Post-hybridization washes, detection, and counterstain

Once the hybridization incubation is complete, the rubber cement and coverslip are carefully removed. The slides are then washed to remove unbound and nonspecifically bound probe, which can cause background fluorescence. Gentle agitation during post-hybridization washes facilitates the loosening of unbound probes and antibodies. The stringency of the wash solution affects the signal specificity and intensity. Stringency – the degree to which conditions affect dissociation of the probe-target complex – can be manipulated by reaction temperature, salt concentration, formamide concentration, and duration of washes: lowering the salt concentration and increasing the temperature, formamide concentration, or duration, elevates stringency. Slides hybridized with the WCP of SKY/M-FISH probe sets consisting of a combination of directly and indirectly labeled probes are generally washed in a low-stringency solution (1× SSC at 42–45 °C) following the 50% formamide/2× SSC wash, since painting probes bind primarily to unique sequences. More stringent wash conditions can be applied if high background is observed upon microscopy (see the troubleshooting section). The next step, which is optional, is incubation at 37 °C with a blocking solution such as bovine serum albumin (BSA), which binds nonspecifically to the charged silica of the glass slide, thereby preventing a nonspecific interaction between the slide and the fluorescent-labeled molecules of the detection reagents [47].

The number of detection steps which follow the formamide and blocking steps is dependent upon the number of indirectly labeled probes. The layering of the detection reagents adds 1–3 hours to the total post-hybridization procedure. Each blocking and detection step is followed by a wash series comprising a salt and a detergent, for example, 4× SSC/0.1% Tween 20. Tween 20 (polysorbate 20) is a nonionic detergent that suppresses nonspecific reactions between antibodies, antigens, and other molecules.

Following completion of the detection process, the slides are counterstained with a DNA stain, usually 4'6-diamidino-2-phenylindole dihydrochloride (DAPI), which fluoresces blue and produces a Q-like banding pattern on the chromosomes by binding strongly to DNA sequences containing three to four adjacent AT base pairs [73]. During the imaging process, the DAPI image is inverted electronically to a brightfield format, producing a black and white image revealing a Q-band-like pattern, thereby facilitating chromosome identification. The slides are then mounted and coverslipped with antifade (1,4-phenylene-diamine in glycerol) to avoid bleaching of the fluorescent dyes during microscopy and storage. Reagents such as antifade act as radical scavengers and/or antioxidants, preventing quenching. A DAPI/antifade combination is available commercially. Note: Old or degraded antifade (amber color) or impurities in the antifade can produce high levels of autofluorescence. When not in use, the slides should be stored at 4°C or –20°C to slow the rate of antifade degradation.

The use of only directly labeled probes (see the Abbott Molecular Vysis procedure, Protocol 17.8) in the probe set eliminates the necessity for the detection procedure. Following hybridization, therefore, the slides are simply washed with different concentrations of a salt/detergent mixture such as SSC/NonidetP 40 (NP-40, a nonionic detergent) to remove the unbound probe. The slides are then stained with DAPI.

Image acquisition

For each metaphase, a spectral image and a DAPI image are captured. The two images are analyzed together to determine the outlines of the chromosomes, the bands involved in breakpoints, and the origin of the chromosome or chromosome segment. In all systems, proper installation, with careful calibration and alignment of the microscope, camera, and filters, is crucial. Proper alignment of the microscope lamp to produce even illumination across the field of view is also important. Procedures for image acquisition and analysis software, along with manuals containing troubleshooting sections, are available from several vendors, including Applied Spectral Imaging (SKYVIEW: SKY), Leica Biosystems (CytoVision M-FISH and CW4000 FISH), Abbott Molecular-Vysis (SpectraVysis: M-FISH), and MetaSystems (Isis: M-FISH). Although it had originally been the intention of this author to present a summary of each of these analysis systems, the length and complexity of each has precluded such an inclusion. The only exceptions are brief discussions of M-FISH acquisition procedures that were submitted along with the hybridization procedures by the laboratories of the Oregon Health and Science University Knight Diagnostic Laboratories and the Mayo Clinic.

Metaphase selection is important in all systems: the best results are obtained from well-spread metaphases with few chromosome overlaps, uniform labeling, and high signal intensity along the entire length of each chromosome. Minimal background fluorescence is also desirable. Long exposures due to weak signal intensity increases background “noise,” while exposures that are too short due to the bright fluorescence of nearby nuclei or brightly fluorescing debris will result in weak chromosome signals. While compact metaphases and many overlapping chromosomes such as those frequently found in solid tumor preparations should be avoided, they can be productive but require more interaction and interpretation. High levels of autofluorescence, which interferes with image acquisition, can be due to a number of factors, including poor hybridization, degraded antifade, impurities in the antifade, fluorescent impurities in the probe or microscope slide, and fluorescent impurities in the optical components of the microscope (the latter can be due to optical cement).

Because it is visually difficult to differentiate between spectrally overlapping fluorochromes, the display colors (the raw images which are viewed under the microscope and captured by the camera) are assigned pseudo colors (classification colors) (Figure 17.2b,c) based on their specific fluorochrome signatures; the pseudo colors are chosen to maximize contrast. It is important, however, to view the raw images in order to assess the quality of the signal across the entire metaphase, including the suppression of repetitive DNA regions. Color blending at translocation junctions and overlapping chromosomes can cause the formation of what appear to be additional bands, called the “sandwich” effect. There are useful analytical tools in the provided software that aid in determining whether the color associated with the junction is real or not. The combinatorial schemes are checked to see if the blending of the fluorochromes from the joined chromosome segments could account for the additional band. In some cases, FISH studies are required to distinguish true insertions from color blending. Centromeres and telomeric regions, areas of repetitive DNA suppression by COT-1, can also be falsely classified and require experience to interpret. Dmin and hsr are frequently composed of material from more than one chromosome, and while multicolor FISH can be very helpful in determining the composition of these structures, it is advisable to confirm the chromosome assignment with WCP FISH probes.

All analytical software programs automatically arrange the chromosomes into a karyogram based on the emission spectra of the chromosomes and the combinatorial table. These karyograms usually require correction by the user, particularly when multiple chromosomal rearrangements are present or both normal homologues of a chromosome are missing from the karyogram.

SKY acquisition

The principle of spectral imaging is shown in Figure 17.1d. SKY combines Fourier spectroscopy (interferometry), CCD imaging, and epifluorescence microscopy to reconstruct spectral information at each pixel and differentially display all chromosomes after a single exposure [2,74]. All dyes are excited and measured simultaneously without any image shifts. The emitted light passes through a custom-made triple band pass filter (Chroma Technologies, Bellows Falls, VT) with narrow excitation and broad emission bands, is sent through a Sagnac interferometer (a special Fourier transform spectrometer), and is then captured with a CCD camera. In an exposure of about two minutes, the camera acquires the image and simultaneously measures the intensity of each pixel in the image at all points in the visible and near-infrared spectral range (400–800 nm). An interferogram is generated and the measured spectra are recovered after Fourier transformation. To view the spectral image in the raw (display) colors, the measured spectrum at each pixel is divided into three spectral ranges: red, green, and blue (RGB). The SKYVIEW software compares the acquired image against the combinatorial reference library stored in the computer and automatically assigns the same pseudo color to all pixels that have the same spectrum.

M-FISH acquisition

M-FISH imaging is based on sequential acquisition through narrow (5–15 nm) bandpass fluorochromes-specific optical filters (Figure 17.1b,c) [75]. The M-FISH system uses five single bandpass excitation/emission filter sets, where each filter set is specific for one of the five fluorochromes used in the combinatorial labeling process. The filters, which are designed to maximize the distinction of the fluorescent dyes, are mounted on a filter wheel, and acquisition is motorized and fully automatic. A DAPI image is captured, in addition to a separate image for each fluorescent dye. The concentrations of the fluorescent dyes in the PCR reaction and the labeled painting probes are adjusted so that all the dye combinations achieve equal fluorescent intensities. The fact that each dye can be viewed separately makes it possible to determine the intensity of each fluorescent label. In the M-FISH labeling schemes currently available from most vendors, five fluorochromes are used (Table 17.5). After all the images have been captured, image correction and calculation of a chromosome segmentation mask based on the DAPI image are performed by the computer software. Finally, a combinatorial labeling algorithm, based on a reference library, allows separation and identification of all chromosomes and produces a color karyogram based on the predominant classification of each chromosome.

It is imperative that the filter sets be matched to the specific probe cocktail, since incorrect filters may produce unacceptable levels of “crosstalk” between different fluorescent labels. The spectral images should be acquired prior to prolonged visual examination through the microscope, since extensive exposure to light can result in low specificity of all probes. The order in which the images are captured is also important, because some dyes emit a different wavelength following ultraviolet (UV) exposure.

Troubleshooting in SKY/M-FISH

Troubleshooting SKY/M-FISH is complicated by the many different steps and reagents employed in the preparation of probes and slides, the hybridization and detection procedures themselves, and the imaging process. There are many places where things can go wrong! Table 17.7 lists various factors that can contribute to poor hybridization results.

17.3 Comparative genomic hybridization

17.3.1 Introduction

CGH is a genome-wide screening technique that identifies and maps DNA copy number imbalances in a single experiment [4,76]. One of the major advantages of CGH, as compared with other cytogenetic methods, is that it only requires genomic DNA from the tissue of interest without the necessity of metaphase preparations from tumor cells, which often grow poorly *in vitro*. It also permits the study of archival material, such as formalin-fixed, paraffin-embedded tissue [77], following tissue microdissection and DNA amplification. It can be applied to premalignant tissue, which frequently has few dividing cells. Since CGH requires no prior knowledge of the cytogenetic picture, it is particularly useful as a tool of discovery: it is a good starting point in the search for novel genes implicated in cancer, and can be followed by other molecular techniques, such as

Table 17.7 Factors contributing to poor hybridization in SKY/M-FISH

Slides
Poor chromosome morphology (chromosomes too light gray, too shiny, or hollow in appearance)
Excess cytoplasm and debris (inhibits probe binding)
Slides too old (hardened or degraded DNA)
Excessive slide pretreatment (RNase and pepsin concentration/duration)
Incorrect denaturation time or temperature
Overdenaturation: destroys DNA
Underdenaturation: DNA does not separate into single strands
Inadequate sealing of coverslips (leads to dilution of probe due to excess moisture under coverslips)
Residual immersion oil on previously G-banded slides
Overtrypsinization of previously G-banded slides
Probe
Poor-quality DNA (from source or from DNA extraction procedure)
Poor hapten or fluorochrome incorporation
Expired or improperly stored probe
Incorrect probe concentration
Incorrect denaturation time or temperature
Hybridization
Incorrect hybridization temperature
Insufficient duration of hybridization
Low humidity in hybridization chamber
Antibodies/DAPI/Antifade
Expired or improperly stored antibodies
Improperly diluted antibodies
Antibodies applied in wrong order
Expired or degraded antifade
Post-hybridization washes
Incorrect temperature during detection
Wash solutions not fresh
Blocking solution (BSA) not properly washed off
No agitation during washes
Blocking/detergent reagents allowed to dry on slide
Imaging
Poor metaphase selection
Bright nuclei or debris close to chromosomes
Incorrect sequence of filters in M-FISH image capture
Incorrect filters
Filters installed improperly
Filters degraded
Poor light source (old lamp)
Lamp not properly centered
Failure to close down field diaphragm
Fluorescent impurities in optical components of microscope

FISH and sequencing, to pinpoint actual genes involved in the development of cancer [78]. This technique has been especially useful for screening potential proto-oncogenes (with amplifications) or tumor suppressor genes (with deletions) in various tumors.

17.3.2 Theory

A quantitative two-color technique, CGH is based on the *in situ* hybridization of differentially labeled tumor (test) DNA and normal (control) reference DNA to normal metaphases. It is a reverse hybridization strategy in which the tumor DNA is used as the probe. Control DNA is used as an internal standard “which allows differentiation between signal intensities that are caused by differences in DNA sequence copy numbers from those that are attributable to experimental parameters” [79]. As in multicolor FISH, COT-1 DNA is added to ensure that only single copy sequences contribute to the hybridization. The tumor DNA is labeled with a green fluorochrome (e.g., avidin or streptavidin-FITC) and the reference DNA is labeled with a red fluorochrome (e.g., anti-digoxigenin-TRITC) by a standard nick translation procedure. Equal amounts of each labeled DNA are pooled and hybridized to normal metaphases from the same species. During hybridization, homologous segments of both DNAs compete to anneal to the same targets on metaphase chromosomes. Where the tumor contains normal chromosome elements, the two probes will be present in equal amounts, and the two dyes will combine to produce a third (blended) color, yellow-orange. Where the tumor contains excess material, such as is seen with gene amplification, the tumor color (green) will predominate. Likewise, deleted tumor genome segments will show a dominance of the normal DNA color, that is, red. Figure 17.3a–d shows the color variation obtained from a cell displaying chromosomal gain and loss, and Figure 17.3e demonstrates the principle of CGH. The ratios of the fluorescence intensities of each fluorochrome are measured along each chromosome by a digital image analysis system with a CCD camera, specific optical filters, and specialized computer software, providing an overview of DNA sequence copy number changes of both gains and losses in the tumor tissue. A karyogram is produced displaying loss and gain along each chromosome as a histogram (Figure 17.2e). In the final step, the FITC/TRITC fluorescence intensity ratio is measured, after background subtraction, and the average ratio profiles are calculated along the medial axis of each individual chromosome, based on the data from 10–15 metaphase spreads. A ratio of 1 indicates equal copy number of the tumor and reference genome, a ratio of 0.5 indicates a deletion (monosomy or hemizygosity), and a ratio of 1.5 indicates a gain (partial or total trisomy). A ratio greater than 2.5 indicates amplification (such as seen with *dmin* and *hsr*). The ratios are shown as three vertical lines to the right of each chromosome idiogram: the middle line represents a balanced state, while the left and right vertical lines define the threshold values for under- or over-representation, respectively.

17.3.3 Advantages and limitations

Since many of the significant chromosomal changes in hematologic disorders are balanced rearrangements, CGH has proven to be most useful in detecting copy number changes in solid tumors, which are marked by chromosomal imbalances. Nonrandom patterns of genomic imbalances have emerged for many solid tumors: tumors of the same type have similar patterns of regional gain and loss. CGH is ideal for studying aberrations accumulated during tumor progression, since it can be applied to previously stained solid tumor sections that have been histologically defined [33]. CGH has even been successfully applied to single cells, in preimplantation analysis, and in minimal residual disease [65].

CGH can only detect imbalances that are present in a substantial proportion of the tumor, that is, >50%, although highly amplified regions may be detected even if only 20–30% of the cells are abnormal. Mosaicism cannot be detected with any degree of confidence. In general, the limit for low copy number changes is about 10 Mb; however, amplicons with high copy numbers may be detectable even when the size is much smaller (perhaps 2 Mb); see Jeuken et al. [78] for a more thorough discussion of sensitivity in detecting minimal loss and amplified DNA. CGH is unable to detect balanced whole genome copy number changes (ploidy) or balanced reciprocal chromosomal rearrangements, since these have no effect on copy number, nor can it identify the partners in unbalanced structural rearrangements. Although good correlations are generally found between cytogenetics and CGH results, Schröck et al. [80] reported that standard karyotyping underestimated the frequency of amplifications in their series on malignant gliomas. See Table 17.1 for a summary of the advantages and limitations of CGH and a comparison with FISH and multicolor FISH.

17.3.4 Applications of CGH

Since the introduction of CGH by Kallioniemi et al. in 1992 [4], it has been applied to thousands of cases of cancer, resulting in the identification of tumor-specific and stage-specific aberrations. The Progenetix online database currently (accessed November 2008) contains the results of over 32,000 cases collected from almost 2500 references [6]. While it has been

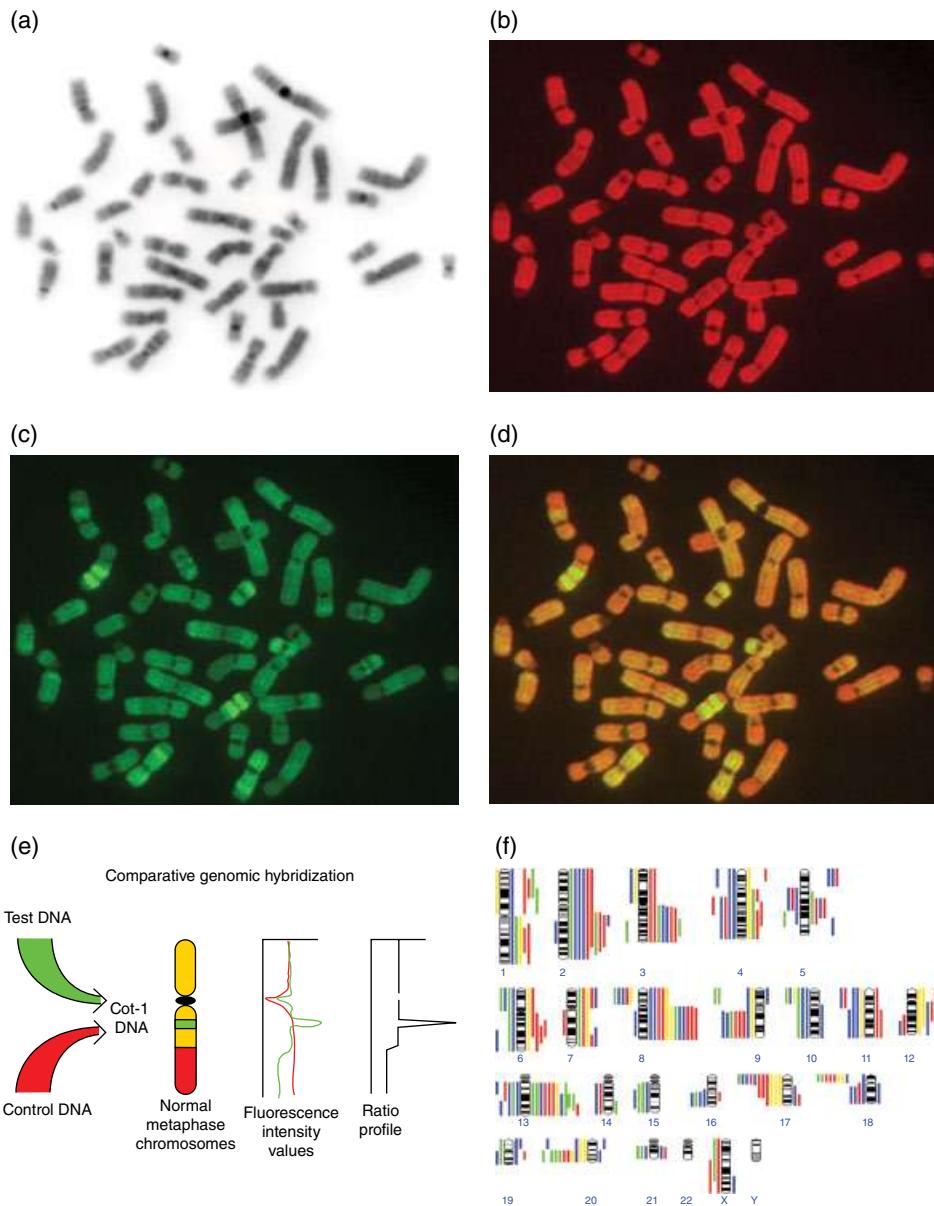


Figure 17.3 Example of acquired metaphase images from CGH of breast cancer cell line SKBR3. (a) Inverted DAPI image. (b) Control (reference) genome specific red fluorescence image (labeled with digoxigenin and detected with TRITC); all chromosomes are labeled homogeneously. (c) Tumor (test) genome specific green fluorescence image (labeled with biotin and detected with FITC). Note different fluorescent patterns indicating chromosomal gains and losses. (d) Electronically merged red and green fluorescence images from (b) and (c). Regions showing predominant green color indicate copy number gain, while regions with predominant red color reflect copy number loss. (e) (CGH) Schematic diagram demonstrating the principle of CGH. Labeled tumor DNA (colored green) and labeled control DNA (colored red) are mixed and co-hybridized under Cot-1 DNA suppression to normal chromosome. A gain of tumor DNA, i.e., amplification in the tumor genome, appears as green regions on the chromosome, and red regions represent a loss of tumor DNA. Gray regions (i.e., centromere) represent regions rich in repetitive sequences which need to be excluded from the analysis due to suppression conditions. Fluorescence intensity values are measured for each fluorochrome along each chromosomal axis. An average ratio profile based on at least 5–10 metaphases is calculated for every chromosome. Reprinted with permission from Veldman et al., *Appl Cytogenet* 1996; 22:117–122. (f) CGH profiles in ovarian cancer: bars to the left of the chromosomes indicate copy number loss and bars to the right indicate copy number gain. Using the CGH Case Comparison tool available on the SKY/M-FISH and CGH Database (<http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi>), the profiles from four different subtypes of ovarian cancer are displayed, each in a different color: mucinous cystadenocarcinoma (green), papillary serous cystadenocarcinoma (red), clear cell adenocarcinoma (blue), and serous cystadenoma of borderline malignancy (yellow). Österberg 2005. Reproduced with permission of Elsevier. See insert for color representation of this figure.

applied to all types of cancer, including leukemias and lymphomas, CGH has proven to be particularly useful in solid tumors. Several large reviews of human CGH studies have been conducted in recent years: Gebhart [81] summarized the results from 8000 cases of solid tumors reflecting ten years of CGH studies, including those from reviews conducted by Zitzelsberger et al. [82], Knuutila et al. [83,84], Rooney et al. [85], Gebhart and Liehr [86], Koschny et al. [87], and Strucki et al. [88]; his results are presented in Table 17.8. The review by Strucki et al. [88] included both solid tumors and hematologic malignancies.

In solid tumors, the average number of copy number aberrations (CNAs) varies by tumor type (and study) from a high of 23 in oral cancer, 17.7 in squamous cell carcinoma, and 13.6 in colorectal cancer to a low of 2.3 in Wilms tumor. According to Rooney et al. [85], the most frequent (in order of prevalence) overall gains involved the long arms of chromosomes 8, 7, 17, 3, 20, 12, and 11, and the short arms of chromosomes 7, 5, 12, and 6. The most common losses were found on 13q, 9p, and 8p. Gebhart and Liehr [86] noted a high average CNA in tumors of the ingestion tract or respiratory airways, leading to speculation of a possible role for ingested or inhaled mutagenic environmental factors. CGH can distinguish histologic subtypes in various connective tissue tumors, clinical stages in colon cancer, tumor classes in head and neck cancer (HNSCC), and primary versus recurrent tumors in prostate cancer [81,85]. It has also been able to distinguish hereditary and nonhereditary forms of breast, ovary, and gastrointestinal tumors [81]. There is usually a considerable similarity between the aberrations of primary tumors and their metastases, but the total of CNAs is usually higher in advanced disease and in metastases. Some abnormalities are seen in many different types of tumors, especially those related to progression, while other abnormalities are specific to certain tumor types. Among the former are gain of 8q (e.g., in cancers of the head and neck, esophagus, colon and rectum, gastrointestinal tract, and prostate), and loss of 8p (e.g., in renal and hepatocellular carcinomas), which appear to be involved in progression in many tumors and are clearly associated with metastatic disease in some tumors. Chromosome arm 17q is amplified in metastases in several tumors (colorectal, renal cell, pancreatic, and gastrointestinal), while 17p loss is associated with progression and metastatic disease (colorectal, breast, lung, liver, and prostate cancer). Some changes are more evident in specific diseases, such as gain of 17q24q25 and loss of 21q in colorectal tumor metastases and loss of 18q in breast carcinomas. In the diagnostic setting, CGH can distinguish between lipoma and liposarcoma (gain of 12q), and gain of 2p and of 3q are of considerable diagnostic significance in rhabdomyosarcoma and cervical cancer [89], respectively. Of prognostic interest, loss of 8p can distinguish between organ-confined and locally advanced prostate cancer [90], and CGH-identified aberrations could be correlated with histology and stage across the spectrum of thymomas [91]. Figure 17.3f shows a comparison of CGH profiles from four different subtypes of ovarian cancer, demonstrating the differences in chromosomal gain and loss observed in various subtypes of this disease.

While CGH is used primarily as a tool in cancer studies, it has been applied in clinical cytogenetics as well. Bryndorf et al. [92] studied 11 fetal samples and resolved seven cases of unbalanced aberrations that were not reliably identified by conventional cytogenetics; four other cases with numerical aberrations were also successfully diagnosed by CGH.

17.3.5 Methodology

The CGH procedure is outlined in Table 17.3. Information on the technical aspects of CGH was obtained primarily from the following sources: Lichter et al. [79], Veldman et al. [93], Montgomery et al. [54], Joos et al. [94], Jeuken et al. [78], Speicher and Carter [65], and the CGH protocols from the National Cancer Institute (USA), Abbott Molecular-Vysis, Leica Biosystems, and Leica Microsystems.

Normal metaphase preparations

Normal metaphase slides to be used for CGH studies can be prepared in large batches using peripheral blood lymphocyte cultures from a normal donor. (Some protocols advocate the use of male-derived cells and reference DNA to ensure the determination of both the X and Y chromosomes, while others recommend using tumor DNA, control DNA, and normal metaphase slides of the same gender) [78]. See Chapter 3 for information on culturing and harvesting techniques.

Slides that are not used within a few days can be stored frozen at -80°C, in the presence of a dessicant, for several months. For quality control purposes, a few of the slides can be tested by *in situ* hybridization with normal genomic DNA. Just as for multicolor FISH, the quality of the slides is very important: they should be free of residual cell debris and the metaphases should be devoid of cytoplasm. The chromosomes should be relatively straight and long (500–800 band resolution; short contracted chromosomes result in lower resolution), and well spread with minimal overlaps. Chromosomes that are curly or overlapped will not produce accurate results. See Karhu et al. [95] for a discussion of slide quality issues in CGH.

Table 17.8 Collection of nearly 8000 tumors examined by CGH grouped according to their entity and ranked on the average number of copy number alterations (CNAs)^{1,2}

Tumor type/site	No. of cases evaluated	Average no. of CNAs	Most frequent imbalances	
			Genomic losses	Genomic gains
HNSCC ³	351	13.6 ⁴	3p, 5q21	3q26q27, 5p15, 11q13
Colorectal carcinomas	223	13.6 ⁴	18q, 8p	20q, 8q24
Lung carcinomas	212	11.8 ⁴	3p, 5q	3q26q29, 5p
Esophageal carcinoma	285	11.2 ⁴	4q21q26, 7p21p22	3q26q27, 8q24
Osteosarcomas	111	11.1 ⁴	10q, 13q	8q, 1p, 1q, 17p, 19
Ovarian carcinoma	362	10.9 ⁴	16q, 17p, 18q	3q, 8q, 1q, 20q
Pancreatic carcinoma	223	10.8 ⁴	9p, 18q	20q, 17q, 7, 8q, 12p
Testicular cancer	100	10.2 ⁴	13q, 18q	12p, 7, 8
Myosarcomas	187	9.6		12q13, 2q, 17p, 7q, 1q21
Adrenocortical carcinoma	106	9.3	4, 18q	12q24, 9q34
Malignant fibrous histiocytoma	141	8.2	13q, 12p	12q15q15, 6q, 5p, 1p21
Breast cancer	725	7.9	16q, 9p, 17p, 18q	1q, 8q, 17q, 11q13, 20q
Gastric cancer	584	7.4	Y, 4q, 17p13	17q, 8q34, 20q, 5q15
Liver carcinoma	523	7.4	4q21q26	1q, 8q
Nasopharynx carcinoma	113	7.4	3p14p21, 9p, 11q23	12p12, 12q13q15, 1q21q22
Urinary bladder carcinoma	520	7.2	9, 8p, 11, Y	1q, 5p, 8q, 17q, 3q, 20q
Neuroblastoma	318	7.0	1p36	17q, 2p23p24, 3q24q26, 8q
Uterine carcinomas	292	6.8 ⁴	3p, 4q, 13q	8q, 3q, 5p
Prostate carcinoma	262	6.8	8p, 13q	8q, 7q, 17q
Mesothelioma	122	6.5	9p21, 22q	1q, 15q, 8q
Astrocytomas	523	6.3	9p, 10q, 13q, 1q	7, 8q, 20q
Kidney carcinomas	410	5.6	3p, 4q, 9p, Y	1q, 7q, 8q, 17q
Malignant melanoma	118	5.6	9, 10q	8q, 6p, 7
Ependymomas	196	4.4	22q, 6q	1q, 7, 9q, 17q
Pheochromocytoma	100	4.3	1p, 3q, 11	19, 17q
Retinoblastoma	200	4.1	16q	6p, 1q, 2p
Thyroid carcinoma	332	3.1	22q, 11q	19, 9q34
Pituitary adenoma	120	2.9	11	19, X
Ewing sarcoma	107	2.3	16q	8, 12, 1q
Wilms tumor	127	2.3	4q, 9p	1q, 8q, 12q

¹ Reprinted from Gebhart E. Comparative genomic hybridization (CGH): ten years of substantial progress in human solid tumor molecular cytogenetics. *Cytogenet Genome Res* 2000; 104:353–358 with permission from the author. The data were gathered from 273 papers reporting on at least 10, but mostly more, cases, each; a complete reference list can be requested from the author.

² Each entity was included only if at least 100 cases were available.

³ Excluding nasopharynx cancer

⁴ There were big differences among the studies with respect to the average CNAs.

See multicolor FISH sections on slide preparation, pretreatment, and storage, above. The mechanisms of action of the various reagents used in slide pretreatment are listed in Table 17.6.

Genomic DNA

The quality of the genomic DNA is very important. Crude or degraded DNA may cause poor nucleotide incorporation during nick translation, resulting in an irregular, speckled hybridization pattern, less pronounced differences in staining intensities, and reduced detection sensitivity. Control DNA can be prepared in batches and stored frozen. It is also available commercially, as is positive control DNA that is prepared from cell lines with known genetic aberrations easily detectable by CGH (e.g., Abbott Molecular-Vysis). A mixture of red and green labeled reference DNA can also be used as a positive control to assess the efficiency of the hybridization. Probe concentration is also important because it affects the signal to noise ratio: too high a concentration can produce nonspecific background hybridization, and with too low a concentration, no signal is detected. A concentration of 0.5–2 µg/µL is ordinarily used for CGH. In general, DNA isolated from frozen tissue is of better quality than DNA from paraffin-embedded tissue, since DNA from the latter can be degraded and cross-linked during formalin fixation [78].

The most common procedure for isolating genomic DNA uses lysis or extraction buffers, proteinase K digestion, and phenol extraction. See the submitted protocols for DNA extraction from fresh, frozen, and paraffin embedded tissue; extraction protocols for other tissues are available at the Ried Lab website (<http://www.riedlab.nci.nih.gov/>). When using paraffin-embedded tissue, the first step involves removal of the paraffin from the block slices by the use of xylene or other such solvents. Solid tumors are thoroughly minced and digested with proteinase K prior to extraction. DNA can also be isolated using a high salt method (see the Ried Lab website protocols) or by using a commercially available kit (e.g., Qiagen DNeasy Blood and Tissue kit, Valencia, CA). A yield of 1 µg of DNA is sufficient for successful CGH. When the yield is small, the genomic DNA can be amplified using DOP-PCR (see SKY/M-FISH Probes, above, and Protocol 17.1); this technique can be applied even when only a few cells are available. It is best to amplify both test and reference DNA to avoid false-negative and false-positive aberrations [78]. See protocol SSCOMP-PCR913 in Joos et al. [94] for obtaining single cells by micromanipulation and amplification of the genomic DNA by DOP-PCR. Physical enrichment of tumor cells can also be accomplished using cell sorting [94]. Microdissection of tumors by micromanipulator-directed fine needle or laser dissection can be used to eliminate contaminating normal cells or to separate different populations of heterogeneous tumors; these samples are then amplified by PCR.

Nick translation

The extracted genomic DNA is labeled using nick translation. This is the preferred method of labeling since the length of the probe fragment can be controlled by DNase digestion. “Double-stranded DNA is labeled enzymatically with labeled nucleotide. DNase I nicks the probe DNA in random locations exposing free 3' OH groups. DNA polymerase I adds nucleotides to these free 3' OH and simultaneously removes nucleotides from the 5' side. As the “nick” proceeds along the DNA, in the presence of labeled dUTP (e.g., digoxigenin or biotin) and other unlabeled nucleotides (dATP, dGTP, and dCTP), labeled nucleotide is incorporated into the DNA to produce a nonisotopic, labeled probe [54]. While direct labeling with coupled fluorochromes is used by some (see the Abbott Molecular-Vysis procedure, Protocol 17.8), the most widely used labels are the reporter molecules biotin (test DNA) and digoxigenin (control DNA), which are detected via fluorochrome-coupled reporter binding molecules.

The extracted DNA (1–2 µg) is combined with the dNTP mixture, nick translation buffer, nick translation enzyme (polymerase), DNase, a β-mercaptoethanol, and water. The mixture is incubated at 15°C for about 2 hours, and the reaction is stopped either with a stop buffer (e.g., EDTA) or by application of heat. The size of the probe fragments is critical to successful uniform hybridization; most procedures recommend a fragment size of 500–1000 base pairs. Before stopping the reaction with EDTA, which inactivates the DNase, the length of the probe is checked with gel electrophoresis using a 1% agarose gel with a suitably sized DNA marker (e.g., Lambda Hind III). If the DNA is too large, more DNase is added and the incubation is continued; this step is repeated until the probe reaches the desired length. If the probe is too small, it may be necessary to repeat the nick translation using less DNase. If the probe fails to be digested, the DNA can be purified with an additional phenol extraction and the nick translation repeated. After stopping the reaction, some protocols call for the purification of the DNA through a Sephadex spin column [94], while others proceed directly to the precipitation protocol. Smaller fragments can produce nonspecific hybridization, while longer fragments can result in halos around the chromosomes and increase background. A universal labeling system (Leica Microsystems/Kreatech Diagnostics, Buffalo Grove, IL) can be used for labeling degraded DNA that is unsuitable for nick translation [78].

Precipitation and denaturation of probe DNA and slide denaturation

The probe DNAs are precipitated in the presence of COT-1 DNA and salmon sperm: 1 µg each of the labeled test and control DNA is combined with COT-1, and precipitated with sodium acetate and ethanol. The DNA is washed with ethanol and then precipitated. It is then re-suspended in the hybridization solution (formamide) and the hybridization buffer. The probe is then denatured at 75 °C for 5 minutes, followed by incubation on ice, and preannealing at 37 °C for 30–60 minutes. (See the SKY/M-FISH probe section, step 4 [(Precipitation of SKY kits), for the action of the various reagents and the purpose of preannealing.) Shortly before the preannealing step is complete, the slides can be denatured for 1.5 to 2 minutes in a formamide/2× SSC solution, either in a Coplin jar or on a slide warmer, and then immediately transferred to cold 70% ethanol, followed by an increasing alcohol series (90%, 100% ethanol); this is done to maintain the chromosomal DNA in its denatured state. See “Slide denaturation” in the multicolor FISH section for a more thorough discussion of slide denaturation.

Hybridization

Once both the probe DNA and the slide DNA have been denatured, the preannealed probe is placed on the target area of the slide, a coverslip is applied, and the edges of the coverslip are sealed with rubber cement. The slide is then placed in a hybridization chamber at 37 °C for 2–3 days.

Post-hybridization washes, detection, and counterstain

See the multicolor FISH section for detailed discussions of the purpose of each step and the effects of stringency in the post hybridization washes and detection steps. The steps involved in detection when using indirect labeling with biotin and digoxigenin are as follows:

1. Remove rubber cement and coverslips.
2. Wash with formamide/2× SSC.
3. Wash with 0.1× SSC.
4. Block with 3% BSA.
5. Detect.
6. Wash with 1% BSA/4× SSC/0.1% Tween 20.
7. Counterstain with DAPI.
8. Mount with antifade.
9. Store slide in a light-free environment at 4 °C until ready to use.

In some published procedures, the detection reagents are combined into one step (e.g., fluorescein-conjugated avidin and rhodamine-conjugated anti-digoxigenin [94]), while in others, the detecting reagents are layered in separate steps (see Ried Lab’s Protocol 17.1).

Procedures employing direct labeling of the fluorochromes require no detection; thus the post hybridization procedure consists solely of washing with a salt/detergent mixture (e.g., SSC/NP-40) to remove unbound probe, followed by staining with DAPI (see the Abbott Molecular-Vysis procedure, Protocol 17.8).

Image acquisition

Image capture, karyotyping, and analysis are outlined in Table 17.9. The information for this section was obtained primarily from the Lichter et al. [79], Joos et al. [94], Applied imaging, Protocol 17.3 and Protocol 17.4 and Leica CGH protocols. High quality imaging is required for CGH: precise calibration of the epifluorescence microscope, mercury lamp (50 W or 100 W), filter sets, and CCD camera is critical for accurate visualization and acquisition of the gray level digital images. The mercury lamp should be precisely adjusted to produce homogeneous illumination (when the bulb is not properly aligned, homologous chromosomes may show different ratio profiles), and the field diaphragm should be closed down to the border of the image acquisition area to reduce blurring and increase contrast. Most laboratories prefer a 63× objective (ultraviolet-transparent plan), which is adequate for visual inspection of the images and small enough so that the images fit into the margins required by the analysis software. The digital CCD camera should have high sensitivity and produce minimal shading. Three high-quality narrow single-band-pass filter sets are used to selectively excite each fluorophore (DAPI, FITC, and TRITC) and to specifically measure the emission light. Consecutive images for each fluorochrome are obtained from each

Table 17.9 Steps in CGH image capture, karyotyping, and analysis

1. Visually examine the slide to evaluate the quality of the hybridization; avoid prolonged exposure.
2. Select cells with well spread chromosomes, few overlaps, and homogeneous staining. Avoid wide variations in chromosome length since this may adversely affect the spatial resolution of the averaged ratio profiles. Generally 10–15 images are acquired for each case, enough to yield 20 copies of each individual chromosome.
3. Center the cell and capture all three images.
4. Subtraction of background fluorescence and normalization of one fluorochrome to the other are done automatically by the software.
5. Threshold values of tests are automatically compared to those of known good cells.
6. Generate mask (border) around each chromosome and accept or reject each chromosome (reject chromosomes that lie outside the slide confidence intervals).
7. Karyotype cells using the inverted DAPI image.
8. Mark correct locations of centromeres and chromosomal axes.
9. CGH profiles are generated for each accepted chromosome.
10. Display profiles for individual chromosomes for each cell, for all cells, and average profiles for each cell and for all cells. If the profile for a particular individual chromosome differs markedly from those seen in the other cells, discard it from analysis.

metaphase (Figure 17.3a–d); the use of motor-driven turrets for automatically shifting the filter sets greatly minimizes optical pixel shift. The optical settings, including exposure times, should be kept constant for all images acquired for a single case. As in multicolor FISH, the DAPI images are electronically inverted (Figure 17.3a) to produce a G-like banding pattern for chromosomal band identification. Software programs designed for CGH imaging and analysis are available from several suppliers, including Abbott Molecular-Vysis, Leica Biosystems, Leica Microsystems, and MetaSystems. The written instructions are too extensive to be included in the protocols section of this chapter, but an example of the CGH process available from Leica Biosystems is outlined in Figure 17.4; a useful option is included in this system: the labeling of select chromosome centromeres (with a fourth fluorochrome) to assist in differentiating between chromosome pairs which are sometimes difficult to distinguish one from the other, e.g., labeling of chromosomes 4 and 19 centromeres to distinguish them from chromosomes 5 and 20, respectively.

Visual inspection will detect gross genomic imbalances and provide a rough assessment of the quality of the hybridization. Bright and homogeneous painting of the chromosomes, with minimal bleed-through and image shifts, is indicative of a successful hybridization, while a speckled, nonhomogeneous pattern with high background fluorescence is indicative of poor quality and can cause considerable variation within ratio profiles. The green fluorescence staining intensity of the tumor DNA is variable, due to gains and losses of the tumor genome, whereas the red staining intensity of the control DNA should be uniform.

The procedure begins with the capture of images from 10–15 cells (at least 15 good chromosomes from each chromosome pair). Once all the fluorochrome images for a cell are captured, the background is automatically subtracted, nonchromosomal artifacts such as nuclei and debris are removed from the image, and touching chromosomes are separated from one another. The software generates the CGH profile using the midline of the chromosome, so this midline should be accurately drawn down the center of the chromosomes. The position of the centromere is also important since it is used to correctly map the profile of each chromosome arm onto the idiogram template. Both the midline and the centromere position can be adjusted manually. Once these tasks have been performed, the software measures the median intensity of each fluorochrome along the chromosome, and the values of these measurements are compared with the threshold values of known cells.

Analysis is performed using fixed thresholds (the default) or statistical thresholds [76,78]. All software currently available automatically threshold the image, and the use of fixed thresholds is generally recommended to avoid false-positive imbalances. Default thresholds are placed symmetrically around a ratio of 1.0. For trisomy the ratio is 1.5 : 1 (3 test : 2 control) and for monosomy the ratio would be 0.5 : 1. However, to compensate for the fact that most tumor samples contain both normal and abnormal cells, default thresholds are set at 1.2–1.25 for gain and 0.75–0.8 for loss. The software ratio thresholds can be recalculated and manually reset. Statistical thresholds are based on standard reference intervals and/or confidence intervals of 95%, 99%, or 99.5% [93]; see the High Resolution CGH software instructions from Applied Imaging. The use of statistical thresholds increases specificity and sensitivity and includes analysis of regions, such as telomeres, that normally are excluded from CGH analysis. Statistical thresholds are based on the calculation of the variability of the average ratio for balanced chromosomes [75,79,96].

The repetitive sequences present in pericentromeric and heterochromatic regions blocked by COT-1 DNA cannot be reliably evaluated and are excluded from analysis. Telomeric regions can also produce unreliable ratios, due to their weak fluorescent

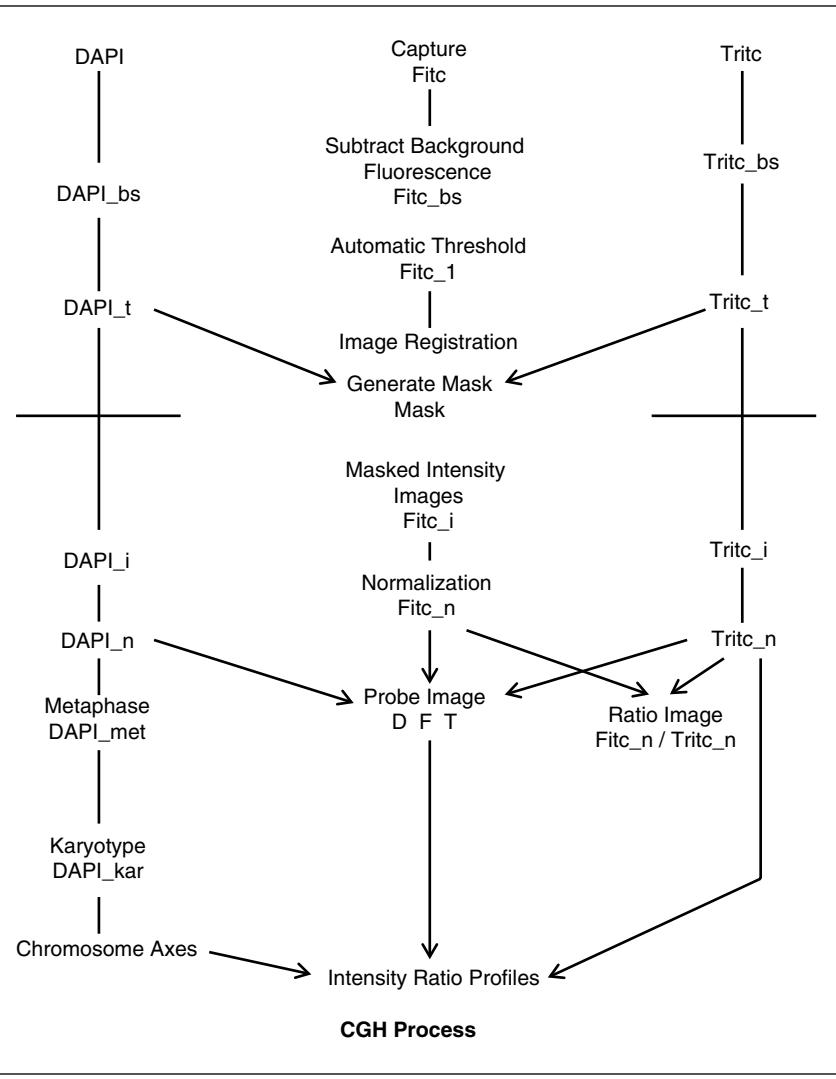


Figure 17.4 Diagram demonstrating the Genetix Corporation protocol for image capture and analysis in CGH. Reproduced with the kind permission of Michael Behme.

Table 17.10 Troubleshooting in CGH

Result	Possible cause
Speckled nonhomogenous staining pattern or high background fluorescence	Poor metaphase spreads Degraded DNA Inappropriate size of labeled DNA probe fragments after nick translation: too large = starry background outside chromosomes; too small = insufficient suppression of repetitive sequences
Bright signals and smooth staining but poor results; strong staining of heterochromatin	Insufficient suppression of repetitive sequences Nonhomogenous illumination of optical field
Variation in profiles impairing spatial resolution of average ratio profiles	Nonhomogenous illumination of optical field Wide variation in chromosome length Failure to reject inadequate images or overlapped or curled chromosomes Incorrect positioning of centromeres or chromosome axes
Blurred image and poor contrast	Failure to close down field diaphragm
No gains or losses detected	Low proportion of tumor cells in sample
Faded or weak fluorescence signals	Bright nucleus or debris within captured image Extended exposure during visual examination
False positive or negative results	Selection of inappropriate threshold values: too narrow → false positive; too wide → false negative

intensities. Small aberrations near the centromere can also be difficult to interpret, since they can be mistaken for centromeric artifacts. According to Jeuken et al. [78], special attention should be paid to the G-C rich regions 1p32-pter, 9p, 16p, 19, and 22, because these areas can show false-positive aberrations in suboptimal CGH experiments.

Troubleshooting

As in multicolor FISH, there are many procedures involved in the CGH process, each with multiple steps and reagents, so troubleshooting can be a complicated process. Table 17.10 lists problematic results and their possible causes. In addition, ploidy differences can be problematic, especially in tumors with mixed populations of multiple ploidies or samples with intermediate ploidy levels, for example, between hyper-triploid or hypo-tetraploid levels. In such cases it is sometimes necessary to perform standard cytogenetic or multicolor FISH studies, if possible, to determine true gain and loss.

17.4 Conclusion

Together, standard G-banded karyotyping, FISH, multicolor FISH, and CGH provide a comprehensive picture of chromosomal aberrations. By combining these traditional and new molecular techniques, cytogenetics has become a powerful diagnostic and research tool in constitutional, prenatal, and implantation settings, and particularly in cancer, where it serves in both the diagnostic and the prognostic arenas. While WCP and unique probe FISH are now standard procedures in virtually all clinical cytogenetic laboratories, and multicolor FISH is applied in the larger institutional setting, CGH is primarily a research tool; it is most often used in large-scale clinical studies of solid tumors and tumor cell lines to identify primary and secondary aberrations. Such studies have revealed that tumors of the same type have similar patterns specific to the tumor entity, and that aberrations related to tumor progression are similar in almost all cancers.

Acknowledgments

The author wishes to extend her most grateful thanks to the people whose expert advice, pertinent information, and helpful suggestions contributed to this chapter. Hesed Padilla-Nash, Linda Barenboim-Stapleton, Nicole McNeil, and Thomas Liehr critically read the manuscript and made valuable comments. Buddy Chen and Tom Ellerman assisted in the preparation of the figures. The following people contributed the protocols of their laboratories or companies: Thomas Ried, Turid Knutsen, and Danny Wangsa (National Cancer Institute); Jane Bayani (Ontario Cancer Institute); Helen Lawce (Oregon Health and Science University Knight Diagnostic Laboratories); Syed M. Jalal, Troy J. Gliem, and Ying S. Zou (Mayo Clinic); Ilse Chudoba and Andreas Plesch (MetaSystems); Michael Behme (Leica Biosystems), F. Scott Cowan, Irit Bar-Am, and Herold L. Rothrock (Applied Spectral Imaging); and Danijela Lucic (Abbott Molecular-Vysis). Michael Behme, Erich Gebhart, Syed Jalal, Evelin Schröck, Thomas Ried, Tim Veldman, and Danny Wangsa provided figures or gave permission for the use of previously published figures or tables.

References

- Pinkel D, Straume T, Gray JW. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proc Natl Acad Sci USA* 1986; 83:2934–2938.
- Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T. Multicolor spectral karyotyping of human chromosomes. *Science* 1996; 273:494–497.
- Speicher MR, Ballard SG, Ward DC. Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nature Genet* 1996; 12:368–375.
- Kallioniemi A, Kallioniemi O-P, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 1992; 258:818–821.
- Knutsen T, Gobu V, Knaus R, Padilla-Nash H, Augustus M, Strausberg RL, Kirsch IR, Sirotnik K, Ried T. The Interactive online SKY/M-FISH & CGH Database and the Entrez Cancer Chromosomes search database: Linkage of chromosomal aberrations with the genome sequence. *Genes Chromosomes Cancer* 2005; 44:52–64.
- Baudis M. An online database and bioinformatics toolbox to support data mining in cancer cytogenetics. *Biotechniques* 2006; 40:269–270. <http://www.progenetix.de/~pgscripts/progenetix/Aboutprogenetix.html>
- Knutsen T, Ried T. SKY: a comprehensive diagnostic and research tool. A review of the first 300 published cases. *J Assoc Genet Technol* 2000; 26:3–15.

8. Bayani J, Squire JA. Advances in the detection of chromosomal aberrations using spectral karyotyping. *Clin Genet* 2001; 59:65–73.
9. Schröck E, Zschieschang P, O'Brien P, Helmrich A, Hardt T, Matthaei A, Stout-Weider K. Spectral karyotyping of human, mouse, rat and ape chromosomes—applications for genetic diagnostics and research. *Cytogenet Genome Res* 2006; 114:199–221.
10. Liehr T, Starke H, Heller A, Kosyakova N, Mrasek K, Gross M, Karst C, Steinhäuser U, Hunstig F, Fickelscher I, Kuechler A, Trifonov V, Romanenko SA, Weise A. Multicolor fluorescence in situ hybridization (FISH) applied to FISH-banding. *Cytogenet Genome Res* 2006; 114:240–244.
11. Müller S, O'Brien PCM, Ferguson-Smith MA, Wienberg J. Cross-species colour segmenting: a novel tool in human karyotype analysis. *Cytometry* 1998; 33:445–452.
12. Schröck E, Padilla-Nash H. Spectral karyotyping and multicolor fluorescence in situ hybridization reveal new tumor-specific chromosomal aberrations. *Sem Hematol* 2000; 37:334–347.
13. Tanke HJ, Wiegant J, van Gijlswijk RPM, Bezrookove V, Pattenier H, Heetebrij RJ, Talman EG, Raap AK, Vrolijk J. New strategy for multi-colour fluorescence *in situ* hybridisation: COBRA: COMBined Binary RAtio labelling. *Eur J Hum Genet* 1999; 7:2–11.
14. Raap AK, Tanke HJ. COmbedded Binary RAtio fluorescence in situ hybridization (COBRA-FISH): development and applications. *Cytogenet Genome Res* 2006; 114:222–226.
15. Chudoba I, Plesch A, Lörrch T, Lemke J, Claussen U, Senger G. High resolution multicolor-banding: a new technique for refined FISH analysis of human chromosomes. *Cytogenet Cell Genet* 1999; 84:156–160.
16. Karhu R, Ahlstedt-Soini M, Bittner M, Meltzer P, Trent JM, Isola JJ. Chromosome arm-specific multicolor FISH. *Genes Chromosomes Cancer* 2001; 30:105–109.
17. Fauth C, Zhang H, Harabacz S, Brown J, Saracoglu K, Lederer G, Rittinger O, Rost I, Eils R, Kearney L, Speicher MR. A new strategy for the detection of subtelomeric rearrangements. *Hum Genet* 2001; 109:576–583.
18. Henegariu O, Bray-Ward P, Artan S, Vance GH, Qumsieh M, Ward DC. Small marker chromosome identification in metaphase and interphase using centromeric multiplex fish (CM-FISH). *Lab Invest* 2001; 81:475–481.
19. Nietzel A, Rocchi M, Starke H, Heller A, Fiedler W, Włodarska I, Loncarevic I, Beensen B, Claussen U, Liehr T. A new multicolor-FISH approach for the characterization of marker chromosomes: centromere-specific multicolour-FISH (cenM-FISH). *Hum Genet* 2001; 108:199–204.
20. Schröck E, Veldman T, Padilla-Nash H, Ning Y, Spurbeck J, Jalal S, Shaffer LG, Papenhausen P, Kozma C, Phelan MC, Kjeldsen E, Schonberg SA, O'Brien P, Biesecker L, du Manoir S, Ried T. Spectral karyotyping refines cytogenetic diagnostics of constitutional chromosomal abnormalities. *Hum Genet* 1997; 101:255–262.
21. Liehr T, Weise A. Frequency of small supernumerary marker chromosomes in prenatal, newborn, developmentally retarded and infertility diagnostics. *Int J Mol Med* 2007; 19:719–731.
22. Warburton D. De novo balanced chromosome rearrangements and extra marker chromosomes identified at prenatal diagnosis: clinical significance and distribution of breakpoints. *Am J Hum Genet* 1991; 49:995–1013.
23. Crolla JA, Long F, Rivera H, Dennis NR. FISH and molecular study of autosomal supernumerary marker chromosomes excluding those derived from chromosomes 15 and 22: I. Results of 26 new cases. *Am J Med Genet* 1998; 75:355–366.
24. Haddad BR, Schröck E, Meck J, Cowan J, Young H, Ferguson-Smith MA, du Manoir S, Ried T. Identification of de novo chromosomal markers and derivatives by spectral karyotyping. *Hum Genet* 1998; 103:619–625.
25. Brecevic L, Michel S, Starke H, Müller K, Kosyakova N, Mrasek K, Weise A, Liehr T. Multicolor FISH used for the characterization of small supernumerary marker chromosomes (sSMC) in commercially available immortalized cell lines. *Cytogenet Genome Res* 2006; 114:319–324.
26. Márquez C, Cohen J, Munné S. Chromosome identification in human oocytes and polar bodies by spectral karyotyping. *Cytogenet Cell Genet* 1998; 81:254–258.
27. Willadsen S, Levron J, Munné S, Schimmel T, Márquez C, Scott R, Cohen J. Rapid visualization of metaphase chromosomes in single human blastomeres after fusion with in-vitro matured bovine eggs. *Hum Reprod* 1999; 14:470–475.
28. Stumm M, Wegner R-D, Bloechle M, Eckel H. Interphase M-FISH applications using commercial probes in prenatal and PGD diagnostics. *Cytogenet Genome Res* 2006; 114:296–301.

29. Weier JF, Ferlatte C, Baumgartner A, Jung CJ, Njuyen H-N, Chu LW, Pedersen RA, Fisher SJ, Weier H-UG. Molecular cytogenetic studies towards the full karyotype analysis of human blastocysts and cytotrophoblasts. *Cytogenet Genome Res* 2006; 114:302–311.
30. Oliver-Bonet M, Benet J, Martin RH. Studying meiosis: a review of FISH and M-FISH techniques used in the analysis of meiotic processes in humans. *Cytogenet Genome Res* 2006; 114:312–318.
31. Veldman T, Vignon C, Schröck E, Rowley JD, Ried T. Hidden chromosome abnormalities in haematological malignancies detected by multicolour spectral karyotyping. *Nature Genet* 1997; 15:406–410.
32. Lestou VS, Gascoyne RD, Sehn L, Ludkovski O, Chhanabhai M, Klasa RJ, Husson H, Freedman AS, Connors JM, Horsman DE. Multicolor fluorescence *in situ* hybridization analysis of t(14;18)-positive follicular lymphoma and correlation with gene expression data and clinical outcome. *Brit J Haematol* 2003; 122:745–759.
33. Ried T, Liyanage M, du Manoir S, Heselmeyer K, Auer G, Macville M, Schröck E. Tumor cytogenetics revisited: comparative genomic hybridization and spectral karyotyping. *J Mol Med* 1997; 75:801–814.
34. Bayani JM, Squire JA. Applications of SKY in cancer cytogenetics. *Cancer Invest* 2002; 20:373–386.
35. Sawyer JR, Lukacs JL, Munchi N, Desikan KR, Singhal S, Mehta J, Siegel D, Shaughnessy J, Barlogie B. Identification of new nonrandom translocations in multiple myeloma with multicolor spectral karyotyping. *Blood* 1998; 92:4269–4278.
36. Rao PH, Cigudosa JC, Ning Y, Calasanz MJ, Iida S, Tagawa S, Michaeli J, Klein B, Dalla-Favera R, Jhanwar SC, Ried T, Chaganti RSK. Multicolor spectral karyotyping identifies new recurring breakpoints and translocations in multiple myeloma. *Blood* 1998; 92:1743–1748.
37. Kearney L. Multiplex-FISH (M-FISH): technique, developments and applications. *Cytogenet Genome Res* 2006; 114:189–198.
38. Ariyama Y, Sakabe T, Shinomiya T, Mori T, Fukuda Y, Inazawa J. Identification of amplified DNA sequences on double minute chromosomes in a leukemic cell line KY821 by means of spectral karyotyping and comparative genomic hybridization. *J Hum Genet* 1998; 43:187–190.
39. Ried T, Dorritie K, Weaver Z, Wangsa D, Difilippantonio MJ, Montagna C. Molecular cytogenetics of mouse models of breast cancer. *Breast Dis* 2004; 19:59–67.
40. Liyanage M, Coleman A, du Manoir S, Veldman T, McCormack S, Dickson RB, Barlow C, Wynshaw-Boris A, Janz S, Wienberg J, Ferguson-Smith MA, Schröck E, Ried T. Multicolour spectral karyotyping of mouse chromosomes. *Nature Genet* 1996; 14:312–315.
41. Coleman AE, Schröck E, Weaver Z, du Manoir S, Yang F, Ferguson-Smith MA, Ried T, Janz S. Previously hidden chromosome aberrations in T(12;15)-positive BALB/c plasmacytomas uncovered by multicolor spectral karyotyping. *Cancer Res* 1997; 57:4585–4592.
42. McCormack SJ, Weaver Z, Deming S, Natarajan G, Torri J, Johnson MD, Liyanage M, Ried T, Dickson RB. Myc/p53 interactions in transgenic mouse mammary development, tumorigenesis and chromosomal instability. *Oncogene* 1998; 16:2755–2766.
43. Weaver ZA, McCormack SJ, Liyanage M, du Manoir S, Coleman A, Schröck E, Dickson RB, Ried T. A recurring pattern of chromosomal aberrations in mammary gland tumors of MMTC-cmyc transgenic mice. *Genes Chromosomes Cancer* 1999; 25:251–260.
44. Coleman AE, Forest ST, McNeil N, Kovalchuk AL, Ried T, Janz S. Cytogenetic analysis of the bipotential murine pre-B cell lymphoma, P388, and its derivative macrophage-like tumor, P388D1, using SKY and CGH. *Leukemia* 1999; 13:1592–1600.
45. Buwe A, Steinlein C, Koehler MR, Bar-Am I, Katzin N, Schmid M. Multicolor spectral karyotyping of rat chromosomes. *Cytogenet Genome Res* 2003; 103:163–168.
46. Ried T, Schröck E, Ning Y, Wienberg J. Chromosome painting: a useful art. *Human Molecular Genet* 1998; 7:1619–1626.
47. Difilippantonio MJ, Ried T. Technicolor genome analysis. In: Lakowicz JR, ed. *Topics in Fluorescence Spectroscopy: Volume 7*. Dordrecht: Kluwer Academic/Plenum Publishers, 2003; 291–316.
48. Solovei I, Kienle D, Little G, Eils R, Savelyeva I, Schwab M, Jäger W, Cremer C, Cremer T. Topology of double minutes (dmins) and homogeneously staining regions (HSRs) in nuclei of human neuroblastoma cell lines. *Genes Chromosomes Cancer* 2000; 29:297–308.

49. Solovei I, Walter J, Cremer M, Habermann F, Schermelleh L, Cremer T. FISH on three-dimensionally preserved nuclei. In: Beatty B, Mai S, Squire J, eds. *FISH: A Practical Approach*. Oxford: Oxford University Press, 2002; 119–157.
50. Popp S, Scholl HP, Loos P, Jauch A, Stelzer E, Cremer C, Cremer T. Distribution of chromosome 18 and X centric heterochromatin in the interphase nucleus of cultured human cells. *Exp Cell Res* 1990; 189:1–12.
51. Walter J, Joffe B, Bolzer A, Albiez H, Benedetti PA, Müller S, Speicher MR, Cremer T, Cremer M, Solovei I. Towards many colors in FISH on 3D-preserved interphase nuclei. *Cytogenet Genome Res* 2006; 114:367–378.
52. Reilly PA. Fluorescent in situ hybridization. In: Kaplan B, Dale K, eds. *The Cytogenetic Symposia*. Burbank, CA: Association of Cytogenetic Technologists, 1994; 16-1–16-6.
53. Swiger RR, Tucker JD. Fluorescence in situ hybridization: A brief review. *Environment Mol Mutagen* 1996; 27:245–254.
54. Montgomery KD, Keitges EA, Meyne J. Molecular cytogenetics: definitions, clinical aspects, and protocols. In: Barch MJ, Knutsen T, Spurbeck J, eds. *The AGT Cytogenetics Laboratory Manual*, 3rd ed. Philadelphia: Lippincott-Raven, 1997; 557–590.
55. Macville M, Veldman T, Padilla-Nash H, Wangsa D, O'Brien P, Schröck E, Ried T. Spectral karyotyping, a 24-colour FISH technique for the identification of chromosomal rearrangements. *Histochem Cell Biol* 1997; 108:299–305.
56. Eils R, Uhrig S, Saracoglu K, Sätzler K, Bolzer A, Petersen I, Chassery J-M, Ganser M, Speicher MR. An optimized, fully automated system for fast and accurate identification of chromosomal rearrangements by multiplex-FISH (M-FISH). *Cytogenet Cell Genet* 1998; 82:160–171.
57. Spector DL, Goldman RD, Leinwand LA. Chromosome analysis by spectral karyotyping. In: Spector DL, Goldman RD, Leinwand LA, eds. *Cells: A Laboratory Manual*. Vol 3: *Subcellular Localization of Genes and Their Products*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1998; 113.1–113.9.
58. Blancato J. Fluorescence *in situ* hybridization. In: Gersen S, Keagle M, eds. *The Principles of Clinical Cytogenetics*. Totowa, NJ: Humana Press, 1999; 443–471.
59. Green GA, Schröck E, Veldman T, Heselmeyer-Haddad K, Padilla-Nash HM, Ried T. Evolving molecular cytogenetic technologies. In: Mark HFL, ed. *Medical Genetics*. New York: Marcel Dekker, 2000; 579–592.
60. Bayani J, Squire JA. Multicolour FISH and spectral karyotyping. In: Beatty B, Mai S, Squire J, eds. *FISH: A Practical Approach*. Oxford: Oxford University Press, 2002; 205–220.
61. Bayani J, Squire JA. Spectral Karyotyping. In: Fan YS, ed. *Methods in Molecular Biology*, Vol 204: *Molecular Cytogenetics: Protocols and Applications*. Totowa, NJ: Humana Press, 2002; 85–104.
62. Beatty BG, Scherer SW. Human chromosome mapping of single copy genes. In: Beatty B, Mai S, Squire J, eds. *FISH: A Practical Approach*. Oxford: Oxford University Press, 2002; 29–54.
63. Teixeira MR. Combined classical and molecular cytogenetic analysis of cancer. *Eur J Cancer* 2002; 38:1580–1584.
64. McNeil N, Montagna C, Difilippantonio MJ, Ried T. Comparative cancer cytogenetics. In: *Atlas Genet Cytogenet Oncol Haematol* 2003; 7:616–636.
65. Speicher MR, Carter NP. The new cytogenetics: blurring the boundaries with molecular biology. *Nature Rev/Genet* 2005; 6:782–792.
66. Mai S, Wiener F. Murine chromosome preparation. In: Beatty B, Mai S, Squire J, eds. *FISH: A Practical Approach*. Oxford: Oxford University Press, 2002; 55–76.
67. Mundy CR, Cunningham MW, Read CA. Nuclei acid labeling and detection. In: Brown TA, ed. *Essentials of Molecular Biology, A Practical Approach*, Vol. II. Oxford: IRL Press, 1991; 57–82.
68. Bray-Ward P. FISH probes and labelling techniques. In: Beatty B, Mai S, Squire J, eds. *FISH: A Practical Approach*, Oxford: Oxford University Press, 2002; 5–28.
69. Carter NP, Ferguson-Smith ME, Affara NA, Briggs H, Ferguson-Smith MA. Study of X chromosome abnormality in XX males using bivariate flow karyotype analysis and flow sorted dot blots. *Cytometry* 1990; 11:202–207.
70. Gray JW, Langlois RG, Carrano AV, Burkhardt-Schulte K, Van Dilla MA. High resolution chromosome analysis: one and two parameter flow cytometry. *Chromosoma* 1979; 73:9–27.
71. Telenius H, Ponder BAJ, Tunnacliffe A, Pelmear AH, Carter NP, Ferguson-Smith MA, Behmel A, Nordenskjöld M, Pfragner R. Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow sorted chromosomes. *Genes Chromosomes Cancer* 1992; 4:257–263.

72. Carter NP. Cytogenetic analysis by chromosome painting. *Cytometry* 1994; 18:2–10.
73. Wilson WD, Tanious FA, Barton HJ, Jones RL, Fox K, Wydra RL, Strekowski L. DNA sequence dependent binding modes of 4',6-diamidino-2-phenylindole (DAPI). *Biochemistry* 1990; 29:8452–8461.
74. Garini Y, Macville M, du Manoir S, Buckwald RA, Lavi M, Katzir N, Wine D, Bar-Am I, Schröck E, Cabib D, Ried T. Spectral karyotyping. *Bioimaging* 1996; 4:65–72.
75. Azofeifa J, Fauth C, Kraus J, Maierhofer C, Langer S, Bolzer A, Reichman J, Schuffenhauer S, Speicher MR. An optimized probe set for the detection of small interchromosomal aberrations by use of 24-color FISH. *Am J Hum Genet* 2000; 66:1684–1688.
76. du Manoir S, Speicher MR, Joos S, Schröck E, Popp S, Döhner H, Kovacs G, Robert-Nicoud M, Lichter P, Cremer T. Detection of complete and partial chromosome gains and losses by comparative genomic in situ hybridization. *Hum Genet* 1993; 90:590–610.
77. Speicher MR, du Manoir S, Schröck E, Holtgreve-Grez H, Schoell B, Lengauer C, Cremer T, Ried T. Molecular cytogenetic analysis of formalin-fixed, paraffin-embedded solid tumors by comparative genomic hybridization after universal DNA-amplification. *Hum Mol Genet* 1993; 2:1907–1914.
78. Jeuken JWM, Sprenger SHE, Wesseling P. Comparative genomic hybridization: Practical guidelines. *Diagnostic Molecular Pathol* 2002; 11:193–203.
79. Lichter P, Bentz M, du Manoir S, Joos S. Comparative genomic hybridization. In: Verma RS, Babu A, eds. *Human Chromosomes: Principles and Techniques*, Second Edition. New York: McGraw-Hill, 1995; 191–210.
80. Schröck E, Thiel G, Lozanova T, du Manoir S, Meffert M-C, Jauch A, Speicher MR, Nürnberg P, Vogel S, Jänisch W, Donis-Keller H, Ried T, Witkowski R, Cremer T. Comparative genomic hybridization of human malignant gliomas reveals multiple amplification sites and nonrandom chromosomal gains and losses. *Am J Pathol* 1994; 144:1203–1218.
81. Gebhart E. Comparative genomic hybridization (CGH): ten years of substantial progress in human solid tumor molecular cytogenetics. *Cytogenet Genome Res* 2004; 104:352–358.
82. Zitzelsberger H, Lehmann L, Werner M, Bauchinger M. Comparative genomic hybridisation for the analysis of chromosomal imbalances in solid tumours and haematological malignancies. *Histochem Cell Biol* 1997; 108:403–417.
83. Knuutila S, Bjorkqvist AM, Autio K, Tarkkanen M, Wolf M, Monni O, Szymanska J, Larramendy ML, Tapper J, Pere H, El-Rifai W, Hemmer S, Wasenius VM, Vidgren V, Zhu Y. DNA copy number amplifications in human neoplasms: review of comparative genomic hybridization studies. *Am J Pathol* 1998; 152:1107–1123.
84. Knuutila S, Aalto Y, Autio K, Bjorkqvist A-M, El-Rifai W, Hemmer S, Huhta T, Kettunen E, Kiuru-Kuhlefelt S, Larramendy ML, Lushnikova T, Monni O, Pere H, Tapper J, Tarkkanen M, Varis A, Wasenius V-M, Wolf M, Zhu Y. DNA copy number losses in human neoplasms. *Am J Pathol* 1999; 155:638–694.
85. Rooney PH, Murray GI, Stevenson DAJ, Haites NE, Cassidy J, McLeod HL. Comparative genomic hybridization and chromosomal instability in solid tumors. *Brit J Cancer* 1999; 80:862–873.
86. Gebhart E, Liehr T. Patterns of genomic imbalances in human solid tumors (Review). *Int J Oncol* 2000; 16:383–399.
87. Koschny R, Koschny T, Froster UG, Krupp W, Zuber MA. Comparative genomic hybridization in glioma: a meta-analysis of 509 cases. *Cancer Genet Cytogenet* 2002; 135:147–159.
88. Struski S, Doco-Fenzy M, Cornillet-Lefebvre P. Compilation of published comparative genomic hybridization studies. *Cancer Genet Cytogenet* 2002; 135:63–90.
89. Heselmeyer K, Schröck E, du Manoir S, Blegen H, Shah K, Steinbeck R, Auer G, Ried T. Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. *Proc Natl Acad Sci USA* 1996; 93:479–484.
90. Chu LW, Troncoso P, Johnston DA, Liang JC. Genetic markers useful for distinguishing between organ-confined and locally advanced prostate cancer. *Genes Chromosomes Cancer* 2002; 36:303–312.
91. Inoue M, Starostik P, Zettl A, Ströbel P, Schwarz S, Scaravalli F, Henry K, Willcox N, Müller-Hermelink H-K, Marx A. Correlating genetic aberrations with World Health Organization-defined histology and stage across the spectrum of thymomas. *Cancer Res* 2003; 63:3708–3715.
92. Bryndorf T, Kirschhoff M, Rose H, Maahr J, Gerdes T, Karhu R, Kallioniemi A, Christensen B, Lundsteen C, Philip J. Comparative genomic hybridization in clinical cytogenetics. *Am J Hum Genet* 1995; 57: 1211–1220.

93. Veldman T, Heselmeyer K, Schröck E, Ried T. Comparative genomic hybridization: A new approach for the study of copy number changes in tumor genomes. *Applied Cytogenet* 1996; 22:117–122.
94. Joos S, Schwänen C, Lichter P. Comparative genomic hybridization on metaphase chromosomes and DNA chips. In: Beatty B, Mai S, Squire J, eds. *FISH: A Practical Approach*. Oxford: Oxford University Press, 2002; 159–182.
95. Karhu R, Kähkönen M, Kuukasjärvi T, Pennanen S, Tirkkonen M, Kallioniemi O. Quality control of CGH: impact of metaphase chromosomes and the dynamic range of hybridization. *Cytometry* 1997; 28:198–205.
96. Kirchhoff M, Gerdes T, Maahr J, Rose H, Bentz M, Döhner H, Lundsteen C. Deletions below 10 Megabasepairs are deleted in comparative genomic hybridization by standard reference intervals. *Genes Chromosomes Cancer* 1999; 25:410–413.

Contributed protocols section

IMPORTANT: No protocol included in this manual should be used for clinical testing unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements for all chemicals used in the laboratory.

General safety warning

Many FISH reagents are biohazardous and should therefore be handled carefully. Gloves and laboratory coats should be worn when using these reagents, both to protect the technologist and to prevent DNA from the skin being transferred into the test system. Painting probes contain formamide which may be harmful by inhalation, ingestion, or skin absorption; it can cause skin irritation and may have reproductive effects. To prevent photo bleaching, handle all reagents and slides containing fluorochromes in reduced light. Centrifuge all tubes prior to opening to assemble the contents at the bottom of the tube.

Protocol 17.1 Spectral karyotyping (SKY)

Submitted by Turid Knutsen, Section of Cancer Genomics, Genetics Branch, National Cancer Institute, Bethesda, Maryland

Note: All protocols from this laboratory are available at <http://www.riedlab.nci.nih.gov/>

Principle

Spectral karyotyping (SKY) [1], a fluorescence *in situ* hybridization technique [2], permits the simultaneous visualization of each chromosome pair in a different color. It is based on the simultaneous hybridization of specific painting probes for each chromosome in the genome of interest (e.g., 24 in humans), labeled with different fluorochromes or fluorochrome combinations. Combinatorial labeling makes it possible to discriminate many more targets than the number of spectrally resolvable fluorophores. Five pure spectrally distinct dyes are used either singly or in combination to create a chromosome cocktail of probes, each with a unique spectral signature for each chromosome. SKY has been made possible by technical advances in fluorescence microscopy, including the development of suitable optical filters and sensitive digital imaging devices. The strength of multicolor FISH (both SKY and M-FISH) [1, 3] lies in their ability to define translocations, marker chromosomes, and complex rearrangements, and to reveal cryptic change; they cannot, however, detect intrachromosomal rearrangements such as duplications, very small deletions, or small paracentric inversions.

I. SKY probe kit (human), Sections A–C below

Materials

Reagents

1. Agarose, ultrapure, Invitrogen #15510-027
2. 5× Buffer D (contains MgCl₂), Invitrogen #K1220-02D

3. 10× Buffer and 25 mM MgCl₂, Perkin Elmer #N808-0010
4. Template DNA (flow-sorted chromosomes)
5. Ethidium bromide, Research Genetics #750007
6. 5× Loading buffer, Quality Biological #51-026-030
7. 10 mM dNTP nucleotide mix, Invitrogen (comes with buffer D above)
8. 100 mM dNTP nucleotides:
 - a. dGTP, pH 7, Roche #11051466001
 - b. dCTP, pH 7, Roche #11051458001
 - c. dATP, pH 7, Roche #11051440001
 - d. dTTP, pH 7, Roche #11051482001
9. Primer “UN1”, Midland Certified Reagent Co. Telenius 6 MW [5'-CCGACTCGAGNNNNNNATGTGG-3']
10. Ampli Taq polymerase 5 U/μL, Perkin Elmer #N808-0158
11. Super Taq polymerase 15 U/μL, CPG #STAQ050H-500U
12. 10× Taq buffer with (NH₄)₂SO₄, Fermentas #B33 (previously 10× TAE buffer, Advanced Biotechnologies #08-514-001)
13. x-dUTP:
 - a. Dy505-green, Dyomics #505-34 (previously Spectrum Green)
 - b. Spectrum Orange, dUTP, Abbott Molecular #02N33-050
 - c. Dy590, Dyomics #590-34 (previously Texas red)
 - d. Biotin-16 – dUTP, pH 7, Roche #11093070910
 - e. Digoxigenin, Roche 11573179910
14. Water, molecular-grade, sterile distilled

Equipment

1. PCR Thermocycler, MJ – Research, Inc. Model PTC – 100
2. Gel system and power source
3. PCR tubes, PGC Scientifics #502-075

Preparation

1. 1× TAE buffer: dilute the 10× TAE with sterile molecular grade dH₂O
2. 1% agarose gel: dissolve 1 g of agarose into 100 mL of 1× TAE buffer by warming the solution.

Sterile techniques and workspace

Sterile techniques are crucial since you will be re-amplifying the product from this reaction and any contaminants will also be amplified.

1. Autoclave molecular grade water using liquid cycle program.
2. Autoclave PCR microcentrifuge tubes (0.5 mL size), 2-mL microcentrifuge tubes, and pipette tips (10, 200, and 1000 μL). Autoclave all tubes using dry cycle program with slow drying time to prevent condensation.
3. Sterilize pipettes (using UV) to be used for PCR and only use that set for PCR (can use Stratalinker or UV light source in tissue culture hood).
4. Workspace: Before starting, make sure there is a steady air flow; if not, then use the tissue culture hood to maintain sterility. Thoroughly wipe down UV hood with 70% ethanol including walls, ceiling, and work area.
5. UV-expose all equipment used including the hood, pipettes, pipette tips, tube racks, microcentrifuge tubes, autoclaved water, waste container, etc. for at least 20 minutes.
6. Use sterile gloves.

A. Amplification of flow sort DNA using DOP-PCR

Flow-sorted chromosomes

Flow-sorted chromosomes should be kept tightly wrapped and sealed at 4 °C until they are ready to be used. Be aware that each 50-μL reaction will be carried out in the original tube that is sent containing the flow-sorted chromosomes.

Method

1. Vortex each tube of flow sorted chromosomes well in order to detach chromosomes from the inner walls. Spin them at 13,000 RPM for at least 3 minutes.
2. Combine the following for each reaction (make sure to change tips every time):

Buffer D	10.0 μ L
dNTP	4.0 μ L
primer	1.5 μ L
dd H ₂ O	2.0 μ L
Super Taq	0.25 μ L
Total	17.75 μ L

Note: Do not mix up and down with pipette.

3. Vortex each tube lightly and spin at 13,000 RPM for 30 seconds.
4. IMMEDIATELY transfer to PCR machine.
5. Run the PCR program as described (Notes 1 and 2).
6. When the program is completed run a 2 μ L aliquot (dissolved in place 0.8 μ L of 5 \times DNA loading buffer) from each reaction on a 1% agarose gel to initially determine the efficiency of the amplification reaction. The resulting smear migrates to around 500 base pairs (bp) (Note 3).

Notes

1. PCR program

Step	Temperature (°C)	Minutes
1 (initial denaturation)	93	10
2	94	1
3	30	1.5
4	ramp 30–70	3
5	72	3
6 repeat steps 2–5, 4 times		
7	94	1
8	62	1
9	72	3 + 1 second/cycle
10 repeat steps 7–9, 34 times		
11	72	10
	4	∞

2. After running the PCR program it is time to take out the samples and evaluate the quality of your amplification. Make sure that you still maintain sterile techniques with this amplified product. You will be using this product as starting material later for secondary amplification or for a labeling PCR. Therefore do not touch the tubes without gloves and open tubes only in the hood to decrease chances of contamination.
3. 1% agarose gel (**Note:** see Ried Lab web site for example of gel)

B. DOP-PCR secondary

Preparation

Stock dNTP 2 mM:

	μL	mM final
dGTP	10	0.2
dCTP	10	0.2
dATP	10	0.2
dTTP	10	0.2
dH_2O	460	—
Total	500	

Template DNA: 150–200 ng (Note 1)

Method

1. Label each 0.5 mL tube with the chromosome number (1 to Y) and date, and include a control tube lacking any DNA.
2. Thaw template DNA at 37°C, vortex and spin briefly in picofuge prior to aliquoting the DNA. Aliquot 2 μL of the appropriate DNA into each PCR tube and place closed PCR tubes at 4°C temporarily.
3. Since you will have a total of 25 reactions you will use two 2 mL microcentrifuge tubes for putting together the Master Mix. One tube will contain enough Master Mix for 13 reactions and the other will contain enough for 12.
4. The Master Mix for one reaction is as follows (multiply each by number of reactions) and specifically in this order:

	1 Reaction	13 Reactions
Sterile dH_2O	65 μL	845
10x buffer	10 μL	130
MgCl_2	8 μL	104
dNTP	10 μL	130
Primer	4 μL for 13 reactions	52
Taq	1 μL (Note 2)	13

5. Vortex the two tubes, spin them down and place on ice. At this point take out the Taq enzyme, mix carefully (tap with finger), spin down, then add appropriate amounts to each Master Mix tube. Vortex the Master Mix tubes, spin, and put back on ice.
6. Put PCR tubes with aliquoted DNA in order and open each tube by handling only the outside of the tube. Pipet 96 μL of Master Mix into each tube (change tips between each tube) and put on ice.
7. Vortex each tube, spin down briefly and put them all into the PCR machine. Make sure that the correct PCR program (Note 3) is selected.
8. After completion, remove the tubes from the PCR machine, vortex, spin down, and place on ice. In a round-bottom ELISA plate, place 0.8 μL of 5x DNA loading buffer into each well to which you will be adding a 2 μL aliquot from each reaction to run on a 1% agarose gel. The resulting smear migrates to around 500 bp (Note 4).

Notes

1. The stock DNA used for this secondary amplification is derived from the primary amplification as described in the primary DOP-PCR protocol. If the primary amplification yield is 50 μL and you use 2 μL per secondary amplification then you should have enough starting material for 25 secondary PCR reactions.

2. The Taq polymerase should always be added last. Since it is more viscous and sticky, it needs to be mixed well before each use. Use the pipette tip you are about to draw with to gently stir the contents as you draw up the enzyme.
3. PCR program:

Step	Temperature (°C)	Minutes
1	94	1
2	56	1
3	72	3 with addition of 1 sec/cycle
4	repeat steps 1–3, 29 times	
5	72	10
6	4	∞

4. 1% agarose gel. See Ried Lab website for example of gel.

C. DOP-PCR labeling

Preparation

Stock dNTP 2 mM

	μL	mM final
dGTP	10.0	0.2
dCTP	10.0	0.2
dATP	10.0	0.2
dTTP	7.5	0.15
dH ₂ O	462.5	—
Total	500.0	

x-dUTPs:

Spectrum Orange (SpO) is diluted 1:5 in sterile H₂O (14 μL SpO + 56 μL H₂O).

Dy590 is diluted 1:2 in sterile H₂O (30 μL Dy590 + 30 μL H₂O).

DY505-green is diluted 1:5 in sterile H₂O (13 μL DY505 + 52 μL H₂O)

Template DNA

400–600 ng of secondary PCR product for each reaction (Note 1)

Method

Use sterile techniques throughout, especially when handling the DNA samples.

1. Label PCR tubes according to labeling scheme (Note 2) (e.g., 1A, 1E, etc.) plus a DNA negative control for each color (Master Mix with no DNA sample).
2. Defrost template DNA at 37°C, vortex and spin briefly prior to aliquoting the DNA. Aliquot 4 μL of the appropriate DNA into each PCR tube, seal lid, and temporarily place at 4°C.
3. Label five 2 mL microcentrifuge tubes (A–E) and make the Master Mix for each fluorochrome according to the table (Table 17.11) below and put on ice. The order for pipetting the Master Mix is as follows: dH₂O, buffer, MgCl₂, dNTP, and primer.
4. Dilute the Dy590, SpO, and Dy505 dUTPs immediately before use. Vortex the labeled fluor dUTPs, spin down briefly and add each to the appropriate Master Mix and place back on ice.

Table 17.11 Master Mix

	SpO A	Dy590 B	Bio (Cy5) C	Dy505 D	DIG (Cy5.5) E
10× PCR buffer	130	120	140	150	130
MgCl ₂ (25 mM)	104	96	112	120	104
1-dNT (2 mM)	65	60	70	75	65
sterile dH ₂ O	819	756	910	945	845
Primer (100 μM)	52	48	56	60	52
PE Tac enzyme	13	12	14	15	13
x-dUTP (dil. 1 : 5)	65	60 (dil. 1 : 5)	42	75	39
No. of reactions	1248: 96=13	1152: 96=12	1344: 96=14	1440: 96=15	1248: 96=13

5. Mix the Taq enzyme carefully (tap with finger), spin down, then add appropriate amounts to each master mix. Vortex the master mix tubes, spin and put back on ice.
6. Put PCR tubes with aliquoted DNA in fluorochrome order (all A's in one row, all B's in the next, etc.)
7. Carefully open each tube in row A by handling only the outside of the tube. Vortex the Master Mix for SpO, pipet 96 μL into each tube (change tips between each tube) and put on ice. Repeat the same procedure for each remaining x-dUTP.
8. Vortex each tube, spin down briefly and put on ice again.
9. Start the PCR machine and select the PCR program (Note 3), arrange the tubes in the PCR machine, close lid, and start the program.
10. After completion, remove the tubes from the PCR machine, vortex, spin down, and place on ice. In a round-bottom ELISA plate, place 0.8 μL of 5× DNA loading buffer in each well to which you will be adding a 2 μL aliquot from each reaction to run on a 1% agarose gel. The resulting smear migrates to around 500 bp (see Ried Lab website for examples of gels).

Notes

1. The stock DNA used for labeling PCR reactions is initially amplified as described in the primary DOP-PCR protocol. This amplified product can then be amplified again as described in the secondary DOP-PCR protocol. It is this secondary amplification product that is then used as the starting material for the SKY labeling PCR reactions.
2. Labeling scheme:
 A – Spectrum Orange
 B – Dy590
 C – Biotin
 D – DY505-green
 E – Digoxigenin
 Chromosome
 1 – AE
 2 – E
 3 – CDE
 4 – BC
 5 – ABDE
 6 – BE
 7 – CD
 8 – D
 9 – ADE
 10 – CE
 11 – A
 12 – BCDE
 13 – AD

14 – B
 15 – ABC
 16 – BCD
 17 – C
 18 – ABD
 19 – AC
 20 – ACD
 21 – DE
 22 – ABCE
 X – BD
 Y – ACDE

3. PCR program

Step	Temperature (°C)	Minutes
1	94	1
2	56	1
3	72	3 with addition of 1 sec/cycle
4	repeat steps 1–3, 29 times	
5	72	10
6	4	∞

D. Precipitation of SKY kits and quality control (QC)

Materials

Reagents

1. Ethanol, absolute
2. Chromosome paints: product of SKY labeling PCR (see protocol)
3. Dextran sulfate (50%), Intergen #S4030
4. Formamide, deionized (pH 7), Ambion #9342
5. Mouse Cot-1™ DNA, Invitrogen Corp. #18440-016
6. Human Cot-1™ DNA, Invitrogen Corp. #15279 – 011
7. Salmon sperm DNA, Sigma Molecular Biology #D-7657)
8. Sodium acetate (3 M)
9. 20× SSC

Preparation

Master Mix

Dextran sulfate, 50%	40mL
20× SSC, pH7	20mL
Sterile dH ₂ O	40mL
Total	100mL

Vortex solution and place on a shaking platform overnight to insure proper mixing. Aliquot, and store at –20 °C.

Method

1. For each SKY probe, combine in a 1.5 mL microcentrifuge tube, 4 μL aliquots of each SKY labeling PCR product. This will give a total of 57 PCR reactions × 4 μL = 228 μL total volume for human, and 45 PCR reactions × 4 μL = 180 μL total volume for mouse.

2. To this add 20 µL of human Cot-1™ DNA or 50 µL mouse Cot-1™ DNA and 1 µL of salmon sperm DNA, bringing the total volume to 249 µL human, or 231 µL mouse.
3. Add 1/10 the total volume sodium acetate.
4. Add 2.5 times the total volume of ethanol (~10 v), 700 µL.
5. Vortex and store overnight at -20 °C or at -80 °C for 30 minutes.
6. Centrifuge precipitated DNA at 13,000 RPM for 30 minutes at 4 °C.
7. Carefully aspirate off the supernatant and spin in speed vac with the tube uncapped for 10 minutes (medium heat) until pellet is dry.
8. Add 5 µL of deionized formamide to each tube and incubate at 37 °C for 30 minutes while shaking in a thermomixer.
9. After the pellet has fully dissolved, add 5 µL of Master Mix, vortex and spin briefly. After this step the probe is now ready to either denature or freeze at -20 °C.

Notes

1. To guarantee that the probe will dissolve successfully in the formamide, the precipitated probe must be thoroughly dried and no ethanol should remain in the tube.
2. In order to minimize photo-bleaching of the probe, minimize exposure of the probe to ambient light.

II. Pretreatment of chromosome slides for FISH/CGH/SKY

Materials

Reagents

1. RNase A, 100 mg, Boehringer #109169
2. Pepsin, 5 g, Sigma #P 6887
3. 1 M MgCl₂
4. 1× PBS
5. 1 M HCl
6. 2× SSC
7. Formaldehyde (37%)
8. Ethanol, absolute

Preparation

1. RNase (Stock solution: 10%)
Dissolve RNase (20 mg/mL sterile water), boil for 15 minutes. Cool to room temperature.
Aliquot and store at -20 °C.
2. 1× PBS/MgCl₂
1 M MgCl₂ 50 mL
1× PBS 950 mL
3. Pepsin (Stock solution)
Dissolve pepsin, 100 mg/mL, in sterile water. Keep on ice. Make 50 µL aliquots, store at -20 °C.
4. 1% Formaldehyde/1× PBS/MgCl₂
Formaldehyde, 37% 2.7 mL
1× PBS/MgCl₂ 97.3 mL
5. 0.01 M HCl
1 M HCl 1 mL
dH₂O 99 mL
Adjust pH to 2.0. Pre-warm to 37 °C in waterbath.

Preparation

1. RNase (Stock solution: 10%)
Dissolve RNase (20 mg/mL sterile water), boil for 15 minutes. Cool to room temperature. Aliquot and store at -20 °C.

2. 1× PBS/MgCl₂
1 M MgCl₂ 50 mL
1× PBS 950 mL
3. Pepsin (Stock solution)
Dissolve pepsin, 100 mg/mL, in sterile water. Keep on ice. Make 50 µL aliquots, store at -20 °C.
4. 1% Formaldehyde/1× PBS/MgCl₂
Formaldehyde, 37% 2.7 mL
1× PBS/MgCl₂ 97.3 mL
5. 0.01 M HCl
1 M HCl 1 mL
dH₂O 99 mL
Adjust pH to 2.0. Pre-warm to 37 °C in water bath.

Method

1. Equilibrate slides in a Coplin jar containing 2× SSC for 5 minutes at RT.
2. Dilute the RNase stock solution (1 : 200) in 2× SSC.
3. Apply 120 µL RNase to 24 × 60 mm coverslip, touch slide to coverslip.
4. Incubate slides in a moist hybridization chamber at 37 °C for 45 minutes.
5. Carefully remove coverslips and wash slides 3 × 5 minutes in a Coplin jar containing 2×SSC at RT, shaking.
6. Add 2–30 µL of pepsin stock solution (see notes) inside an empty, clean 100 mL glass beaker, then add 100 mL pre-warmed 0.01 M HCl; mix well. Transfer to a clean Coplin jar.
7. Incubate slides in Coplin jar for 2–5 minutes (see notes) at 37 °C.
8. Wash 2 × 5 minutes in 1× PBS at RT, shaking (vigorously for first wash).
9. Wash 1 × 5 minutes in 1× PBS/MgCl₂ at RT, shaking.
10. Place slide in 50 mL Coplin jar containing 1% formaldehyde/1× PBS/MgCl₂, 10 minutes at RT (not shaking).
11. Wash slide 5 minutes in 1× PBS at RT, shaking.
12. Dehydrate slide in ethanol series: 70%, 90%, 100% ethanol, 3 minutes each.
13. Air dry slide.
14. Check slides for chromosome morphology, which should be similar to starting material. Select area for hybridization.

Notes

1. The time of pepsin treatment and amount of pepsin stock solution to be used is dependent on (a) the amount of cytoplasm surrounding the metaphase spreads, as observed with a light microscope using phase objectives BEFORE slide pretreatment, and (b) the age of the slide. Slides with excess cytoplasm, seen as a gray particulate haze around the chromosomes, or older than six months may require longer treatment with pepsin (3–5 minutes) and higher concentrations of pepsin ranging from 10–30 µL.
2. After exposure to the pepsin, one can place the slide into a Petri dish containing 1× PBS and look at the slide under an inverted microscope to see if longer pepsin treatment is required. If so, place the slide back into the Coplin jar containing the pepsin/acid mixture.
3. It is very important that the pepsin be added to the clean beaker first and NOT directly into the acid solution. If the pepsin is added to the acid solution it causes the pepsin to precipitate and it will not dissolve properly into the acid solution.

III. Hybridization

Materials

Reagents

1. dH₂O
2. Ethanol solutions (70%, 90%, and 100%)
3. Formamide, deionized, Ambion #9342
4. HCl, 1 N
5. Rubber cement
6. 20× SSC

Preparation

1. 70% Formamide/2× SSC:

20× SSC	10mL
dH ₂ O	20mL
deionized formamide	70mL

Adjust to pH 7.25 with 1 N HCl. Aliquot and store at –20 °C. Pre-cool 70% ethanol to 0 °C.

Method

1. If SKY-Kit was stored at –20 °C, prewarm at 37 °C for 5–10 minutes, shaking; vortex, spin briefly. If stored at 4–5 °C, pre-warm 5 minutes; vortex, spin briefly.
2. Denature SKY-Kit at 80 °C for 5 minutes in a thermomixer or water bath.
3. Preanneal at 37 °C for 1 hour.
4. For slide denaturation apply 120 µL of 70% deionized formamide/2× SSC to a 24 × 60 mm coverslip. Touch the slide to the coverslip (Note 1).
5. Denature slide at 80 °C on a hot plate for 1 minute, 30 seconds (Note 2).
6. Immediately let coverslip slide off and place slide in 70% ethanol (0 °C) for 3 minutes, followed by 90% ethanol (RT) and 100% ethanol (RT) for 3 minutes each.
7. Let slide air dry.
8. After pre-annealing, add SKY-Kit (10 µL) to the denatured slide and cover with 18 × 18 mm coverslip.
9. Seal coverslip with rubber cement, being sure that all edges are covered (Note 3).
10. Hybridize at 37 °C in a humidified hybridization chamber for 48 hours (Note 4).

Notes

1. The slide should be pretreated prior to the denaturation step (see *II. Pretreatment of chromosome slides for FISH/CGH/SKY*).
2. The denaturation time and temperature depends on the age of the slide, the species, and cell type. For example, you may need to reduce time and temperature for mouse chromosome preparations.
3. After applying the SKY-Kit and before sealing the coverslip with rubber cement, all air bubbles should be removed by gently applying pressure to the coverslip (e.g., with forceps). In order to prevent the probe from drying out during the 48-hour hybridization time, it is important that the coverslip is completely sealed with rubber cement, and that the chamber is sufficiently moist but not over-saturated.
4. Hybridize mouse chromosome preparations for 48–96 hr.

IV. Detection

Materials

Reagents

1. Anti-mouse IgG [H&L] (Goat) antibody CY5.5 Conjugated (1 mg), Rockland™ #610-113-121
2. Antifade (1,4-phenylene-diamine), 100 g, Sigma-Aldrich #P1519
3. Bovine serum albumin (BSA), Roche Diagnostics #100-350
4. Streptavidin Cy5-conjugated, 1 mg, Rockland™ #S000-06
5. DAPI (4'-6-Diamidino-2-phenylindole), Sigma-Aldrich #18860
6. Ethyl alcohol, anhydrous
7. Formamide (FA), Fluka BioChemika #47671
8. HCl, 1 N
9. Monoclonal anti-digoxin (mouse IgG1), Clone DI-22 (0.1 mg/mL), Sigma-Aldrich #D8156
10. 20× SSC
11. Tween 20, Sigma #P-1379
12. dH₂O

Preparation

1. 50% Formamide/SSC (FA/SSC)
20× SSC 20 mL
dH₂O 80 mL
Formamide 100 mL
Adjust pH to 7.25 using 1 N HCl. Pre-warm to 45 °C.
2. 1× SSC
20× SSC 25 mL
dH₂O 475 mL
Pre-warm to 45 °C.
3. 4× SSC/0.1% Tween 20
20× SSC 200 mL
dH₂O 799 mL
Tween 20 1 mL
Pre-warm to 45 °C.
4. Blocking solution (3% BSA/4× SSC/0.1% Tween 20)
BSA 0.3 g
4× SSC/0.1% Tween 20 10 mL
Vortex until dissolved. Pre-warm to 37 °C.
5. DAPI stock solution (final concentration = 0.2 mg/mL)
DAPI 2 mg
dH₂O 10 mL
Aliquot and store at -80 °C.
6. DAPI staining solution (final concentration = 80 ng/mL)
DAPI (stock solution) 40 μL
2× SSC 100 mL
Store at 4 °C in a light-tight Coplin jar.

Method

1. Carefully remove rubber cement surrounding coverslip with forceps. Pre-soak slide in formamide/2× SSC if rubber cement is difficult to remove.
2. Wash slide in 50% formamide/2× SSC for 3 × 5 minutes each, shaking, preferably in 45 °C water bath.
3. Wash slide in 1× SSC for 3 × 5 minutes, shaking.
4. Dip slide in 4× SSC/0.1% Tween 20; do not let it dry.
5. Add 120 μL of blocking solution (3% BSA/4× SSC/0.1% Tween 20) to a 24 × 60 mm coverslip, invert slide onto coverslip and incubate in a moist hybridization chamber at 37 °C for 30 minutes.
6. Wash slide in 4× SSC/0.1% Tween 20 to wash off blocking solution, 5 minutes, shaking.
7. Spin all fluorescent dyes for 1 minute at 13,000 RPM.
8. Combine the two antibodies, mouse anti-dig and streptavidin Cy⁵-conjugated, into the same Eppendorf tube, and apply 150 μL of antibody solution to a 24 × 60 mm coverslip. Each antibody should be diluted 1:200 in 4× SSC/0.1% Tween 20 (Note 4). Invert the slide onto the solution. Incubate the slides in a moist hybridization chamber at 37 °C for 45–60 minutes.
9. Wash slide in 4× SSC/0.1% Tween 20, 3 × 5 minutes, shaking.
10. Add 150 μL of the CY5.5 antibody (diluted 1:200 in 4× SSC/0.1% Tween 20). Incubate slide in a moist hybridization chamber at 37 °C for 45–60 minutes.
11. Wash slide in 4× SSC/0.1% Tween 20, 3 × 5 minutes, shaking.
12. Stain slide for 5 minutes in the DAPI staining solution in a light-protected Coplin jar at room temperature.
13. Wash slide with 2× SSC 3–5 minutes.
14. Dehydrate slide in ethanol series of 70%, 90%, and 100% for 3 minutes each; air-dry slides.
15. Apply 35 μL of antifade solution, cover each slide with a 24 × 60 mm coverslip, and store in a light-protected container at 4 °C until slide is imaged.

Notes

1. Exposure of slides to ambient light should be minimized during all procedures.
2. Carefully remove coverslips during all procedures to minimize scratches.
3. Do not let the slide dry out between washing steps.
4. BSA may contribute to nonspecific background. If this is the case, dilute the antibodies in 4× SSC/0.1% Tween 20 (do not use BSA or goat serum).
5. Maintaining correct temperatures for detection washes is important for reducing background.
6. Expiration dates of antibodies require continuous monitoring. Generally, the antibodies stocks should be stored at -30°C and expire within a year after aliquots are made.
7. The concentration of the antibody dilution should be altered depending on the quality of the antibody.

Reagents

1. 1,4-phenylene-diamine, 100 g, Sigma #P1519
2. Glycerol, 86%
3. Phosphate buffered saline (PBS), 1×
4. Sodium bicarbonate
5. Sodium carbonate

Preparation

Carbonate-bicarbonate buffer (pH 9.0)
 Sodium bicarbonate, 0.5 M (pH 8.13) 4 mL
 Sodium carbonate, 0.5 M (pH 11.32) 1 mL
 Filter sterilize.

Method

1. Prepare carbonate-bicarbonate buffer (pH 9.0).
2. Dissolve 50 mg 1,4-phenylenediamine in 2 mL 1× PBS.
3. Adjust pH to 8.0 with carbonate-bicarbonate buffer.
4. Add 1× PBS to 5 mL. Important: Add 2 mL buffer and check pH, then add dropwise until pH is 7.99–8.00. If pH exceeds 8.0, the procedure must be started over.
5. Mix with 45 mL 86% glycerol. Leave on inverter for at least 1 hr.
6. Aliquot and store at -20°C.

V. SKY/FISH of previously G-banded slides

Materials

Reagents

1. Acetic acid, glacial
2. Deionized formamide, Ambion #9342
3. dH₂O
4. Ethanol, absolute
5. 1 M MgCl₂
6. Methanol, anhydrous, Mallinckrodt AR (ACS) #3016
7. Phosphate buffered saline (PBS) 1×
8. Rubber cement
9. 2× SSC
10. Xylene

Preparation

1. Fixative (3 : 1 methanol–acetic acid (Vol : Vol))
 - Methanol 45 mL
 - Acetic acid 15 mL
2. FA/SSC
 - Deionized formamide (FA) 70 mL
 - 2× SSC 30 mL
 - Adjust pH to 7.0.

Method: G-band slide pretreatment

1. If required for analysis, first image G-banded slides and record X and Y coordinates from the microscope so that one can relocate the identical metaphase spreads that will subsequently be hybridized with SKY probes (Note 1).
2. Remove immersion oil, if any, from the previously G-banded slide by washing the slide in a Coplin jar containing xylene for 2 minutes.
3. Rinse the slide in a 50 mL Coplin jar containing methanol for 2 minutes.
4. De-stain the slide by immersing it into a 50 mL Coplin jar containing fixative (methanol–acetic acid).
5. Rinse the slide in ddH₂O twice for 5 minutes each.
6. Rinse the slide in 1× PBS twice for 5 minutes each.
7. Dehydrate the slide in ethanol series: 70%, 90%, 100% ethanol, 2 minutes each.
8. Air dry slide.

Method: G-band slide denaturation and hybridization

1. First fill a 50 mL Coplin jar with FA/SSC (pH 7, pre-warmed to 70 °C) which is then placed inside a water bath set at 70 °C (Note 2).
2. To denature the slide, place it in the Coplin jar for approximately 10–30 seconds. Quickly remove slide and place into a new Coplin jar containing ice cold ethanol (70%) for 2 minutes, followed by washes in 90% and 100% ethanol for 2 minutes each; air dry slide.
3. Apply the probe to the dry slide, carefully place a glass coverslip (18 × 18 mm) over the probe and seal all edges with rubber cement.
4. Hybridize the slide for 48–72 hours at 37 °C in a moist light-tight container (Note 3).

Notes

1. Since these slides have been previously G-banded, the slide pre-treatment using pepsin is eliminated, as the chromosomes have already been partially digested with trypsin. G-banding enhances the DAPI banding and often results in brighter hybridization signals than the slides pre-treated with pepsin.
2. The timing of slide denaturation is dependent on the age of the slide and the extent of digestion by the enzyme (trypsin) used in the G-banding procedure. Older slides (>2 months) require longer denaturation times.
3. Proceed with detection of SKY hybridization according to protocols outlined in *Detection for SKY*, located under SKY Protocols.

References

1. Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T. Multicolor spectral karyotyping of human chromosomes. *Science* 1996; 273:494–497.
2. Pinkel D, Straume T, Gray JW. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proc Natl Acad Sci USA* 1986; 83:2934–2938.
3. Speicher MR, Ballard SG, Ward DC. Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nature Genet.* 1996; 12:368–375.

Protocol 17.2 Spectral karyotyping (SKY)

Submitted by Jane Bayani, Ontario Cancer Institute, Department of Applied Molecular Oncology, Toronto, Canada

Materials

Day 1

1. Metaphase slides aged to at least 2 days
2. Small forceps/tweezers
3. 50 mL 0.01 M HCl prewarmed to 37°C in a Coplin jar and maintained in an incubator or water bath
4. 10% pepsin (w/v)
5. 2 Coplin jars containing 1× PBS at room temperature
6. 1 Coplin jar containing 1× PBS/50 mM MgCl₂ at room temperature
7. 1 Coplin jar containing 1% formaldehyde/1× PBS/MgCl₂ at room temperature in a fume hood
8. 70% formamide/2× SSC prewarmed at 72°C in a water bath
9. 1 Coplin jar each of 70%, 80% and 100% ethanol
10. SKY Kit (Applied Spectral Imaging, Carlsbad CA) containing probe cocktail, block reagents, detection reagents and counterstain
11. 22 × 22 mm glass coverslips
12. Rubber cement
13. Hybridization box (such as a black plastic video tape box)
14. 37°C oven

Day 2

1. Hybridized slides from Day 1
2. Hybridization box
3. 37°C oven
4. Small forceps/tweezers
5. 22 × 50 mm glass coverslips
6. 3 Coplin jars containing 50% formamide/2× SSC prewarmed and maintained in a 45°C water bath (see MATERIALS, above)
7. 3 Coplin jars containing 1× SSC maintained in a 45°C water bath
8. 7 Coplin jars containing 0.1% Tween-20/4× SSC maintained in a 45°C water bath
9. Detection reagents
SKY Kit (Applied Spectral Imaging, Carlsbad CA) containing probe cocktail, block reagents, detection reagents and counterstain
10. DAPI/Antifade counterstain (see Reagents and Solutions)
11. Fluorescent microscope and associated Applied Spectral Imaging Acquisition hardware and software (Applied Spectral Imaging)

Method

Day 1

1. Under phase-contrast microscopy, determine the extent of cytoplasmic residue on the slide preparations.
2. To 50 mL of prewarmed 0.01 M HCl, add 10–15 µL of 10% pepsin (w/v). Allow the slides to incubate for 5–10 minutes at 37°C.
3. Wash the slides in 1× PBS for 5 minutes at room temperature.
4. Wash the slides in 1× PBS/50 mM MgCl₂ for 5 minutes at room temperature.
5. Incubate the slides in 1% formaldehyde/1× PBS/50 mM MgCl₂ for 10 minutes at room temperature in a well-ventilated area or fume hood.

6. From the commercially available SKY kit, aliquot 10 µL of probe cocktail (Vial 1) for each slide to be hybridized. Heat denature the probe at 72 °C for 10 minutes, then place at 37 °C for 1 hour. This is sufficient probe to cover a 22 × 22 mm area.
7. Wash the slides in 1× PBS for 5 minutes at room temperature.
8. Pass the slides through an ethanol dehydration series: 70%, 80%, 100% for 5 minutes each and allow the slides to air dry after the final ethanol wash.
9. Denature the slides for 1.5–2 minutes in 70% formamide/2× SSC at 72 °C. The time will vary according to the age and quality of the slide.
10. Promptly place the slides into 70% ethanol following denaturation and proceed through the dehydration series as in step 8.
11. Add the preannealed probe to the denatured slide. Coverslip and seal with rubber cement. Transfer the slides to a hybridization container that has been slightly dampened with a wet paper towel or gauze and place at 37 °C for 48 hours.

Day 2

12. After 48 hours, carefully peel off the rubber cement from the slides and immerse in the first Coplin jar of 50% formamide/2× SSC at 45 °C. Allow the coverslip to fall off and let stand for 5 minutes. Remove slides and transfer to second Coplin jar for 5 minutes. Repeat with third solution.
13. Wash the slides in 1× SSC at 45 °C for 5 minutes in each of the 3 washes.
14. Briefly wash the slides in 0.1% Tween-20/4× SSC at 45 °C.
15. Drain excess solution, but do not allow to dry, and add 80 µL of blocking reagent (Vial 2) as supplied by ASI to the slides. Coverslip and place back in hybridization box at 37 °C for 40 minutes.
16. Remove coverslip and add 80 µL of detection reagent (Vial 3) as supplied by ASI to the slides. Coverslip and place back in hybridization box at 37 °C for 40 minutes.
17. Remove coverslip and wash slides in three washes of 0.1% Tween-20/4× SSC at 45 °C for 5 minutes each, with gentle agitation.
18. Drain excess solution, but do not allow to dry, and add 80 µL detection reagent (Vial 4) as supplied by ASI to the slides. Coverslip and place back in hybridization box at 37 °C for 40 minutes.
19. Remove coverslip and wash slides in three washes of 0.1% Tween-20/4× SSC at 45 °C for 5 minutes each, with gentle agitation.
20. Drain excess solution, but do not allow the slides to dry. Add sufficient DAPI/Antifade counterstain (Vial 5) as supplied by ASI or from other source. Coverslip. The slides are now ready for visualization. When not in use, store slides at –20 °C.

Protocol 17.3 DNA spectral karyotyping

Submitted by F. Scott Cowan, Applied Spectral Imaging, Migdal Haemek, Israel

Materials

Reagents

Analyte-Specific Reagents: analytical and performance characteristics are not established. The DNA Spectral Karyotyping Reagents are designed to enable simultaneous visualization of all chromosomes in different colors. The distinction between the dyes can be performed only with the SKY® spectral imaging system from Applied Spectral Imaging.

Intended use

The following procedure is intended for hybridization of the Spectral Karyotyping Reagents on a normal metaphase slide preparation. Slide quality is one of the most important factors affecting the degree of hybridization. It is highly recommended that sample slides are viewed under phase contrast before, during, and after pretreatment steps to ensure a successful hybridization. Sample slides that are sparse, have visible cytoplasm surrounding the metaphase spreads, or were aged at room temperature for more than 2 weeks are not recommended for use. For long term storage, dehydrate and store slides with a desiccant at –20 °C, or store the cells in fixative at –20 °C and drop slides 1–3 days before hybridization.

Analyte specific reagents supplied by ASI

1. Vial 1 Spectral Karyotyping (Human/Mouse/Rat) reagent 10 µL/slides
2. Vial 2 Blocking reagent 80 µL/slides
3. Vial 5 Anti-fade-DAPI reagent 20 µL/slides

Store all reagents at +4 °C or at –20 °C.

Reagents required/not supplied

1. Cy 5 staining reagent (vial 3 from Applied Spectral Imaging's #CAD03 or prepare according to Appendix A)
2. Cy 5.5 staining reagent (vial 4 from Applied Spectral Imaging's #CAD04 or prepare according to Appendix A)
3. 20× SSC (prepare 1× SSC, 2× SSC, 4× SSC)
4. Distilled water
5. Pepsin, 10% stock solution, Sigma #P6887
6. 1× PBS
7. HCl
8. 1 M MgCl₂, Sigma #M1028
9. 37% Formaldehyde, Sigma #F1268
10. Formamide (molecular biology grade), Sigma #F7503
11. 70%, 80%, 100% Ethanol (room temperature and -20 °C)
12. Tween 20, Sigma #P-9416

Reagent preparation

Day 1

1. 0.01 M HCl
Add 0.5 mL 1 M HCl to 49.5 mL distilled water.
Heat solution to 37 °C in a glass Coplin jar.
2. Pepsin stock
Prepare a 10% stock solution (100 mg/mL) in sterile water. Dissolve completely and aliquot. Store at -20 °C.
3. Phosphate buffered saline (1× PBS)
Prepare a 1× PBS solution. Store at room temperature.
4. 1× PBS/MgCl₂
Add 50 mL of 1 M MgCl₂ to 950 mL of 1× PBS.
5. 1% Formaldehyde
Add 2.7 mL of 37% formaldehyde to 100 mL of 1× PBS/MgCl₂.
6. Ethanol series
Prepare 70%, 80% and 100% ethanol and place in Coplin jars (room temp). Prepare same series and place at -20 °C.
7. Denaturation solution
Add 35 mL formamide, 10 mL distilled H₂O, 5 mL 20× SSC (final concentration is 70% formamide/2× SSC). Adjust pH to 7.0 using HCl; heat to 72 °C.

Day 3

1. Washing solution I (50% formamide/2× SSC)
15 mL 20× SSC
60 mL distilled water
75 mL formamide
Total: 150 mL
Adjust pH to 7.0 using HCl and heat to 45 °C.
2. Washing solution II (1× SSC solution)
12.5 mL 20× SSC
237.5 mL distilled water
Total: 250 mL
Mix well and heat to 45 °C.
3. Alternative wash: rapid washing (0.4× SSC)
2 mL 20× SSC
98 mL distilled water
Total: 100 mL
Mix well and heat to 72 °C.
4. Washing solution III (4× SSC/0.1% Tween 20)
100 mL 20× SSC
400 mL distilled water

0.5 mL Tween 20
 Total: 500 mL
 Mix well and heat to 45 °C.

Reagents quality control protocol

Please note that the hybridization time for Spectral karyotyping reagents is 24–36 hours.

Method

Day 1

A. Pepsin treatment

Prepare and select samples for hybridization. Look at slides under phase and note cytoplasm. Select the best area of the slide and mark it. If no cytoplasm is observed and the slides look clean, continue with the denaturation step.

1. Pre-warm 50 mL of 0.01 M HCl to 37 °C in a glass Coplin jar. Add 5–15 µL of pepsin stock solution and mix well. Start with 6 µL stock pepsin solution in 50 mL of 0.01 N HCl solution.
2. Incubate slides at 37 °C in the pepsin solution for 3–5 minutes. Under normal conditions, a typical metaphase preparation will require 3–5 minutes in a pepsin solution containing 5–10 µL of pepsin.
3. Wash slides in 1× PBS at room temperature (RT) for 5 minutes. Repeat with a second 1× PBS wash for 5 minutes.
4. Wash slides in 1× PBS/MgCl₂ at RT for 5 minutes.
5. Place slides in a Coplin jar containing 1% formaldehyde and incubate for 10 minutes at RT.
6. Wash the slide in 1× PBS for 5 minutes.
7. Dehydrate the slides in 70%, 80%, and 100% ethanol for 2 minutes each. Air-dry the slides.

Alternatively: Instead of using pepsin, a short pretreatment using trypsin is also recommended:

Protocol for trypsin pretreatment

1. Wash slides briefly in Earl's medium.
2. Put 0.2–0.4 mL of trypsin/EDTA (5 g/L trypsin and 2 g/L EDTA) in 50 mL Earl's medium at RT.
3. Incubate the slides for 20–40 seconds in the trypsin solution.
4. Wash in water and dehydrate in ethanol series: 70%, 80%, and 100% for 2 minutes each wash.
5. Air-dry the slides and continue with denaturation.

B. Chromosome denaturation

1. Heat 40 mL of denaturation solution to 72 °C (±2 °C) in a glass Coplin jar. Place slides in the solution for 1.5 minutes. DO NOT OVERDENATURE: some samples denature in 60 seconds. A slide warmer can also be used for denaturation: put 100 µL of the denaturation solution on the slide, cover with a cover glass and put on a slide warmer at 74 °C for 1.5 minutes.
2. Immediately place slides in cold 70%, 80%, and 100% ethanol, 2 minutes each. Air-dry.

C. Probe denaturation and hybridization

1. Centrifuge briefly the contents of the Spectral Karyotyping reagent (vial 1 supplied by ASI).
Note: Some red precipitation or clumps may normally be visible in this vial.
2. Mix well the content of the vial, including the red precipitation, by pipetting up and down several times. Take 10 µL for each slide, put in an Eppendorf tube and denature the probe by incubation at 80 °C in a water bath for 7 minutes.
3. Put in a water bath at 37 °C for 30–60 minutes.
4. Add 10 µL from the denatured Spectral Karyotyping Reagent to the denaturized chromosome preparation.
5. Place an 18 × 18 mm glass coverslip over the probe mix, being careful not to trap air bubbles under the coverslip. Seal the edges with rubber cement. Transfer the slide to a humidified chamber or container and place in incubator or baking oven set at 37 °C for 24–36 hours.

Day 3

D. Detection

1. Remove the slides from the humidified chamber and carefully remove the rubber cement.
2. Transfer the slides to a Coplin jar containing washing solution I (50% formamide in 2× SSC); wash slides 3 times for 5 minutes each wash in a water bath at 45 °C.
3. Wash slides twice in washing solution II (1× SSC) for 5 minutes each wash in a water bath at 45 °C.
Alternatively, a rapid wash procedure can be used: Instead of steps 2 and 3, put slides in a Coplin jar containing rapid washing solution (0.4× SSC) at 72 °C (± 2 °C) for 2–5 minutes. Continue with step 4.
4. Dip slides in washing solution III (4× SSC/0.1% Tween 20) for 1 minute.
Optional step: Apply 80 µL of blocking reagent (vial 2 – supplied by ASI), cover with a plastic coverslip (24 × 60 mm), and incubate at 37 °C for 30 minutes.
5. Tilt slides and allow fluid to drain. Apply 80 µL of Cy 5 staining reagent. Cover with a plastic coverslip (24 × 60 mm) and incubate at 37 °C for 40 minutes.
6. Wash slides 3 times in washing solution III (4× SSC/0.1% Tween 20) for 2 minutes each wash in a water bath at 45 °C.
7. Apply 80 µL of Cy 5.5 staining reagent, place a plastic coverslip (24 × 60 mm) and incubate at 37 °C for 40 minutes.
8. Repeat step 6.
9. Tilt slide and allow fluid to drain. Put 20 µL from the Antifade-DAPI reagent (vial 5 supplied by ASI); place a cover glass (24 × 60 mm) over the surface. Try to remove any air bubbles that may have formed.
10. The slides are now ready for imaging with the SkyVision® spectral imaging system from Applied Spectral Imaging.

Appendix A: Preparation of Cy 5 staining reagent (Vial 3) and Cy 5.5 staining reagent (Vial 4)

Materials

1. Anti digoxigenin, 0.1 mL, Sigma #D8156
2. Cy 5 streptavidin, 1 mg, Rockland #S000-06 or Amersham #PA45001
Stock solution: 1 mg/mL; dissolve the content of the bottle in 1 mL sterile water and store in small aliquots at –20 °C.
3. Cy 5.5 sheep anti-mouse, 1 mg, Rockland #610-113-121
Stock solution: 1 mg/mL; dissolve the content of the bottle in 1 mL sterile water and store in small aliquots at –20 °C.

Method

1. Vial 3: Take 1 mL of 4× SSC, add 5 µL of anti digoxigenin and 5 µL of Cy 5 streptavidin.
2. Vial 4: Take 1 mL of 4× SSC, add 5 µL of Cy 5.5 anti-mouse.
3. Use the diluted vials 3 and 4 according to the regular SKY protocol.
4. Discard the diluted antibodies at the end of the day.
5. Should longer storage of the diluted vials 3 and 4 be needed, add 1% of BSA fraction V (Roche #735078 or, for USA only, Roche #100062) to the 4× SSC solution, e.g., add 0.1 g BSA to pre-warmed (37 °C) 10 mL 4× SSC. Vortex well and leave at room temperature until dissolved. Store at 4 °C.
6. When using this buffer, the diluted vials 3 and 4 will stay stable for several days or up to a month.
This material is subject to proprietary rights of Amersham Biosciences Corp and Carnegie Mellon University, and is made and sold under license from Amersham Biosciences Corp. This product is licensed for sale for research purposes only.

Protocol 17.4 Multicolor-FISH method (M-FISH) I

Submitted by Helen Lawce, Clinical Cytogenetics Laboratory, Clinical Genetics Laboratories at Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

Principle

Multicolor FISH is designed for the simultaneous identification and examination of the 24 human chromosomes in metaphase cells. The SpectraVysis Assay from Abbott Molecular-Vysis employs a 24-color chromosome paint panel that provides information to define chromosome abnormalities through molecular cytogenetics. It should be used in conjunction with

traditional G- or R-banding and is particularly useful in situations where these banding techniques do not yield definitive results. Examples include translocations where banding patterns of the participating chromosomes are similar, complex translocations involving more than two chromosomes, cryptic translocations, and marker chromosomes.

Limitations: Multicolor-FISH does not detect intrachromosomal changes or very small rearrangements.

The SpectraVysis Assay from Abbott Molecular-Vysis is designed for use on metaphase cells derived from a variety of human tissues. It consists of a 5-label, 52-probe mixture of whole chromosome paint (WCP) DNA probes that paint and cover the 24 human chromosome homologues. The WCP probes are directly labeled with the five different fluorophores in a combinatorial labeling format to provide distinct colors when analyzed. The fluorophores that are directly attached to the probes include: SpectrumAqua™, SpectrumGreen™, SpectrumGold™, SpectrumRed™, and SpectrumFRed™. COT-1 DNA is included in the probe mixture to suppress sequences that are common to various chromosomes. The labeled probes are hybridized to target DNA in specimens that are fixed and mounted to slides using standard cytogenetic techniques. The assay also utilizes DAPI counterstaining of chromosomes. Combinations of fluorophores are assigned pseudo colors for analysis and karyotyping.

Materials

Materials required but not provided

1. 100% Ethanol
2. 1 N HCl
3. 1 N NaOH
4. 20× SSC to make 2× SSC, 0.4× SSC, and 10× SSC.
5. DAPI III Counterstain, Vysis #32-804932
6. Formamide
7. Forceps
8. Formaldehyde fixation solution. Mix together 57.6 mL of purified water, 3.5 mL of 1 M MgCl₂, 7 mL of 10× PBS, and 1.89 mL of 37% formaldehyde. Mix well. Place in a Coplin jar. (*Prepare fresh each month*).
9. NP-40, U.S. Biologicals #N3500
10. 1× PBS
11. Pepsin, Sigma #P7012. Prepare a 10% (w/v) pepsin stock solution by mixing 0.1 g pepsin in 1 mL water prewarmed to 37°C. Aliquot into 35 μL volumes and store at -20°C until ready to use. When ready to use, prepare a 0.005% pepsin working solution by mixing 35 μL of the 10% pepsin stock into a solution of 69.3 mL purified water and 0.7 mL 1 N HCl that has been warmed to 37°C in Coplin jar. Discard the pepsin solution after each use.
12. pH meter
13. Purified water
14. DNase-free RNase A. Prepare a stock of 10 mg/mL RNase A in 2× SSC. If RNase is not labeled as DNase-free, then place it in a boiling water bath for 10 minutes to inactivate DNase. Aliquot into 10 μL volumes and store at -20°C. At time of use, prepare RNase working solution by thawing the stock solution in the tube and directly adding 990 μL of 2× SSC pH 7.0-7.4 to the 10 μL contained in the tube. Vortex to mix. Do not freeze-thaw. Discard after use.
15. Denaturation solution. Prepare by combining 35 mL formamide, 10 mL 10× SSC, and 5 mL distilled water; adjust pH to 7.0. Refrigerate. Discard after one week.
16. Water baths (37°C and 72°C)

Method

1. Age the slide on an aluminum tray in an oven at 90°C for 10 minutes.
2. Apply 20 μL of the RNase working solution to the target region on the slide. Apply a 22 × 22 mm glass or flexible plastic coverslip. Place the slide in a humidified chamber (sealed box containing moistened paper towel) at 37°C for 30 minutes.
3. Remove the coverslip. Wash two times for 5 minutes each in 2× SSC.
4. Meanwhile, prepare pepsin working solution. Place slides in the pepsin working solution at 37°C for 5 minutes (time carefully so that 5 minutes are not exceeded).
5. Wash two times for 5 minutes each in PBS.
6. Prepare a fresh solution of formaldehyde fixation solution. Place the slides in this fixation solution for 2 minutes at ambient temperature (time carefully so that 2 minutes are not exceeded).

7. Wash two times for 5 minutes each in 1× PBS at ambient temperature.
8. Dehydrate the slides by placing in 70%, 85%, and 100% ethanol for 1 minute each.
9. Air dry the slides and proceed to specimen denaturation. Slides may be used immediately or stored at –20 °C under desiccation until ready to use. If stored at –20 °C, repeat steps 8 and 9 prior to use.

Denaturation of the specimen slide

1. Warm the **denaturation solution** to room temperature and then to 72 °C prior to use.
2. Immerse the slides in the denaturation solution at 72 °C for 1–3 minutes (typically 2 minutes). Note: Immerse no more than four slides in the denaturation solution simultaneously.
3. Dehydrate slides for 1 minute each in 70%, 85%, and 100% ethanol.
4. Proceed to hybridization.

Hybridization of the SpectraVysion probe to the target chromosomes

1. Add 10 µL of the SpectraVysion probe to a 0.5 mL Eppendorf tube with cap.
2. Denature probe by floating the tube containing the probe in a 72 °C water bath for 5 minutes. Time the denaturation of the probe so that it is completed approximately the same time as the specimen slide denaturation.
3. Apply 10 µL of the SpectraVysion probe to the target area of the specimen slide and apply a 22 × 22 mm glass coverslip.
4. Seal the coverslip with rubber cement to prevent evaporation during hybridization.
5. Place slides in a prewarmed humidified box and place box in a 37 °C incubator for 12–18 hours (overnight).

Washing the slide

1. Remove the coverslip from one slide and immediately immerse the slide in 0.4× SSC/0.3% NP-40 at 72 °C. Agitate slides for 1–3 seconds. Repeat with other slides. Note: To maintain the proper temperature of the 0.4× SSC/0.3% NP-40, wash a maximum of four slides simultaneously.
2. Wash slides in the above solution for 2 minutes.
3. Immerse the slides in 2× SSC/0.1% NP-40 at ambient temperature. Agitate the slides for 1–3 seconds. Remove slides after 5–60 seconds.
4. Drain the slide by tilting. Complete the air drying of the slides in darkness.
Note: Occasionally after air drying, there will be a visible film on the slide: this film is not usually visible under fluorescence and will not interfere with the assay results.
5. Apply 10 µL of DAPI III counterstain to the target area of the slide and apply coverslip. (Alternative: Dilute DAPI II with antifade solution 1:3.)

Microscopy

Visual analysis of the assay is not feasible since the analysis must be performed via a composite image of 6 fluorophore image captures and computerized pseudo-coloring of the chromosomes.

1. Using the Cytovision software from Leica Biosystems, click on the M-FISH icon under Capture Mode.
2. The window for the list of filters will pop up, and you are now ready to capture the metaphases. Capture in this order: Spectrum Gold first, Spectrum FRed second, then Aqua, Red, and Green, followed by DAPI. Failure to capture FRed early will degrade its fluorescence.
3. If you change the order of the filters, it will be necessary to re-enter the filter assignment of each chromosome under the Fluomap window. The filter assignment for each chromosome is the following:

Chromosome 1: SpectrumGold

Chromosome 2: SpectrumRed

Chromosome 3: SpectrumAqua

Chromosome 4: SpectrumGreen and SpectrumRed

Chromosome 5: SpectrumFRed and SpectrumGold

Chromosome 6: SpectrumGreen

Chromosome 7: SpectrumFRed

Chromosome 8: SpectrumFRed and SpectrumRed
 Chromosome 9: SpectrumGold and SpectrumRed
 Chromosome 10: SpectrumFRed, SpectrumAqua, and SpectrumGold
 Chromosome 11: SpectrumAqua and SpectrumRed
 Chromosome 12: SpectrumGreen and SpectrumGold
 Chromosome 13: SpectrumAqua and SpectrumGreen
 Chromosome 14: SpectrumGreen, SpectrumGold, and SpectrumRed
 Chromosome 15: SpectrumAqua, SpectrumGold, and SpectrumRed
 Chromosome 16: SpectrumFRed and SpectrumGreen
 Chromosome 17: SpectrumFRed, SpectrumGreen, and SpectrumRed
 Chromosome 18: SpectrumFRed, SpectrumGold, and SpectrumRed
 Chromosome 19: SpectrumFRed, SpectrumGreen, and SpectrumGold
 Chromosome 20: SpectrumFRed, SpectrumAqua, and SpectrumRed
 Chromosome 21: SpectrumAqua, SpectrumGreen, and SpectrumGold
 Chromosome 22: SpectrumAqua, SpectrumGreen, and SpectrumRed
 Chromosome X: SpectrumFRed and SpectrumAqua
 Chromosome Y: SpectrumAqua and SpectrumGold

4. Following the capture of metaphases, separate overlapping chromosomes in the Analysis screen.
5. Automatically karyotype via Color Classifier.
6. Correct errors using editing tools, use reverse DAPI function to check orientation, classification, etc.
7. If there is a small chromosome or piece of chromosome that is not large enough for precise color determination, use spectral analysis to match against other chromosomes' spectral profiles to determine the most likely candidate(s). Use paint probes to follow up.
8. Labeling M-FISH chromosomes: Click on Fluomap. Click on the class number you are interested in. The arrows will move to that group. Click on one of the arrows. You can only use this feature if "M-FISH class" is turned on in "Customize."

Note: Any abnormality detected by multicolor FISH should be confirmed by whole chromosome paints and/or other chromosome-, arm-, or region-specific probes for the chromosomes involved in the detected rearrangement.

Protocol 17.5 Multicolor FISH (M-FISH) or 24-color FISH II

Submitted by Syed M. Jalal, Ying Zou, and Troy J. Gliem, Cytogenetics Laboratory, Mayo Clinic, Rochester, Minnesota

Principle

Whole chromosome painting by fluorescent labeled probes for each human chromosome became available in the late 1980s. By selectively using 120 DNA fluorescent probes specific to chromosome 4 and fluorescence *in situ* hybridization (FISH), the first "painted" chromosome was produced [1]. The whole chromosome painting probes (wcp) for each human chromosome have been commercially available since the early 1990s in two colors (green and orange). This development was immensely helpful in resolving chromosomal abnormalities as a complement to the banded technique.

The shortcoming of painted chromosome analysis was that it was restricted to the analysis of only one or two chromosomes at one time. For abnormalities involving multiple chromosomes, this approach was cumbersome and time consuming. Thus, it was a "dream come true" when it became possible to "paint" the entire human genome simultaneously with each chromosome fluorescing in a unique and distinct color [2]. The procedure has been referred to commonly as multicolor FISH (mFISH) or more descriptively as 24-color FISH [3]. The principle is to label the set of DNA probes unique to each chromosome in a particular combination of one or more fluorophores from a group of five. Thus, a unique color (spectral signature) converted by computer software is produced for each chromosome simultaneously.

It is customary for WCP and mFISH methods to suppress highly repetitive DNA sequences (Cot-1™ DNA) by using excess unlabeled complementary DNA. This helps to reduce background cross-hybridization. The procedural details were originally provided in Chapter 8 of *Molecular Cytogenetics: Protocols and Applications*, 2004, edited by Yao-shan Fan and by Law and Jalal [4]; the latter publication includes information on trouble-shooting. An updated protocol and analysis procedure are provided here.

Materials

1. Coverslips (12 mm circle), Thickness 1. 1 ounce lots, Fisher Scientific. Use within 3 years.
2. Coverslips (24 × 50 mm), Thickness 1.5. 1 ounce lots, Fisher Scientific. Use within three years.
3. DAPI Counterstain: 4', 6'-diamidino-2-phenyl indole dihydrochloride (1000 ng/mL in antifade mounting solution), Vysis. 1000 mL lots. Store at -20°C. See product label for expiration date.
4. DAPI working solution (10%): Mix 1 part DAPI counterstain with 9 parts Vectashield. Store at -20°C. Use within 6 months after date of preparation.
5. Ethanol (70%): Add 300 mL of distilled water to 700 mL of 100% ethanol. Mix well and store at room temperature. Use within one year of date of receipt.
6. Ethanol (85%): Add 150 mL of distilled water to 850 mL of 100% ethanol. Mix well and store at room temperature. Use within 1 year of date of receipt.
7. Ethanol (100%): Pure grain alcohol (ethanol). Store a 1000 mL aliquot at room temperature. Use within 1 year of date of receipt.
8. Formaldehyde (37% stock solution), 500 mL lots, Sigma. Store at room temperature. See product label for expiration date.
9. Formaldehyde working solution (1%): Add 0.18 g magnesium chloride and 1 mL of formaldehyde (37% stock solution) to 37 mL of PBS working solution in a Coplin jar. Store at room temperature. Use within three months after date of preparation.
10. Formamide (CH_3NO), 500 g lots, US Biochemicals. Store at 4°C. Use within one year.
11. Formamide solution (70%): Mix 7 parts formamide, 2 parts distilled water, 1 part 20× SSC solution. Adjust pH to 7.0. Store at 4°C. Use within three months after date of preparation.
12. HCl (1 N): Hydrochloric acid, 1 quart lots, Ricca Chemical Co. Store at 4°C. See product label for expiration date.
13. HCl solution (0.01 N): hydrochloric acid. Add 1 mL of 1 N HCl to 99 mL of distilled water. Store at 4°C. Use within one year after date of preparation.
14. Nonidet P40 (NP-40), 500 μL lots, Vysis. Store at 20°C. See product label for expiration date.
15. 0.1% NP-40/2× SSC solution: Add 500 μL nonidet P40 to 500 mL 2× SSC solution (pH 7.0). Store at room temperature. Use within two months after date of preparation.
16. Phosphate buffered saline (PBS), Sigma. 10 mM phosphate buffered saline pH 7.4. Box of 10 packets. Store at room temperature.
17. PBS working solution: Add 1 packet of PBS to 1 L distilled water and mix thoroughly. Store at room temperature. Use within six months after date of preparation.
18. Pepsin (crystallized and lyophilized), 2,500–3,500 units/mg protein (solid). 100 g lots, Sigma. Store at -20°C. See product label for expiration date.
19. Pepsin stock solution (10%): Add 1 g pepsin to 10 mL distilled water. Aliquot into 20 μL lots. Store at -20°C. Use within six months after date of preparation.
20. Pepsin working solution (0.005%): Add 20 μL pepsin stock solution (10%) to 40 mL HCl solution (0.01 N). Make fresh for each batch of slides processed each day.
21. Rubber cement, 4 fl. oz. lots, Mayo Inventory Center. Store at room temperature. 0.4× SSC solution (pH 7.0): Add 20 mL of 20× SSC solution (pH 6.3) to 980 mL distilled water, mix thoroughly and adjust pH to 7.0. Store at room temperature. Use within six months after date of preparation.
22. 2× SSC solution (pH 7.0): Add 100 mL 20× SSC solution (pH 6.3) to 900 mL distilled water and adjust pH to 7.0. Store at room temperature. Use within six months after date of preparation.
23. 20× SSC solution: Add 132 g 20× SSC powder to final volume of 500 mL distilled water and adjust pH to 6.3. Store at room temperature. Use within six months after date of preparation.
24. 20× SSC (powder), 500 g lots, Abbott Molecular-Vysis. Store at room temperature. See product label for expiration date.
25. SpectraVysis probe, 50 μL lots, Abbott Molecular-Vysis. Store at -20°C. See Note 1, M-FISH probes.
26. Vectashield, Vector Laboratories. Store at 4°C. See product label for expiration date.

See Note 2, Quality control is essential to assure consistency of results.

Method

The methods are based on the manufacturer's guidelines and our experiences.

Slide preparation and pre-treatment

1. Prepare slides based on your standard procedure for each tissue type (blood, amniotic fluid, fibroblast culture or bone marrow).
2. Find suitable metaphases using phase contrast microscopy and etch areas to be hybridized.
3. Place slides in 90 °C oven for 10 minutes to artificially age. However, this step is skipped if the slides are 24 hours old and have been stored at room temperature.
4. Incubate slides in 2× SSC at 37 °C for 1 hour.
5. Transfer slides to 0.005% pepsin solution (20 µL of 10% pepsin stock solution in 40 mL of 0.01 N HCl) at 37 °C for 1 minute.
6. Wash slides in a Coplin jar filled with PBS working solution for 5 minutes at room temperature.
7. Fix slides in 1% formaldehyde for 5 minutes at room temperature.
8. Wash slides in PBS working solution for 5 minutes at room temperature.
9. Dehydrate slides in 70%, 85% and 100% ethanol for 2 minutes each at room temperature.
10. Jet air dry slides.

Denaturation, hybridization and post-hybridization wash

1. Denature slides in 70% formamide solution at 74 °C for 2 minutes.
2. Dehydrate slides in 70%, 85% and 100% ethanol for 2 minutes each and air dry slides at room temperature.
3. Mix SpectraVysion probe well, then pipet 3 µL of probe working solution for each hybridization site into a labeled microcentrifuge tube.
4. Denature the probe working solution for 5 minutes at 76 °C by floating the microcentrifuge tube containing the probe mixture in a water bath.
5. Apply 3 µL of the probe mix onto the selected hybridization area of the slide preparation, place a coverslip (12 mm circle) over the hybridization area, and cover all edges of the coverslip with a continuous bead of rubber cement to provide a seal.
6. Incubate slides in a humidified incubator at 37 °C for up to 20 hours (minimum of 16 hours).
7. Remove slides from the incubator, then carefully remove and discard the rubber cement and the coverslip without scratching the hybridization area.
8. Place slides in a 0.4× SSC solution at 72 °C for 2 minutes. Wash no more than six slides at a time.
9. Rinse slides in fresh 0.1% NP40/2× SSC for 30 seconds at room temperature.
10. Remove slides and touch the edges of the slides with a paper towel to remove excess 0.1% NP40/2× SSC solution.
11. Apply 10 µL of DAPI working solution to the hybridization area.
12. Place a coverslip (22 × 22 mm) over the hybridization area.
13. Carefully remove air bubbles with gentle pressure on top of the coverslip with a paper towel.
14. The slides are now ready for analysis, but should be stored in a darkened area to avoid prolonged exposure to “direct light” to prevent premature fading of probe signals. If the slides need to be stored for longer than 1 day, they should be stored at –20 °C.

Analysis

1. Use a fluorescence microscope equipped with a 100 watt mercury lamp and filter cubes suitable for M-FISH analysis (see Note 1) using the following filter sets with a 100× oil immersion objective: (1) single pass DAPI filter to capture DAPI counterstain, (2) single pass Gold filter to capture gold fluorochrome, (3) single pass spectrum Cy 5 filter to capture far red fluorochrome, (4) single pass spectrum Green filter to capture green fluorochrome, (5) single pass spectrum Red filter to capture red fluorochrome, and (6) single pass Aqua filter to capture aqua fluorochrome.
2. The analysis is initiated with a 40× objective lens and DAPI filter for selecting suitable metaphases. After a metaphase is selected, check if fluorochromes have a good hybridization by switching to individual filters. Then the metaphase is focused with a 100× objective lens. Multiple images are captured using the manufacturer’s instructions. When capture software is initiated, the barrier filter is moved to the correct setting for the first fluorochrome plane. After the first exposure, the barrier filter is moved to the next fluorochrome and captured. This process is repeated until all five fluorochromes and the DAPI counterstain have been exposed separately. Then the computer software merges all the images to a single image. Each chromosome is individually classified according to the assigned fluorescence signature (provided by the manufacturer). Images can be immediately analyzed and karyotyped, or saved for later analysis. We typically capture and analyze ten metaphases and karyotype two for each case and/or clone. Individual chromosome pairs

- can be pseudocolored differently when necessary to highlight their fluorescence pattern. This feature is especially useful to accentuate chromosome rearrangements between two chromosomes where default pseudocolors are similar.
3. Cells can be harvested using standard cytogenetic techniques, both for constitutional (PHA-stimulated blood, amniotic fluid, fibroblast, chorionic villus, or abortus tissue) or oncologic (bone marrow, pleural effusions, or solid tumors) tissues. For consistency of chromosome spreading and morphology, we use a Thermatron drying chamber for harvesting. The optimum setting of the chamber for blood harvest is 25°C and 50% relative humidity. At these settings, a single drop of fixed cell suspension is placed on a dry microscope slide in the drying chamber. The settings are different for each tissue and may vary somewhat depending on the ambient temperature and humidity. It is desirable to check the first slide under phase microscopy for inadequate or over spreading and adjust the temperature and humidity settings accordingly.
 4. We have successfully used sequential G-banding followed by M-FISH. The sequential staining requires greater care for optimum results. Our G-banding procedure includes artificial aging for 1 hour at 90°C, and trypsinization and staining with Leishman's Giemsa stain. Immersion oil is removed by two 10-minutes washes in 2× SSC/0.1% NP40 solution (all reagents are at room temperature). Following removal of immersion oil, slides are destained in 100% ethanol for 2 minutes twice. Then the slide is dehydrated in an ethanol series of 95% for 2 minutes, 70% ethanol/1% HCl for 3 minutes, 95% ethanol for 2 minutes, and air dried. The slide is treated with 1% formaldehyde for 20 minutes and 2× SSC/0.1% NP40 for 20 minutes, dehydrated in an ethanol series (70%, 85%, and 100%) for 2 minutes in each solution, and air dried. The hybridization procedure and post-hybridization washes are the same as provided earlier.
 5. The M-FISH probe (Abbott Molecular-Vysis, Des Plaines, IL) and slide are denatured separately whether one is using a freshly prepared slide or a slide that has previously been G-banded. This procedure is preferred over the HYBrite in as much as it allows for better morphology and decreases the "halo effect" or "bleeding" of the fluorochrome. A 16-hour hybridization is optimal. The M-FISH results are usually much better from fresh preparations than from preparations which have been G-banded, due to the artificial aging necessary for G-banding.
 6. One of the most important considerations when using the M-FISH assay is the contrast between specific and nonspecific binding. If the contrast is poor, the chromosome classification will not be sharp. The contrast is especially critical for the far red fluorochrome. Increasing the digestion time of pepsin solution during the pretreatment procedure can help to improve the contrast. However, exposure to pepsin for too long can cause a loss of chromosomal DNA and hybridization signals that can lead to poor or weak hybridization signals. Low humidity in the hybridization chamber can cause the hybridization reagents to be dehydrated, thus increasing the concentration of the solution, which could cause the probes and the chromosomal DNA to hybridize randomly, negatively affecting the contrast.
 7. Another important factor for optimum results is chromosome morphology. Poor morphology can be a problem because it makes DAPI bands, when converted to gray scale, difficult to interpret. It can also cause "bleeding" of colors from one chromosome to another. The most frequent cause of poor morphology is inadequate aging. Artificially aging or treating the slide with hot 2× SSC for 2 minutes may improve the morphology. Rinsing the slide briefly in deionized water at room temperature and counterstaining with DAPI solution may also improve morphology but may reduce the intensity of hybridization signals. Over-aging and/or 2× SSC over-pretreatment can also reduce hybridization intensity. Poor morphology may also result from over-denaturation. Good morphology and poor hybridization is usually indicative of over-aging, excessive pretreatment with 2× SSC, and/or under-denaturation. A "dirty" preparation with a lot of background debris can also compromise results. Brightly fluorescent debris, especially prevalent in Texas red, can decrease the exposure duration and cause problems with chromosome classification. This problem can be corrected by exposing the Texas Red plane with debris for longer periods of time using the manual setting.
 8. Sometimes an optimum result may be elusive when there is nothing wrong with the reagents or the procedure. It is then necessary to examine each fluorochrome image plane individually and compare them with the table provided by the manufacturer. For instance, chromosome 22 (aqua, red, and green) is sometimes misclassified as chromosome 3 (aqua). One can examine the green and red fluorochrome planes individually to make sure that these spectra are present to confirm the identity for chromosome 22.
 9. At the junctions of break and fusion points of interchromosomal rearrangements and chromosomal overlaps, chromosome classification sometimes indicates the involvement of a "third chromosome" owing to overlapping fluorochromes. This is particularly critical when translocated segments are small. All possible chromosomes should be considered for the color classification observed. For instance, a derivative chromosome 7 (Red) with a small translocated segment of chromosome 12 (Gold and Green) may actually be classified as chromatin from chromosome 19 (Gold, Red and Green). To resolve the problem, the sample must be analyzed by a wcp 12 probe. If the segment is positive for wcp 12, it is concluded to be from chromosome 12. If the result is negative, then it must be from chromosome 19. Nonspecific colored areas also appear when a combination of fluors occurs that is outside of the range of the classification system. For instance, if an overlap occurs between chromosomes 20 (Far Red, Aqua and Red) and 21 (Gold and Aqua) resulting in four colors (Far Red, Aqua, Red and Gold), a nonspecific colored area will be encountered since no chromosome is assigned more than three colors [5].

10. Small translocations, especially insertions, can be misread, especially if the derivative chromosome's fluorophore signature contains all the fluorophores of the translocated chromosome. An example would be a small segment of chromosome 1 (Gold) translocated to chromosome 5 (Gold and Far Red). The entire length may appear to be chromosome 5. Again, this can be resolved by use of individual whole chromosome painting probes. Thus, it is important to check the individual fluor images to assess what fluors are hybridizing to the chromosome or marker of interest to determine if pseudo-colors are properly assigned.
11. Metaphase selection is also important. Avoid metaphases that are too tight or have many overlaps. Tight metaphases are difficult to analyze due to the “bleeding” of the fluors and their subsequent pseudocolors. Metaphases that have bright debris or nuclei close to or touching individual chromosomes should also be avoided. These chromosomes will be subtracted from the composite image by the software as noise, along with the nuclei.
12. Constitutive heterochromatin, including segments rich in alpha satellite and acrocentric p-arms rich in repetitive DNA sequences, are either blocked by using unlabeled Cot-1™ DNA or not having complementary DNA sequences in the probes. These segments are also randomly assigned a pseudocolor and classified. It is especially important to bear this in mind when analyzing markers or derivative chromosomes. Other limitations of M-FISH include the inability to detect intra-chromosomal abnormalities such as inversions and small inter-chromosomal anomalies, especially those that are 3 Mb or less in size.

Notes

1. **M-FISH probes.** The M-FISH probes, filter set, and computer software are commercially available as accessories to fluorescent image analysis from several companies. We use the Abbott Molecular-Vysis system. It has the following five fluors and their respective absorption and emission peaks (nm): Far Red (655/675), Red (592/612), Gold (530/555), Green (497/524) and Aqua (433/480).
2. **Quality control is essential to assure consistency of results.** We take the following steps for quality control: new reagents are tested on a control specimen before use in clinical practice, all test failures are investigated and documented to establish the source of the problem, and a monthly result summary report is prepared to assess test performance.

References

1. Pinkel D, Landegent J, Collins C, Fuscoe J, Segraves R, Lucas J, Gray J. Fluorescence *in situ* hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocation of chromosome 4. *Proc. Natl Acad Sci USA* 1988; 85:9138–9142.
2. Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bay-Am I, Soenksen D, Garini Y, Ried T. Multicolor spectral karyotyping of human chromosomes. *Science* 1996; 273:494–497.
3. Adeyinka A, Jalal SM. Clinical applications of 24-colour FISH. In *Encyclopedia of Medical Genomics and Proteomics*. Jurgen Fuchs, Maurizio Podda, eds. Marcel Dekker, New York City, 2005; pp. 471–473.
4. Jalal SM, Law ME. Multicolor FISH. In *Methods in Molecular Biology*. Vol. 204. *Molecular Cytogenetics: Protocols and Applications*. Y.S. Fan, ed. Humana Press, Totowa NJ, 2002; pp. 105–120.
5. Law M, Jalal SM. M-FISH technique: How to set up and analyze. *J Assoc Genet Tech* 2000; 26:51–53.
6. Jalal SM, Law ME. Utility of multicolor fluorescent *in situ* hybridization in clinical cytogenetics. *Genet Med* 1999; 1(5):181–186.

Protocol 17.6 Multicolor FISH (M-FISH) III

*Submitted by Ilse Chudoba, MetaSystems, Altlussheim, Germany
For research use only!*

Safety warning

Painting probes contain formamide. Handle carefully. Avoid contact with skin, and wear gloves while handling the reagents. To prevent photo bleaching, handle all reagents and slides containing fluorochromes in reduced light. Centrifuge all tubes prior to opening to assemble the contents at the bottom of the tube.

I. Pretreatment and denaturation of chromosome slides

Careful chromosome slide preparation is the first essential of a successful hybridization. Slides should not be older than two weeks; we recommend preparing the slides one day prior to hybridization. For long term storage keep slides at -20°C .

Check slides for spreading and morphology under phase contrast before hybridization.

A. Protein digesting pretreatment prior to hybridization (optional)

Cytoplasmic proteins surrounding the chromosomes may impair the hybridization. Pretreat slides if necessary.

Materials

1. Pepsin stock solution: Dissolve 1 g pepsin, Sigma #P 7012, in 50 mL sterile distilled H_2O ; store in 500 μL aliquots at -20°C .
2. 1 N HCl
3. 1 \times PBS
4. Post-fixation solution: 1% formaldehyde in 1 \times PBS + 50 mM MgCl₂
5. Ethanol series: 70%, 90%, and 100% ethanol

Method

1. Add 1 mL 1 N HCl to 99 mL H_2O ; prewarm to 37°C .
2. Add 500 μL of the pepsin solution; mix well.
3. Immediately immerse the slides into the pepsin solution and incubate 2 minutes for amniocytes and lymphocytes, and up to 5 minutes for bone marrow.
4. Wash slides in 1 \times PBS for 3 minutes.
5. Apply 100 μL of the post-fixation solution to each slide, overlay with a 24 \times 60 mm coverslip, and incubate for 10 minutes at room temperature.
6. Wash slides in 1 \times PBS for 3 minutes.
7. Dehydrate slides in 70%, 90%, and 100% ethanol for 2 minutes each.
8. Let air dry.

Note: If the protein digestion pretreatment is done immediately prior to the prehybridization washing, the ethanol series for dehydration and rehydration are not necessary. Transfer slide from 1 \times PBS directly into preheated 2 \times SSC.

B. Slide denaturation materials

1. 0.1 \times SSC, pH 7.0–7.5
2. 2 \times SSC, pH 7.0–7.5
3. NaOH 0.07 M
4. Ethanol series: 100%, 70%, 50%, and 30% ethanol

Method

1. Put a Coplin jar with 0.1 \times SSC and 2 \times SSC into the refrigerator.
2. Prewarm a Coplin jar with 2 \times SSC to 70°C in a water bath.
3. Rehydrate slide: 100%, 70%, 50%, and 30% ethanol, 1 minute each.
4. Transfer slide into 0.1 \times SSC at room temperature for 1 minute.
5. Incubate slide for 30 minutes in 2 \times SSC at 70°C .
6. Remove Coplin jar from water bath, let cool down to 37°C (takes about 20 minutes).
7. Transfer slide to 0.1 \times SSC at room temperature, 1 minute.
8. Denature slide in 0.07 N NaOH at room temperature, 1 minute.
9. Put slide into 0.1 \times SSC, 4 $^{\circ}\text{C}$, 1 minute.
10. Put slide into 2 \times SSC, 4 $^{\circ}\text{C}$, 1 minute.
11. Dehydrate slide: 30%, 50%, 70%, and 100% ethanol, 1 minute each.
12. Let air dry.

II. Probe denaturation and hybridization

Note: Start the probe denaturation during pretreatment and denaturation of chromosome slides. Time the procedure so that the prepared slide has just dried as the probe prehybridization is completed.

A. Method

1. Probe cocktail per hybridization:

Use 7 µL for an 18 × 18 mm coverslip, 10 µL for a 22 × 22 mm coverslip, or 12 µL for a 24 × 24 mm coverslip.

Note: see Table 17.5 in this chapter for MetaSystems' probe combinatorial labeling scheme.

2. Denature the probe by incubating at 75 °C for 5 minutes.
3. Put on ice briefly.
4. Incubate at 37 °C for 30 minutes.

III. Post-Hybridization washing steps

A. Materials

1. 1× SSC, pH 7.0–7.5, 75 °C
2. 4× SSCT = 4× SSC, pH 7.0–7.5, containing 0.05% Tween™20, room temperature
3. 1× PBS, room temperature

B. Method

1. Carefully remove rubber cement and coverslips.
2. Place slides in prewarmed (75 °C) 1× SSC, 5 minutes.
3. Incubate slides for 5 minutes in 4× SSCT.

IV. Detection of the biotin-labeled probes with Cy 5 using metasystems' B-tect detection kit

A. Blocking step

1. Apply 50 µL of blocking reagent to each slide, overlay with a 24 × 60 mm coverslip and incubate 10 minutes at 37 °C.
2. Flip off coverslips, put slides into 4× SSCT and continue with the next step.

B. Detection step 1

1. For each slide mix 50 µL blocking reagent with 1 µL detection 1 + 3 reagent.
Apply 50 µL to each slide, overlay with a 24 × 60 mm coverslip and incubate 15 minutes at 37 °C.
2. Wash 2 × 3 minutes in 4× SSCT, shaking at room temperature.

C. Detection step 2

1. For signal amplification, mix 50 µL blocking reagent with 1 µL detection 2 reagent. Apply 50 µL to each slide, overlay with a 24 × 60 mm coverslip and incubate 15 minutes at 37 °C.
2. Wash for 2 × 3 minutes in 4× SSCT, gently shaking at room temperature.

D. Detection step 3

1. See Detection step 1.
2. Wash for 2 × 3 minutes in 4× SSCT, shaking at room temperature.
(Detection steps 2 and 3 are optional and are only necessary if the fluorescence signal is weak and you want to amplify the signal.)

E. Counterstaining

1. Wash for 3 minutes in 1× PBS, shaking at room temperature.
2. Drain fluid off and blow dry with a rubber ball or let air dry.

3. Apply 20 µL of the DAPI/antifade.
4. Overlay with a 24 × 60 mm coverslip.

V. Notes

1. Denaturation procedure adapted from Fritz et al., *Hum Genet* 1998; 103:441–449 and Rieder et al., *Leukemia* 1998; 9:1473–1481.
2. Tween™ is a trademark of ICI America, Inc.; Spectrum Orange™ is a trademark of Vysis, Inc.; Cy™ is a trademark of Amersham Pharmacia Biotech UK Limited; Texas Red® is a registered trademark of Molecular Probes, Inc.

Protocol 17.7 Comparative genomic hybridization I

Submitted by Turid Knutsen and Danny Wangsa, Section of Cancer Genomics, National Cancer Institute, Bethesda, Maryland
 (All protocols from this laboratory are available at <http://www.riedlab.nci.nih.gov/>)

Principle

CGH is a genome-wide screening technique that identifies and maps DNA copy number imbalances in a single experiment [1]. A quantitative two-color technique, CGH is based on the *in situ* hybridization of differentially labeled tumor (test) DNA and normal (control) reference DNA to normal metaphases. It is a reverse hybridization strategy in which the tumor DNA is used as the probe. Control DNA is used as an internal standard. The tumor DNA is labeled with a green fluorochrome (e.g., avidin or streptavidin-FITC) and the reference DNA is labeled with a red fluorochrome (e.g., anti-digoxigenin-TRITC) by a standard nick translation procedure. Where the tumor contains normal chromosome elements, the two probes will be present in equal amounts, and the two dyes will combine to produce a third (blended) color, yellow-orange. Where the tumor contains excess material, such as is seen with gene amplification, the tumor color (green) will predominate. Likewise, deleted tumor genome segments of the tumor that are deleted will result in a dominance of the normal DNA color, i.e., red. A karyogram is produced displaying loss and gain along each chromosome as a histogram. One of the major advantages of CGH, as compared with other cytogenetic methods, is that it only requires genomic DNA from the tissue of interest without the necessity of metaphase preparations from tumor cells, which often grow poorly *in vitro*. It also permits the study of archival material, such as formalin-fixed, paraffin-embedded tissue, after following tissue microdissection and DNA amplification. It can be applied to premalignant tissue, which frequently has few dividing cells. Disadvantages of CGH include its inability to detect balanced, whole-genome copy number (ploidy) changes, balanced rearrangements, and imbalances present in less than 50% of the sample.

DNA Extraction

(see Ried Lab website for DNA extraction from other tissues and by other methods)

I. DNA preparation from paraffin tissue

Materials

Reagents

1. Chloroform, Mallinckrodt #4440
2. EDTA, 0.5 M
3. Ethanol, absolute
4. Isoamyl alcohol, Sigma #I-3643
5. Phenol
6. Proteinase K
7. RNase A, Boehringer #109 169
8. Sodium acetate, pH 5.2
9. Sodium chloride, 5 M
10. Sodium thiocyanate (NaSCN), 1 M, 250 g, Sigma #S 7757
11. TE Buffer (Tris-EDTA), pH 7.4
12. Tween 20
13. Xylene

Preparation

1. Chloroform/Isoamyl alcohol 24:1
Chloroform 24 mL
Isoamyl alcohol 1 mL
2. DNA extraction buffer
5 M NaCL 1.5 mL
0.5 M EDTA 5.0 mL
Tween 20 0.5 mL
Fill up to 100 mL with sterile water.
3. Proteinase K (10 mg/mL)
Dissolve 100 mg Proteinase K in 10 mL TE for 30 minutes at RT.
Aliquot and store at -20°C.
4. RNase A (20 mg/mL)
Dissolve 200 mg RNase A in 5 mL sterile water.
Boil for 15 minutes.
Cool to room temperature.
Aliquot and store at -20°C.

Method

1. Cut 50 µm slices of formalin-fixed and paraffin-embedded tumor samples.
(Note: Before and after each 50 µm slice, cut 4 µm slices for analysis with hematoxylin–eosin staining to insure that the tissue is still representative.)
2. Incubate in xylene at 45 °C for 15 minutes.
3. Centrifuge 10 minutes at 14,000 RPM.
4. Pipet off supernatant.
5. Repeat steps 2–4.
6. Add 1 mL 100% ethanol to tissue pellet, vortex, centrifuge for 10 minutes at 14,000 RPM, and pipet off supernatant.
7. Add 1 mL 90% ethanol to tissue pellet, vortex, centrifuge for 10 minutes at 14,000 RPM, and pipet off supernatant.
8. Add 1 mL 70% ethanol to tissue pellet, vortex, centrifuge for 10 minutes at 14,000 RPM, pipet off supernatant, and dry pellet in speed vac.
9. Resuspend pellet in 1 mL NaSCN (1 M) and incubate at 37 °C overnight.
10. Centrifuge for 10 minutes at 14,000 RPM; pipet off supernatant.
11. Resuspend pellet in 1 mL of DNA extraction buffer.
12. Centrifuge for 10 minutes at 14,000 RPM; pipet off supernatant.
13. Repeat steps 11–12, twice.
14. Add 5 µL RNase (20 mg/mL) and incubate for 1 hour at 37 °C (RNase treatment is optional since paraffin material often does not contain large amounts of RNA).
15. Add 40 µL of proteinase K (10 mg/mL), vortex briefly, and incubate at 55 °C overnight (if tissue is not completely dissolved, add additional proteinase K and continue incubating; tissue should be dissolved).
16. Add 440 µL of phenol, shake vigorously by hand for 5 minutes, and centrifuge for 5 minutes at 8000 RPM.
17. Pipet supernatant into a new tube, add a solution of 220 µL phenol plus 220 µL chloroform/isoamyl alcohol (24:1), shake vigorously by hand for 5 minutes, and centrifuge for 5 minutes at 8000 RPM.
18. Pipet supernatant into a new tube, add 440 µL chloroform/isoamyl alcohol (24:1), shake vigorously by hand for 5 minutes, and centrifuge for 5 minutes at 8000 RPM.
19. Pipet supernatant into a new tube (2 mL Eppendorf tube), add 1/10 volume of sodium acetate (pH 5.2), add 3 volumes of ice cold 100 % ethanol, and store tube for 1 hour at -80 °C or overnight at -20 °C.
20. Centrifuge for 30 minutes at 14,000 RPM at 4 °C.
21. Remove and save supernatant (optional, in case you are not sure of the precipitation).
22. Wash pellet in 70% ethanol, spin for 15 minutes at 14,000 RPM at 4 °C, remove supernatant.
23. Dry pellet in speed vac.
24. Add 20–50 µL sterile H₂O (depending on the amount of DNA you expect, which is subject to experience).
25. Shake gently in thermomixer at 37 °C for 2 hours (DNA should be dissolved, but if you have doubts, put the sample on a rotating shaker in the cold room overnight).
26. Measure DNA concentration with a spectrophotometer and run around 200 ng on a 1% agarose gel.

II. DNA preparation from fresh/frozen tissue

Materials

Reagents

1. Agarose, ultrapure, Invitrogen #15510-027
2. Chloroform
3. EDTA, 0.5 M
4. Ethanol, absolute
5. Isoamyl alcohol, Sigma #I-3643
6. Phenol
7. Phosphate buffered saline (PBS), 1×
8. Proteinase K, 100 mg, EM Science, Gibbstown, WV #24568-2
9. RNase A, Boehringer Mannheim #109 169
10. Sodium acetate, 3 M
11. Sodium dodecyl sulfate (SDS) solution, 10%, Digene Diagnostics, Beltsville, MD #3400-1016
12. 10× TAE buffer, Advanced Biotechnologies #08-514-001

Preparation

1. DNA buffer (Tris-EDTA)
1 M Tris pH 8.0 20 mL
0.5 M EDTA 20 mL
Sterile water 100 mL
2. Proteinase K (10 mg/mL)
Dissolve 100 mg proteinase K in 10 mL TE for 30 minutes at room temperature.
Aliquot and store at -20 °C.
3. RNase A (20 mg/mL)
Dissolve 200 mg RNase A in 10 mL sterile water, boil for 15 minutes, and cool to room temperature. Aliquot and store at -20 °C.
4. 1× TAE buffer: dilute the 10× TAE with sterile molecular grade dH₂O.
5. 1% agarose gel: dissolve 1 g of agarose into 100 mL of 1× TAE buffer by warming the solution.

Method

1. Put 100 mg of tissue in a Petri dish with culture media, and divide the tissue into two pieces.
2. Put each tissue sample into a homogenizer with 3 mL of pure culture media. Grind well, then transfer into sterile 15 mL tubes and centrifuge for 5 minutes at 4 °C at 1000 RPM.
3. Remove the supernatant, wash the pellet with 5 mL 1× PBS, and centrifuge for 5 minutes at 1000 RPM.
4. Remove the supernatant, add 5 mL of DNA buffer, and centrifuge for 5 minutes at 1000 RPM. (To store the pellet at this stage, resuspend the pellet in 1 mL 1× PBS. Using a cryo-tube, centrifuge at 1,500 RPM for 2 minutes at 4 °C. Remove the supernatant and store the pellet at -80 °C.)
5. Remove supernatant, and resuspend the pellet in 3 mL DNA-buffer.
6. Add 100 µL of proteinase K (10 mg/mL), mix thoroughly by flicking the tube with your finger before adding 240 µL 10% SDS; shake gently, and incubate overnight at 45 °C in a water bath.
7. If there are still some tissue pieces visible, add proteinase K again, shake gently, and incubate for another 5 hours at 45 °C.
8. Add 3 mL of phenol, shake by hand for 5–10 minutes, and centrifuge at 3000 RPM for 15 minutes at 10 °C.
9. Pipet the supernatant into a new tube, add 1.5 ml phenol and 1.5 mL chloroform/isoamyl alcohol (24:1); shake by hand for 10 minutes and centrifuge at 3000 RPM for 15 minutes at 10 °C.
10. Pipet the supernatant into a new tube, add 3 mL chloroform/isoamyl alcohol (24:1), shake by hand for 10 min, and centrifuge at 3000 RPM for 15 minutes at 10 °C.
11. Pipet the supernatant into a new tube, add 1/10 of 3 M sodium acetate (pH 5.2) and 2.5 volume 100% ethanol (-20 °C), and shake gently until the DNA precipitates.
12. Heat a glass pipette over a gas burner, allow it to cool, and use it to fish the DNA threads out of the solution; transfer DNA into 70% ethanol and rinse well by inverting for 2 hours.
13. Transfer DNA into new Eppendorf tube (1.5 mL) and centrifuge for 30 minutes at 14,000 RPM.

14. Pour off the supernatant and invert Eppendorf tube so that the remaining solution drains off onto paper; dry pellet in a speed vac for 5 minutes.
15. Dissolve the DNA in 100–300 µL sterile water overnight at 37°C in Eppendorf Thermomixer (or longer if necessary).
16. Measure the DNA concentration: (1) gel electrophoresis method: run 1–5 µL (approximately 200 ng) on 1% agarose gel in 1× TAE buffer; (2) spectrophotometer method: measure the DNA with a NanoDrop instrument and print the results for future reference.

Tissue (mg)	10	20	40	50–60	75–80	100
DNA buffer	600 µL	1200 µL	2400 µL	3000 µL	4800 µL	6000 µL
Proteinase	20 µL	40 µL	80 µL	100 µL	160 µL	200 µL
10% SDS	44 µL	88 µL	176 µL	220 µL	352 µL	440 µL

III. Nick translation

Materials

Reagents

1. Biotin-16-dUTP, Boehringer Mannheim #1093070
2. Bovine serum albumin (BSA)
3. Dig-11-dUTP, Boehringer Mannheim #1093088
4. dATP, dTTP, dGTP, dCTP, Boehringer Mannheim #105 1440, 105 1458, 105, 1466, 105 1482
5. DNase I from bovine pancreas, Boehringer Mannheim #104 159, 100 mg
6. EDTA, 0.5 M
7. Glycerol
8. Lambda HindIII DNA marker
9. Magnesium chloride ($MgCl_2$), 2 M
10. β -Mercaptoethanol, 99%
11. Polymerase (Kornberg), Boehringer Mannheim #104 485
12. NaCl, 1 M
13. Tris-HCl, 1 M, pH 8.0
14. Water, sterile

Preparation

1. dNTP

	Final conc.
100 mM dATP, dCTP, and dGTP	5 µL of each
100 mM dTTP	1 µL
Sterile water	984 µL
Total	1000 µL

(equals 0.5 mM each of dATP, dCTP, and dGTP, and 0.05 mM dTTP)

Aliquot and store at –20°C.

2. DNase I stock solution, 1 mg/mL

	Final conc.
DNase I	10 mg
NaCl, 1 M	1.5 mL
Glycerol	5 mL
Sterile water	bring up to 10 mL
Total	10 mL

Aliquot and store at –20°C.

3. 10× NT-Buffer

		Final conc.
Tris-HCL, 1 M, pH8.0	500 µL	0.5 M
MgCl ₂ , 2 M	25 µL	50 mM
BSA, 10mg/mL	50 µL	0.5 mg/mL
Sterile water	425 µL	
Total	1 mL	

Aliquot and store at -20 °C.

4. 0.1 M β-Mercaptoethanol

		Final conc.
99% solution (14.4 M)	34.7 µL	0.1 M
Sterile water	bring up to 5mL	
Total	5mL	

Aliquot and store at -20 °C.

Method

- For each DNA sample, add to an Eppendorf tube:
 2 µg DNA
 10 µL 10× NT-Buffer
 10 µL dNTP mix
 10 µL 0.1 M β-Mercaptoethanol
 4 µL BIO-16-dUTP or 4 µL DIG-11-dUTP (1 mM)
 X µL sterile water
 (The total volume including reagents added in step 3 should be 100 µL.)
- Vortex, centrifuge, and place tubes on ice.
- Add 2 µL polymerase (Kornberg) first, and then 3–8 µL DNase (1 mg/mL) 1:1000 (DNase amount is variable because DNase concentration varies by lot).
- Vortex and centrifuge.
- Incubate at 15 °C for 2 hour (1.5–2 hours).
- Prepare gel electrophoresis.
- Run about 5 µL of each sample and the Lambda HindIII DNA marker; ideally the length of the DNA should be 500–900 bp after nick translation.
- If DNA is too large, add more DNase and incubate at 15 °C for 10–30 minutes.
- Stop the nick translation with 1 µL of 0.5 M EDTA and incubate at 65 °C for 10 minutes.
- Store DNA at -20 °C or precipitate the same day.

IV. DNA precipitation and hybridization

Materials

Reagents

- Dextran sulfate (50%), Intergen #S4030
- Ethanol, absolute
- Formamide, deionized
- Mouse Cot-1™ DNA, 500 mg, Invitrogen #18440-016
- Human Cot-1™ DNA, 1 mg/mL, 500 µg, Invitrogen #15279-011
- Salmon testes DNA, 9.7 mg/mL, 1 mL, Sigma Molecular Biology #D-7657
- 20× SSC

8. Sodium acetate (Na-Acetate), 3 M
9. Water, sterile

Preparation

1. Master Mix

Dextran sulfate, 50%	40mL
20× SSC	20mL
Sterile H ₂ O	40mL
Total	100mL

Vortex solution and place tube on a shaking platform overnight to insure proper mixing. Aliquot, and store at -20 °C.

2. 70% Formamide/2× SSC

Deionized formamide	70µL
20× SSC	10µL
Sterile water	20µL
Total	100µL

Adjust to pH 7.0.

Method

1. Add to an Eppendorf tube:
10–25 µL nick-translated test or tumor probe DNA (500–1000 ng DNA)
Equal amount of nick-translated control whole genomic DNA as probe DNA
(Note: can use 1–2 µg DNA if tumor DNA is isolated from paraffin material)
30–60 µL human Cot-1™ DNA (1 mg/mL)
1 µL salmon sperm DNA (10 mg/mL)
Note: Usually the test DNA is nick translated with biotin-16-dUTP and the control DNA is nick translated with digoxigenin-11-dUTP.
2. Add 1/10 volume Na-Acetate (3 M).
3. Add 2.5–3.0 × total volume of absolute ethanol.
4. Vortex, store at -20 °C overnight, or at -80 °C for at least 15–30 minutes.
5. Centrifuge (14,000 RPM) precipitated DNA at 4 °C for 30 minutes.
6. Pour off supernatant and speed vac for 5–10 minutes to dry pellet.
7. Add 5 µL pre-warmed deionized formamide (pH 7.5), incubate at 37 °C for 30 minutes, shaking; vortex a few times during the 30 minutes incubation.
8. Add 5 µL pre-warmed Master Mix, vortex, and centrifuge briefly. Incubate at 37 °C for 15–30 minutes.
9. Denature probe DNA at 80 °C for 5 minutes and centrifuge briefly. Probe can be kept at 37 °C until ready to denature.
10. Preanneal at 37 °C for 1–2 hours.
11. For slide denaturation apply 120 µL 70% formamide/2× SSC to a 24 × 60 mm coverslip and place inverted slide onto coverslip.
12. Incubate slides at 75 °C for 1.5 minutes on a slide warmer.
13. Remove coverslip and immediately place slide in ice cold 70% ethanol for 3 minutes, followed by 90% ethanol and 100% ethanol for 3 minutes each; air dry.
14. Add pre-annealed probe DNA to denatured slides and cover with 18 × 18 mm or 22 × 22 mm coverslips; seal coverslips with rubber cement.
15. Hybridize at 37 °C in a humidified chamber for 72 hr.

V. Detection

Materials

1. Antibodies
 - Avidin-FITC, Vector #A-2011
 - Biotinylated anti-avidin, Vector #BA0300
 - Goat anti rabbit TRITC, Sigma #T-5268
 - Mouse anti-DIG, Sigma #D-8156
 - Rabbit anti mouse TRITC, Sigma #T-2402
2. Antifade (1,4-phenylene-diamine)
3. Bovine serum albumin (BSA)
4. DAPI
5. Formamide
6. HCl, 1 N
7. 20× SSC
8. Tween 20
9. Water, sterile

Preparation

1. FA/SSC
 - 20× SSC 30 mL
 - dH₂O 120 mL
 - Formamide 150 mL
 - Adjust pH to 7–7.5 with 1 N HCl
 - Prewarm to 45 °C.
2. 4× SSC/Tween 20
 - 20× SSC 100 mL
 - dH₂O 400 mL
 - Tween 20 0.5 mL
 - Prewarm to 45 °C.
3. 0.1× SSC
 - 20× SSC 2.5 mL
 - Add H₂O to 500 mL
 - Prewarm to 60 °C.

Note: For the above wash solutions, you need 70 mL for glass Coplin jars and 50 mL for plastic jars per step.

4. Blocking solution (3% BSA)
 - Bovine serum albumin (powder) 0.3 g
 - Pre-warmed (37 °C) 4× SSC/Tween 20 10 mL
 - Keep at 37 °C to dissolve albumin.
5. DAPI (80 ng/mL DAPI in 2× SSC)
 - a. Stock solution:

		final conc.
DAPI	2 mg	0.2 mg/mL
Sterile water	10 mL	

- b. Working solution:

Stock solution	40 μL	80 ng/mL
2× SSC	100 mL	

6. Antibodies

- Layer 1 avidin-FITC (1:200) + mouse-anti-DIG (1:200)
- Layer 2 biotinylated anti-avidin (1:200) + rabbit anti mouse TRITC (1:200)
- Layer 3 avidin-FITC (1:200) + goat anti rabbit TRITC (1:200)

Note: Make sure solutions are at the correct temperatures before using; check with a thermometer.

Method

1. Remove rubber cement and coverslips from hybridized slides.
 2. First dip the slides into FA/SSC to remove coverslips; wash slides in FA/SSC (use Coplin jars), 3 × 5 minutes, shaking.
 3. Wash slides in 0.1× SSC, 3 × 5 minutes, shaking.
 4. Dip slides in 4× SSC/Tween 20; do not let them dry.
 5. Add 120 µL of blocking solution to 24 × 60 mm coverslips, touch slides to coverslips, and incubate in hybridization chamber at 37°C for about 30 minutes.
 6. Dip slides in 4× SSC/Tween 20; do not let them dry.
- Note:** Spin all fluorescent dyes for 3 minutes at 14,000 RPM before use.
7. Antibodies: Add 100 µL of prepared antibody solution (antibodies should be diluted in 1% BSA) to coverslip (use 24 × 60 mm), touch slide to coverslip, and incubate in hybridization chamber for 45–60 minutes at 37°C.
 8. Wash slides in 4× SSC/Tween 20, 3 × 5 minutes, shaking.
 9. Apply second and third antibody layers by repeating steps 7 and 8.
 10. DAPI staining: stain for 2–5 minutes in foil-covered Coplin jar.
 11. Wash in 2× SSC for 2 × 5 minutes, shaking.
 12. Shake off excess buffer and apply 35 µL antifade, cover with 24 × 60 mm coverslip, and store in the dark at 4°C.

Reference

1. Kallioniemi A, Kallioniemi O-P, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 1992; 258:818–821.

Protocol 17.8 Comparative genomic hybridization II

Submitted by Technical Services, Abbott Molecular-Vysis, Des Plaines, Illinois

Nick translation

Materials

1. 0.2 mM SpectrumGreen™ or SpectrumRed™ dUTP
Add 10 µL of 1 mM dUTP to 40 µL nuclease-free water.
2. 0.1 mM dTTP
Add 10 µL of 0.3 mM dTTP to 20 µL nuclease-free water.
3. 0.1 mM dNTP mix
Mix together 10 µL each of 0.3 mM dATP, 0.3 mM dCTP, and 0.3 mM dGTP.
4. Extracted genomic DNA
Prepare a 0.2 µg/µL to 1 µg/µL solution of extracted genomic DNA in Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 8.5) buffer.

Assay procedure

This procedure labels approximately 1 µg of extracted genomic DNA. This is enough for five CGH hybridizations.

1. Place a microcentrifuge tube on ice and allow the tube to cool.
2. Add these components to the tube in the order listed:
(17.5-x) µL nuclease-free water

- x μ L for 1 μ L extracted genomic DNA
 2.5 μ L 0.2 mM SpectrumGreen or SpectrumRed dUTTP
 5 μ L 0.1 mM dTTP
 10 μ L dNTP mix
 5 μ L 10 \times nick translation buffer
 10 μ L nick translation enzyme
 50 μ L total volume
3. Vortex the tube briefly.
 4. Incubate 2–4 hours at 15°C.
 5. Stop the reaction by heating in a 70°C water bath for 10 minutes.
 6. Chill on ice.

Determining the probe size

Determining the probe size is an essential part of the CGH procedure. For detailed instruction on preparing and running an agarose gel see [1] Maniatis T, Fritsch EF, and Sambrook J. Gel electrophoresis of DNA. In: *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring, NY: Cold Spring Harbor Laboratory, 1989 or [2] Ausubel FM, ed. Preparation and Analysis of DNA. In: *Current Protocols in Molecular Biology*. New York: Greene Publishing Associates and John Wiley & Sons, 1989.

1. Prepare a 1% agarose gel by adding 1 g agarose to 100 mL TAE buffer. Heat the solution in the microwave until the agarose is melted. This volume is enough for three minigels.
2. Cool agarose solution to 55°C and add 10 μ L ethidium bromide (final concentration is 0.01% (v/v)).
3. Pour the melted agarose into a minigel (10 \times 6.5 cm) apparatus with combs. Let the agarose cool to solidify.
4. Pour enough TAE running buffer into the minigel apparatus to cover the gel by approximately 1 mm.
5. Remove 9 μ L of the reaction mix containing the nick translated DNA and add 1 μ L of gel loading buffer.
6. Run the nick translated sample in one lane and a sample of the DNA size marker in another lane to size the probe.
7. Electrophorese the gel at 10V/cm until the dye in the gel loading buffer is 2–3 cm from the end of the gel.
8. Estimate the size range of the probe from the gel. The majority of the DNA smear should be in the 300–3000 bp range. Probe fragments that are larger will give a diminished fluorescence intensity when used in a CGH analysis.

As you increase the amount of enzyme and the incubation time, the size distribution shifts to progressively smaller probe fragments. To produce smaller probe fragments use these conditions that are listed in order of decreasing fragment size: 5 μ L enzyme mix/2 hour incubation, 5 μ L enzyme mix/4 hour incubation, 10 μ L enzyme mix/2 hour incubation and 10 μ L enzyme mix/4 hour incubation. Adjust the amount of nuclease-free water added to keep the total reaction volume at 50 μ L.

Reagent preparation

1. 20 \times SSC, pH 5.3
 Mix thoroughly 66 g 20 \times SSC in 200 mL purified H₂O. Adjust to pH 5.3 at ambient temperature using concentrated HCl and QS to final volume of 250 mL. Store at ambient temperature. Discard stock solution after six months, or sooner if solution appears cloudy or contaminated.
2. Denaturation solution
 Add 49 mL formamide, 7 mL 20 \times SSC (pH 5.3) and 14 mL purified H₂O to a glass Coplin jar and mix thoroughly. Verify that the pH is 7.0–7.5 by measuring the pH at ambient temperature. Between use, store covered at 4°C. Discard after 7 days.
3. Ethanol wash solutions (70%, 85% and 100%)
 Dilute 100% ethanol (v/v) with purified H₂O to prepare the wash solutions. Between uses, store covered at ambient temperature. Discard stock solutions after six months.
4. 0.4 \times SSC/0.3% NP-40 wash solution
 Mix thoroughly 20 mL of 20 \times SSC with 950 mL purified H₂O. Add 3 mL NP-40. Mix thoroughly until NP-40 is dissolved. Adjust pH to 7.0–7.5 with NaOH. Add purified H₂O to bring final volume to 1 L. Store at ambient temperature. Discard stock solution after six months, or if solution appears cloudy or contaminated.

5. 2× SSC/0.1% NP-40 Wash Solution

Add 100 mL of 20× SSC to 850 mL purified H₂O. Add 1 mL NP-40. Adjust pH to 7.0–7.5 with NaOH. Add purified H₂O to bring final volume to 1 L. Store at ambient temperature. Discard stock solution after six months, or if solution appears cloudy or contaminated.

6. Test DNA

Direct-label the test (and unlabeled control) DNA with SpectrumGreen™ dUTP. See the Vysis® CGH Nick Translation Kit for the procedure.

CGH procedure

Materials

1. Controls

Positive and negative controls provide comparisons for evaluating the hybridization and interpretation of the CGH data. For a negative control, use normal DNA for both the test and reference DNA (Abbott #32-804024, #32-802024). The intensity profiles for this experiment should be within the threshold values as determined by image analysis. For a positive control, use test DNA (Abbott #32-800227) that is extracted from a cell line with known genetic aberrations that are easy to detect by CGH analysis.

2. Normal Metaphase CGH Target Slides

Do not pretreat slides.

Slides are prepared using standard cytogenetic slide preparation methods that are optimized for CGH. The slides are made from phytohemagglutinin (PHA)-stimulated lymphocytes cultured for 48–72 hours before thymidine is added to synchronize the cells. The chromosome length is 400–550 bands. The lymphocytes are derived from a karyotypically normal male donor.

3. Preparing the Probe Mix

To produce a hybridization with signal intensities equivalent for both SpectrumGreen™ and SpectrumRed™ labeled DNA, the amount of SpectrumGreen labeled DNA is increased.

Method

1. Combine the following in a 1.5 mL microcentrifuge tube:

- a. 10 µL (200 ng) SpectrumGreen (nick translated labeled) test DNA
- b. 1 µL (100 ng) SpectrumRed total genomic reference DNA (#32-804023 or #32-804024)
- c. 10 µL (10 µg) Human Cot-1™ DNA (#32-800028)

2. Add 2.1 µL (0.1 volume) 3 M sodium acetate, then add 52.5 µL (2.5 volumes) of 100% ethanol to precipitate the DNA. Vortex briefly and place on dry ice for 15 minutes.

3. Centrifuge at 12,000 RPM for 30 minutes at 4 °C to pellet the DNA.

4. Remove the supernatant and dry the pellet for 10–15 minutes under a vacuum at ambient temperature.

5. Resuspend the pellet in 3 µL purified H₂O and 7 µL CGH hybridization buffer.

6. Denature the probe by heating the probe mix for 5 minutes in a 73 °C water bath.

Note: Denature the probe while the slide is being dehydrated (step 3 in Hybridizing the Probe to the Target Metaphase).

Hybridizing the probe to the target metaphase

1. Mark hybridization areas on the slide using a diamond tipped scribe.

2. Ensure that the temperature of the denaturation solution is at 73 ± 1 °C. Immerse the slide containing normal metaphase spreads into the solution for 5 minutes.

3. Dehydrate the slide for 1 minute in the 70% ethanol solution, followed by 1 minute in the 85% ethanol solution, and 1 minute in the 100% ethanol solution.

4. Dry the slide by touching the bottom edge to a blotter and wiping the underside with a paper towel.

5. Place the slide on a 45–50 °C slide warmer to allow remaining ethanol to evaporate.

6. Apply 10 µL of denatured probe mix to the slide.

7. Immediately apply the coverslip and seal with diluted rubber cement.

8. Place slide in a sealed, humidified box and place in a 37 °C incubator for 48–72 hours for hybridization.

Washing the slide

1. Place the wash tank containing 0.4× SSC/0.3% NP-40 wash solution in a 74 ± 1 °C water bath for at least 30 minutes prior to use. Discard after using one day. Prepare a second wash tank of 2× SSC/0.1% NP-40 at ambient temperature.
2. Remove the rubber cement seal and the coverslip and immediately place the slide into the 0.4× SSC/0.3% NP-40 wash solution at 74 ± 1 °C. Agitate the slide for 1–3 seconds.
3. Repeat step 2 for each slide—do not wash more than four slides at once—and then let the slides stand in the wash solution for 2 minutes.
4. Place the slide in the 2× SSC/0.1% NP-40 wash solution at ambient temperature. Agitate the slide 1–3 seconds and then let stand in the wash solution for 5–60 seconds.
5. Air dry slide in darkness.

Visualizing the hybridization

Apply 10 µL of DAPI II counterstain and a coverslip to each hybridization location.

CHAPTER 18

Genomic microarray technologies for the cytogenetics laboratory

Bhavana J. Davé¹ and Warren G. Sanger²

¹*Human Genetics Laboratory, Munroe Meyer Institute for Genetics and Rehabilitation, University of Nebraska Medical Center, Omaha, NE, USA*

²(deceased*) formerly, *Human Genetics Laboratory, Munroe Meyer Institute for Genetics and Rehabilitation, University of Nebraska Medical Center, Omaha, NE, USA*

18.1 Introduction

In the past decades, innovative technical advances in the field of molecular cytogenetics have greatly enhanced the detection of chromosomal alterations and have facilitated the diagnostic potential of cytogenetic studies in constitutional and acquired diseases. In the 50 years since Tijo and Levan recognized the correct chromosome number, cytogenetics has served as a powerful diagnostic and research tool. From banding and recognizing individual chromosomes for “chromosomal” karyotyping with a resolution of about 5–10 megabase (Mb), to utilizing the genomic microarray, also known as chromosomal microarray (CMA) or array-based comparative genetic hybridization (aCGH) technology for “molecular” karyotyping with a resolution of 2 kilobase (kb) to 1 Mb, the application of molecular technologies onto cytogenetics have enhanced the analytical resolution and expanded the diagnostic capacity. Some noteworthy innovations that have altered the landscape of clinical and research investigations include the use of various fluorescence in situ hybridization (FISH) techniques, the use of multicolor FISH to identify the chromosomal alterations unresolved by classical karyotyping, and yet another major advancement of chromosomal CGH which offers genome-wide screening by determining DNA content differences and revealing the chromosomal imbalances even when fresh specimen and chromosome preparations are unavailable. More recently, the development of array technology, which involves the use of an ordered set of defined nucleic acid sequences derived from various sources, immobilized on glass slides, allows the detection of much smaller genomic imbalances. The principle of genomic array technology was first described in 1997 by Solinas-Toldo et al. [1]. The first applications were published by Pinkel et al. [2], Snijders et al. [3], and Buckley et al. [4] in the following years. Rauch et al. [5] and Vermeesch et al. [6] proposed to call this novel technology “molecular karyotyping.” Depending on the specific investigational requirements, the majority of landmark procedures have and will continue to be utilized in clinical cytogenetics laboratories; however, CMA is finding its niche as a complementary technique in the clinical diagnostic and medical research arena.

18.1.1 Principle and advances

Genomic microarray was developed on the same principle as CGH on chromosomes except that metaphase chromosomes are substituted by cloned DNA segments as targets for hybridization. As a genome-wide screen, the single most valuable and advantageous aspect of chromosomal CGH over standard karyotyping was that DNA from the specific tissue replaced the

* Editors' note: Warren Sanger, PhD, October 6, 1945 to February 5, 2015. Dr. Warren Sanger, director of the Human Genetics Laboratory and director of Clinical Genetics at the Munroe Meyer Institute for Genetics and Rehabilitation (MMI) at the University of Nebraska Medical Center in Omaha, Nebraska, died unexpectedly on February 5, 2015, at the age of 69. Dr. Sanger was a professor of the departments of Pediatrics, Pathology and Microbiology, and MMI. He was also a founding Fellow of the American Board of Medical Genetics and author or co-author of over 300 peer-reviewed publications. We are forever in appreciation to him for his contribution to this manual.

requirement of a fresh specimen from the patient, which is a primary prerequisite for chromosomal studies. Chromosomal CGH was developed by Kallioniemi et al. [7] and has been successfully used especially in cytogenetic investigations of solid tumors, where optimal chromosomal preparations are difficult to achieve, and also in performing retrospective studies, where fresh tissue for chromosome preparations is unavailable. Briefly, for CGH, a test DNA (patient) and a reference DNA (genomic DNA from a clinically normal individual) are differentially labeled and co-hybridized on metaphase spreads of a normal individual. Copy number difference between test DNA and reference DNA alters the fluorescence ratio, thus providing the ability to determine gains and losses of chromosomes and/or chromosomal segments. The major constraint of CGH on metaphase chromosomes, however, was that the resolution level did not exceed 3 Mb [8, 9]. To overcome this limitation, Solinas-Toldo et al. [1] spotted an array of an ordered set of defined nucleic acid sequences on glass slides and demonstrated that it can be used as a substitute for metaphase chromosomes. They named the spotted clones as “matrix array.” In ensuing studies, Pinkel et al. [2] constructed a 3-Mb interval array of chromosome 20 and showed the use of DNA derived from various sources, like cosmids, bacterial artificial chromosomes (BAC), P1-derived artificial chromosome (PAC), and other large insert clones (LICs), as targets. Thereafter, PCR-generated sequences [3, 10, 11], cDNA clones [12, 13] or oligonucleotides [14] have also been used for constructing array for CGH. Single nucleotide polymorphisms (SNP) containing microarrays have been used to detect subtle constitutional imbalances [5, 15]. More recently, copy number changes at a resolution of individual exons have been detected [16]. Genomic microarray covering a whole chromosome [17, 18], a chromosome segment [19–24], all subtelomeric regions [25], or the entire genome at a 1 Mb [3, 10] and more recently at a 10–100-kb resolution level [26, 27] have been developed and validated. Tiling path arrays with overlapping clones have been constructed for some chromosomes, chromosomal segments or more recently the whole genome. The array platforms vary in terms of the genomic elements spotted and their coverage of the genome [28]. Although

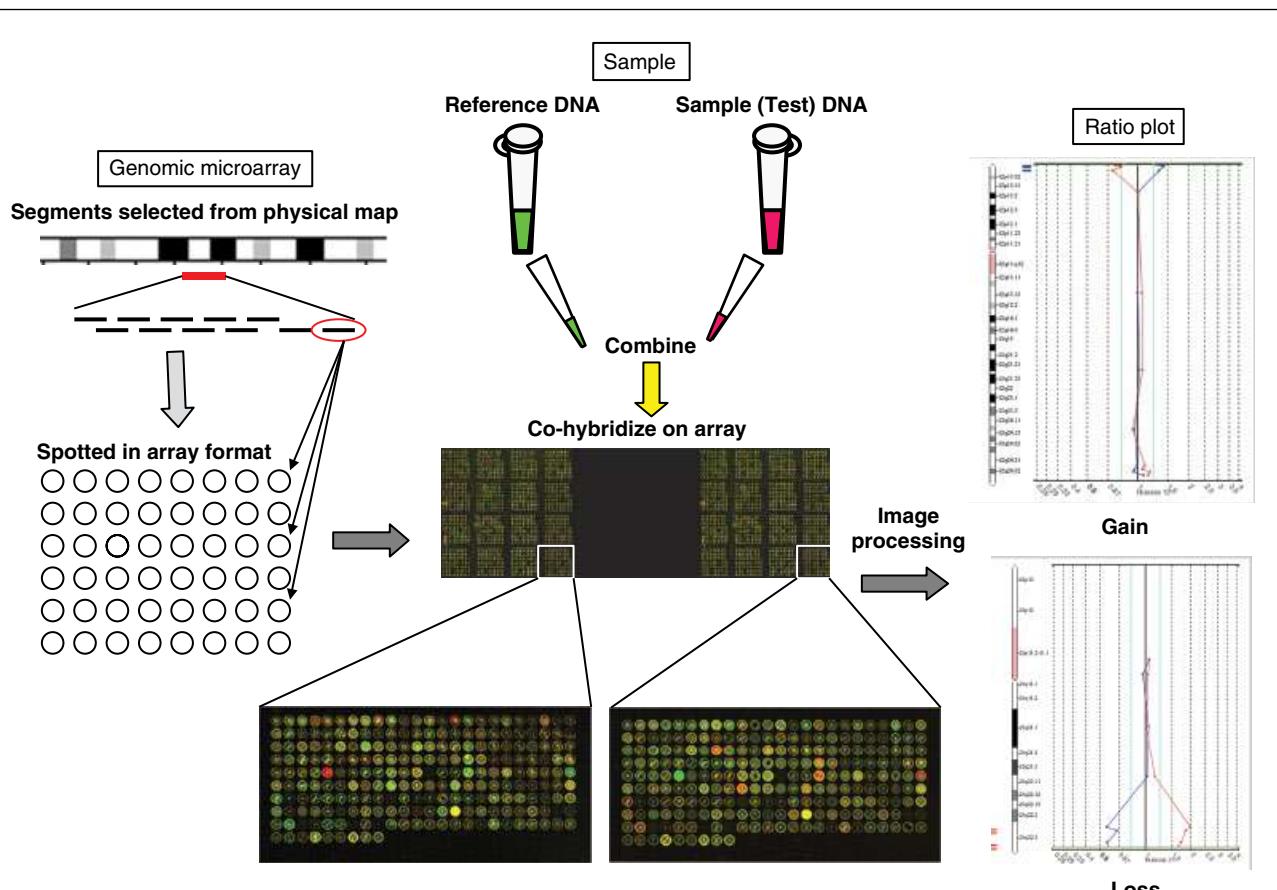


Figure 18.1 Genomic microarray hybridization profile construction. This illustration shows the array comparative genomic hybridization profile construction using differentially labeled test DNA and control DNA co-hybridized to genomic microarray. The differential labeling may be performed twice using a dye reversal procedure. The array is comprised of selected DNA targets spanning either a whole chromosome, or targeted chromosomal regions, or the entire genome. Typically the DNA targets are spotted in duplicates or triplicates. Comparative ratios of the two fluorescent signal intensities reflect the relative copy number of the test DNA. The ratio for each spot is plotted against its corresponding position in the human genome to generate a profile, thus determining any possible gain or loss of the DNA target. See insert for color representation of this figure.

Table 18.1 Genomic microarray platforms available for use in genome-wide CMA

Array elements ¹	Manufacturer ²	Website
LIC	Sanger	www.sanger.ac.uk/Projects/Microarrays
LIC	UCSF	cc.ucsf.edu/microarray
LIC	DKFZ	www.dkfz-heidelberg.de/kompl_genome
LIC	AFCRI	cma.afcri.upenn.edu
LIC	VUMC	www.vumc.nl/microarrays
LIC	BCCRC	www.bccrc.ca/cg/ArrayCGH_Group.html
cDNA	BD Biosciences	www.bdbiosciences.com
cDNA	Bioscience Corp.	www.superarray.com
cDNA	UHN	www.microarray.ca
oligo	Affymetrix	www.affymetrix.com
oligo	VUMC	www.vumc.nl/microarrays
oligo	Illumina Biotechnology Co.	www.illumina.com
oligo	Bioscience Corp.	www.superarray.com
oligo	Agilent Technologies	we.home.agilent.com/USeng/home.html
oligo	Oxford Gene Technology	www.ogt.com

¹ All of these array platforms require standard microarray scanners except that Affymetrix scanner and custom services are required for the Affymetrix platform. LIC, large insert clones.

² UCSF, University of California, San Francisco; DKFZ, Deutsches Krebsforschungszentrum; AFCRI, Abramson Family Cancer Research Institute; BCCRC, British Columbia Cancer Research Centre; UHN, University Health Network; VUMC, Vancouver University Medical Center.

numerous platforms have been developed to support CMA investigations, they all revolve around a common principle of detecting copy number alterations between two samples (Figure 18.1). Table 18.1 (adapted from [29]) summarizes the different array platforms and their relevant features.

In short, technological advancements in genomic analysis have enhanced the resolving power of conventional chromosome analysis from the megabase to the kilobase level. Tools that have mediated these developments include (a) the assembly of the finished human genome sequence and the integration of genome-wide clone libraries into the genomic map, (b) the development of high-throughput microarray platforms including the spotting and *in situ* synthesis technologies, and (c) the optimization of CMA protocols and development of software for data analysis. Together these developments have given rise to “molecular karyotyping,” a technology that allows a sensitive and specific detection of single copy number changes at the submicroscopic level throughout the entire human genome [30]. Genomic microarray detects genomic imbalances by comparing “test” DNA to differentially labeled “control” DNA when hybridized to known genomic sequences. Deviations from the normal (1 : 1) ratio determine gains and losses of chromosomes or deletions and duplications of regions thereof and allow the screening of the whole genome in a single experiment (Figure 18.1). Traditionally, the resolution of CMA has been defined by the genomic distance between each DNA target represented on the array [31, 32]. Quantitating imaging methods and analytical software are required for image acquisition and data processing. Various types of software have been developed allowing to process the data, facilitate the interpretation and to generate a graph of the results automatically [33–45]. Detailed technical aspects of CMA have been elaborately discussed or reviewed by many authors [46–50]. The quality of microarray data depends heavily upon the physical quality of the arrayed DNA sequences, and the control variables that govern the microarray construction process. The key parameters of array quality are the size of the DNA clones, the density of the genomic regions of interest and optimized probe content to allow for the best possible survey of the genome.

18.1.2 Advantages

The CMA technology offers some unique advantages that have rapidly increased its popularity and application in the clinical diagnostic and research arena. Some of the advantages listed here are not exclusive to CMA technology; however, because of its qualities of being comprehensive, amenable to very high resolution, sensitive, and fast, CMA holds great potential in the clinical and research laboratories. Table 18.2 outlines the usefulness of various cytogenetic and molecular cytogenetic techniques in detecting different abnormalities.

Table 18.2 Identification of various abnormalities by different cytogenetic and molecular cytogenetic techniques

	G-band	FISH (specifics required)	SKY/MFISH	CGH	CMA
Whole genome screen	+	-	+	+	+
Resolution	~5–10 Mb	~1–200 kb	~5–10 Mb	~3 Mb	~10 kb to 1 Mb
Polyplloid	+	-	+	-	-/+ ¹
Aneuploid	+	+	+	-	+ ²
Interstitial deletion/duplication	+/-	+	-	+	+
Reciprocal translocation	+	+	+	-	-
Nonreciprocal translocation	+	+	+	+	+
Amplification (dmin and hsr)	+	+	+	+	+
Amplification distributed insertion	-	+	-	+	+
Inversion	+	+	-	-	-
Recessive Gene Mapping	-	-	-	-	+

¹ Triploidy can be detected using specific “control.”² Requires specific “control” to determine sex chromosome abnormalities.

The CMA technology presents the following advantages:

- Provides a high-resolution means for identifying genomic imbalances across the entire genome in one assay.
- Facilitates genome-wide analysis of samples from which chromosome preparations are difficult or impossible.
- Has high throughput, high coverage, and allows direct mapping of aberrations to the genome sequence.
- Represents simultaneous performance of hundreds to thousands of FISH experiments per sample.
- Is applicable to formalin-fixed paraffin-embedded archival material, allowing for the exploration of large clinical tissue archives.
- Offers rapid analysis time due to (semi) automated analysis system.
- Genomic microarrays allow the detection of aneuploidies (e.g., gains and losses of entire chromosomes or segments of chromosomes), interstitial or terminal deletions, and are preferred by some, in investigations of idiopathic diseases.
- Target gene arrays help in identification of abnormalities within a specific gene.

18.1.3 Methods

The technique of CMA was developed with the intent of being able to detect genomic imbalances throughout the entire genome with a single assay. Specifically, CMA is based on competitive hybridization of differentially labeled patient DNA and control DNA onto arrayed-known genomic sequences in order to detect gains or losses of DNA sequences in the patient. Numerous CMA platforms have been developed and are continually being updated for clinical diagnosis. The CMA platforms currently utilized for diagnosis by clinical laboratories include the targeted array which encompasses the known microdeletion regions, the 41 subtelomeric regions, and other DNA segments sparsely covering the genome and the high density array which consists of numerous densely spotted DNA segments to fully cover the entire genome. Thus, CMA technology has much greater multiplexing capabilities than targeted FISH studies and offers much higher resolution for a genome-wide screening than conventional chromosome studies; which allows the accurate recognition of deletions/duplications in a timely fashion. These methods described here do not elaborate on the SNP array which does not utilize concurrent control DNA for analysis.

Depending on the various types of array platforms used for the testing, the procedures may vary; however, the basic steps involved in performing the testing for genomic microarray include

- the isolation of high-quality DNA from the “test” and “control” specimens;
- digestion of DNA to produce smaller fragments for better labeling (this step may be preferentially eliminated depending on the laboratory procedures);
- post-digestion cleanup or re-purification of DNA using preferred column;
- analysis the DNA quality by gel electrophoresis before proceeding for random prime labeling of genomic DNA;

- labeling of “control” and “test” specimens with random prime reaction procedures;
- analysis of post labeled “control” and “test” specimens by gel electrophoresis to ensure appropriate labeling;
- probe precipitation;
- denaturation and hybridization of “prepared” specimens to microarray “chip”;
- incubation of the hybridized microarray “chips” for appropriate time and at optimized temperature and conditions;
- post-hybridization wash;
- scanning an image of the washed microarray “chip”;
- analysis using suitable software programs;
- translation of the results into graphs/scatter plots expressing the results for each chromosome using appropriate software.

18.2 Applications

Genomic microarray has been used for a variety of approaches. Some of the applications pertinent to cytogenetics laboratories are discussed in the following section.

- identification of congenital genetic defects;
- characterization of acquired genetic changes;
- use in prenatal diagnostics;
- determination of genomic variations and polymorphisms;
- evolutionary characterization.

18.2.1 CMA for identification of congenital genetic defects (constitutional abnormalities)

In a clinical genetics clinic sometimes an explanation for the cause of individual developmental or intellectual disability or the etiology of multiple congenital anomalies (MCAs) seen in a newborn is an issue. If there are no stigmata of a known chromosomal syndrome and if family history and phenotype fail to provide clues, the studies will depend heavily on screening methods in the laboratory. Multiple targeted FISH studies with conventional cytogenetics are usually performed. Clinical cytogenetics laboratories are evolving and continually incorporate better and precise methods to provide an answer to the currently eluding diagnosis and in improving the results.

Clinical cases most suited for CMA investigations:

- Unexplained intellectual disabilities (ID), developmental delay (DD), and MCA with or without dysmorphic features.
- Cases with normal chromosome/FISH findings but phenotype suggestive of submicroscopic genomic imbalance.
- Cases that would be candidates for multiple FISH tests and/or subtelomeric FISH screen.
- Cases with known cytogenetic abnormality – to refine deletion duplication area and search for candidate genes.
- Abnormal outcome of prenatal cases with normal cytogenetics.

Several reports of the analysis of constitutional chromosomal aberrations using genomic arrays have appeared recently [51–58]. The array data were not only found to be concordant with previous G-banding or FISH analysis, but they provided higher resolution definition of the involved aberrations. In some cases, CMA revealed additional aberrations, highlighting the value of the whole genome scan as compared to FISH, which can only provide information on those specific targeted loci.

The following section describes the various applications of CMA in delineation of constitutional abnormalities.

Subtelomeric regions

Insight into the molecular basis of ID came from the finding that nearly 5–10% of previously unexplained ID cases can be attributed to submicroscopic, subtelomeric deletions [59–63]. Unbalanced chromosomal abnormalities in subtelomeric regions may be particularly difficult to visualize cytogenetically. The subtelomeric FISH panel, which has been in use since the report of subtelomeric imbalances in patients with unexplained ID involves labor-intensive individual FISH analysis and scoring of multiple subtelomeric regions. CMA detects abnormalities in a single assay and offers a comprehensive view of the submicroscopic deletion and duplication of the related chromosome. Subsequently, arrays designed to study subtelomeric rearrangements were developed. Thus, one of the first applications of CMA was to screen for subtelomeric rearrangements [3, 25, 64]. Deletions, duplications and cryptic subtelomeric imbalances were detected and the cytogenetic diagnosis was enhanced.

Genome-wide arrays for apparently normal chromosomes and apparently balanced chromosomal aberrations (intellectual disabilities, multiple congenital anomalies, developmental delay)

Chromosomal aberrations are found in patients with ID, DD, and MCAs. It has long been assumed that a considerable proportion of these patients could have submicroscopic chromosomal imbalances, not detectable by routine karyotyping. Subtelomeric FISH studies have helped unveil nearly 5% of these patients [59, 62, 65, 66]. However, the introduction of CMA [1, 2], especially the genome-wide high-resolution analysis, has made it feasible to detect genomic DNA copy number alterations. In several recent studies as much as 15–24% segmental aneuploidies were detected in patients with idiopathic ID and dysmorphism [56, 57]. A few additional studies reported detection rates between 10–25% [67, 68]. A more recent and comprehensive analysis of 140 MCA/ID patients reported submicroscopic imbalances in 20% cases including deletions, duplications and unbalanced translocations [69]. Excluding subtelomeric imbalances, this study [69] identified 8% cases with clinically relevant interstitial submicroscopic imbalances. Taking into consideration the reported studies, CMA screening, with a resolution of at least 1 Mb, has been performed on 432 patients with MCA/ID. Most imbalances are nonrecurrent and are spread across the genome. In at least 8.8% of these patients, de novo intrachromosomal alterations have been identified. Menten et al. [69] also illustrated that CMA may detect segmental chromosomal aneuploidies as well as low level mosaicism in as little as 7% of the cells, which may be overlooked in standard karyotyping. Screening of selected patients with normal karyotypes thus seems desirable and feasible. At present it remains unclear which will be the optimal resolution of the array to screen MCA/ID patients. Considering the large percentage of inherited chromosomal imbalances, establishing both benign copy number variations as well as developing a comprehensive morbid map of the human genome will be of major importance in order to understand which imbalances are causative.

Some carriers of apparently balanced de novo or inherited rearrangements present abnormal phenotypic features [70, 71]. The abnormal phenotype may be explained by the disruption of a gene, a positional effect or a cryptic genomic imbalance at the breakpoint or in another region of the genome [72–74]. High-resolution CGH has been successfully used to detect cryptic imbalances at the chromosomal breakpoints [75]. In the studies using CMA to detect chromosomal imbalances in patients with apparently balanced chromosome rearrangement and an abnormal phenotype, most anomalies detected were smaller than 3–5 Mb and would easily be missed by routine karyotyping. In a larger series of cases, unexpected complexity can be revealed, as observed by Gribble et al. [76] who found patients with rearrangements more complex than expected loss, gain or inversion at or near the translocation breakpoints, cases with chromosomal imbalance apparently unrelated to the translocation, and cases with no genomic alteration [76–80].

Critical region for specific diseases

Many recent studies support the usefulness of CMA in determining the critical region of specific diseases. Some initial investigations that made use of such an approach included the studies by Veltman et al. [81]. Deletions in the long arm of chromosome 18 have been implicated in congenital aural atresia (CAA) [82]. Using chromosome-specific CMA analysis in a series of 20 patients with CAA, Veltman et al. [81] found that a common genomic region of 5 Mb on chromosome 18q22.3-q23 was deleted in all patients and thus a critical region for CAA. In another study on cardio-facio-cutaneous (CFC) syndrome, Rauen et al. [83] uncovered that chromosomal region 12q21.2-q22 may be a possible critical region for CFC.

Search for candidate genes

Mendelian cytogenetics refers to the association between structural chromosomal anomalies and single gene disorders, either alone or in contiguous gene syndromes. However, the majority of genomic deletions or duplications remain below the detection limit of traditional karyotyping (5–10 Mb). In addition, the contribution of individual genes to disease may not always be apparent in patients with complex phenotypes due to cytogenetically visible alterations. It is becoming increasingly clear that many so-called microdeletion syndromes are largely or completely due to haploinsufficiency for single genes [84–86]. Microdeletions and microduplications occur at various frequencies in many monogenic diseases with a known genetic cause. Copy number changes of the gene have been refined by using microarray analysis in certain known deletion/duplication syndromes [87–89]. Genomic microarray can also be useful in examining complex rearrangements to unveil the critical regions/genes involved in specific diseases [78–80]. Such studies can also reveal some unanticipated results as was seen by Veltman et al., who found subtle chromosome imbalances in three patients with an inv(X) [77].

The availability of CMA for detecting small chromosomal deletions and duplications enhances the possibility of straightforward mapping of the genes underlying the disease. Localization of specific causative genes in pleiotropic disorders such as CHARGE association (*C*=coloboma, *H*=heart defects, *A*=atresia of the choanae, *R*=retardation of growth and development,

G=genital and urinary abnormalities, *E*=ear abnormalities and/or hearing loss. The diagnosis of CHARGE is based on finding several of these and possibly other features in a child) has been achieved by CMA studies [90]. However, one must consider the possibility that certain monogenic diseases may be caused simply by mutations and not by deletion/duplication of the gene; or that other monogenic diseases could be manifested by various alterations including deletions/duplications or mutations in the same gene. Furthermore, mutations, deletions and duplications of the same gene may cause diverse results in different diseases as is the case with *PMP22* gene duplication resulting in hereditary pressure palsies and mutation causing Charcot Marie-Tooth Type 1A [30]. These diseases may not be suitable for investigation by CMA.

Genotype phenotype correlations

In order to confer a certain genetic alteration within a particular chromosomal region to a specific phenotypic feature, details of genotypic alteration and phenotypic features of multiple patients with the same clinical syndrome and genetic defect need to be put together. However, it is necessary that the chromosomal change be described as precisely as possible. Genomic microarray is very useful in order to precisely map and measure the size of the aberration and has thus helped in establishing genotype-phenotype correlations. Many studies have now been performed focusing on chromosomal regions and whole chromosomes to delineate the genotype-phenotype correlations [18, 21, 22, 24, 52]. However, as described previously in this chapter, such an approach was originally taken to define the critical region for congenital aural atresia and also localize the gene within a 5-Mb critical region on 18q [52]. Subsequently, genotype-phenotype correlation studies were performed by Van Buggenhout et al. [21] to refine the phenotypic map of 4p deletion cases; by Bruder et al. [18] for chromosome 22q in Neurofibromatosis 2 (NF2) patients; by Yu et al. [22] for 1p36 deletion cases; by Shaw et al. [91] with chromosome 17p-specific array; by Sahoo et al. [92] for a detailed characterization of 15q11-q14 region which is frequently altered in Prader–Willi/Angelman syndromes and some autism spectrum diseases and also by various other investigators who have used CMA to characterize various chromosome anomalies [93–102]. As more and more studies attempt to precisely delineate the genetic alteration using CMA technology, more genotype-phenotype correlations will be evidenced. Comprehensive reviews of multiple detailed studies that have used CMA to precisely define a specific chromosomal abnormality for a particular syndrome will help in streamlining and associating a characteristic phenotypic feature with the genotype.

18.2.2 CMA for characterization of acquired genetic changes

When the aim of a study is discovery rather than diagnosis, the approach is much more open ended. Amplifications of oncogenes and deletion of tumor suppressor genes are hallmark events of progressive malignancies. Standard CGH technique has already made a significant impact on cancer cytogenetics as a powerful tool for detection of chromosomal copy-number aberrations, even in epithelial solid tumors where conventional cytogenetic techniques are pushed to elucidate tumor-specific genomic alterations. However, restricted resolution is the major constraint of the chromosomal CGH technique. Genomic microarray compensates for this limitation and allows for much higher resolution, revealing a detailed profile of tumors for the identification of genomic alterations. In addition, since the spotted DNAs in the CMA contain sequence information directly connected with the genome database, one can easily focus on the particular biological aspects of the genes that lie within regions involved in copy number changes; this in turn facilitates identification of genes responsible for the tumors investigated. Thus CMA analysis can be applied to both detection and mapping of amplified genes as well as homozygous deletions. Genomic arrays have been successfully used to detect segmental genomic amplifications and deletions in virtually all types of human tumors.

Using CMA technology, various approaches have been taken to determine the genetic imbalances in different types of cancers. Disease specific arrays have been constructed for cancer diagnostics. These arrays are enriched for the coverage of multiple cancer gene loci [103–109]. Analyses of tumor genomes by CMA have employed arrays focused on a particular region of the genome [110–112], selected regions known to be frequently aberrant in tumors [113–117] or genome-wide arrays [118–123]. In all cases, the enhanced resolution possible with CMA compared with chromosomal CGH has been demonstrated by the fact that CMA found copy number aberrations that were not seen using chromosomal CGH.

Chromosome region-specific arrays

Investigators have focused on chromosomal regions that are known to be altered in specific malignancies, including chronic lymphocytic leukemia, mantle cell lymphoma, and carcinomas, such as oral, pancreatic, lung and other cancers [103, 104, 106–109]. Target clones spanning a region often associated with the copy number changes in certain neoplasms have been specifically scrutinized using CMA, which yields greater details regarding the genes located in the target region.

To determine the target gene commonly amplified in early stages of lung and oral cancers, a specifically dense array was developed for 8q21-q24 region by Garnis et al. [108, 124, 125] which helped uncover the amplification of a novel gene, distinct from the MYC oncogene. Owing to limited resolution by standard CGH, the amplification had been previously thought to be the MYC oncogene located in the chromosomal region. This illustrated the need and the utility of high resolution CMA.

Chromosome-specific arrays

Comprehensive arrays for specific chromosomes and chromosome arms which are frequently altered in a variety of tumors have facilitated the fine mapping of the segmental genomic alterations in oral squamous cell carcinoma, small cell and non small cell lung carcinoma. Arrays with complete coverage of 1p, 3p, and 5p arms have been created and used to study different types of cancers [126–129]. A chromosome 20 array has been used to investigate breast tumors [2], which suggested that multiple regions of the chromosome were involved and that a high resolution, high density array helps reveal much more than has been previously appreciated in cancer chromosome studies. An array for chromosome 22, which has some repeat-rich regions, was created using technical precision for accurate assessment of the genome region that is repeat rich or contains duplication [130].

Genome-wide arrays

Regional and chromosomal microarrays require a prior knowledge of these regions of interest and are thus biased to specific areas. Pollack et al. [12] developed the first array-based genome-wide profiling technique using cDNA target clones for human cancer genomes. The technical complexities in the resolving power of the cDNA arrays limit its utility. Also, only exonic regions of the genome are covered and hence alterations in promoter regions and other protein binding sites are undetectable [16]. Genome-wide arrays using BAC and P1 clones were first constructed by Snijders et al. [3] demonstrating the feasibility and accuracy of these types of arrays. Following this study, many other megabase interval arrays have been widely used [10, 105]. Oligonucleotide arrays are now popular [131, 132]; however, cross hybridization is an issue. To improve hybridization, kinetics whole-genome sampling assay was used by Kennedy et al. [133]; a 100-k SNP array [134], which will also allow for LOH (**l**oss **o**f heterozygosity) information, is another step forward in the array technology and will greatly improve our understanding of carcinogenesis. Another representative oligonucleotide microarray analysis (ROMA) method [27] aims to determine genomic alterations at 30 kb resolution. These technologies may not be best suited for archival specimens since PCR amplification of selected DNA also introduces noise into the assay. Brennan et al. [135] have labeled total genomic DNA and used it to detect single copy alterations on an oligonucleotide array without genomic reduction step. Marker based genome-wide assays have been used to identify somatic genetic changes in tumors as well as large scale copy number variations in human populations [136, 137]. Since marker-based whole genome microarrays have large gaps due to the distance between target probes, Ishkanian and colleagues [26] developed a sub-megabase resolution tiling path array. By using overlapping clones, the resolution of the array has increased beyond the size of a single BAC clone and gains and losses as small as 40–80 kb are detectable. These types of arrays have been used for lung tumor genome investigations [45]. Bertone et al. [138] have created a tiling path array using nonrepetitive fractions of the human genome. As CMAs are being created with higher resolutions, they have been used to redefine the genomic alterations that have not been identified with lower resolution arrays. The multiple investigations performed in mantle cell lymphomas, lung, head and neck, breast, and prostate cancers have illustrated the need for CMAs with higher resolution to discover the genes involved in disease initiation as well as progression. High-density, submegabase arrays can reveal nonrandom chromosome copy-number aberrations that are masked under complex tumor karyotypes and are responsible for neoplastic transformation. As CMA continues to increase resolution, cancer genes will continue to become easier to discover. Chromosomal regions discovered by these arrays can be used to create smaller, higher resolution diagnostic arrays, thus making it practical for diagnosis of cancer or genetic diseases in the clinical setting.

Overall studies

A number of solid tumor investigations have taken advantage of the higher resolution afforded by CMA, including breast cancer [118, 139–141]; glioblastoma [142]; rhabdomyosarcoma [143]; nasopharyngeal carcinoma [144]; ovarian cancer [145]; gastric cancer [146, 147]; bladder tumors [11]; lymphoma [110, 148–150]; sarcomas and adenocortical tumors [115, 151]; head and neck cancers [152]; hepatocellular carcinoma [153, 154]; and neuroblastoma [155–158].

Cancer progression and clinical translation

The CMA technology has also been used to understand the genetic changes that correspond to disease progression from an indolent to an aggressive disease, as evidenced by investigations among follicular lymphoma cases that transformed to more aggressive diffuse large cell lymphomas [159]. Other studies have focused on using the CMA to delineate the genetic changes taking place during the pre-malignant to malignant conditions in gastric carcinoma, breast cancers and lung cancers [122, 129, 160].

The correlation of cytogenetic aberrations with clinical outcome has already been proven in many hematological malignancies and some solid tumor cancers. As a substitute for a panel of FISH assays, some investigators have used CMA in mantle cell lymphoma as well as chronic lymphocytic leukemia and have demonstrated the potential in adapting CMA for clinical correlations [106, 109, 148].

18.2.3 Use of CMA in prenatal diagnostics

Cytogenetic analysis has been the gold standard for prenatal diagnosis since the development of chromosome banding techniques in the late 1960s. Although highly reliable, this procedure has a number of limitations including lower resolution, requirement of cell culture and delay in results. Lately a possibility of using CMA for prenatal diagnosis has been in consideration [161–168]. Chromosomal microarray analysis of fetuses with multiple malformations identified genomic rearrangements in around 16% of cases, which had not been observed by karyotype analysis [169]. In a study of products of conception from spontaneous miscarriages using a low density array containing targeted clones of clinical significance, CMA was able to detect all abnormalities previously identified by karyotype analysis, and detected additional abnormalities in approximately 10% of cases [170]. A recent study specifically designed to detect aneuploidy made use of a Klinefelter male cell line as a control for CMA analyses on products of conception [171]. This approach facilitated the detection of common trisomies and monosomies of the sex chromosomes and ploidy by refining the analysis to the identification of single copy gains or losses. Furthermore, in a blinded study, careful interpretation of the microarray results with particular attention to the sex chromosome ratios between the patient sample and the control allowed for the detection of some common triploidies [172]. Attempts have also been made towards determining the use of array in pre-implantation aneuploidy detection [173]. The technique therefore holds some promise of combining the speed, sensitivity, and potential for partial automation of a DNA based test, with the genome screening characteristics of microscopic karyotyping [174]. Although CMA may address many of the limitations of both conventional microscopic cytogenetic analyses, and has the potential to be used for prenatal diagnosis, careful consideration of many issues need to be addressed before implementing CMA for general clinical use in prenatal diagnosis.

18.2.4 CMA in determination of genomic variations and polymorphisms

The study of human genetic variation at the DNA level constitutes a major challenge and has received considerable attention in the post-genomic era. The dominating type of variation explored so far in the genome has been single nucleotide polymorphisms (SNPs), overshadowing the issue of benign copy number variations (CNVs), also termed as copy-number polymorphisms (CNPs), or large-copy variation (LCVs) [175]. A fundamental step towards identifying benign CNVs was the development of CMA [1, 2]. DNA copy-number variation was previously observed in the human genome, and their importance in health and disease was ascertained by independent comparison studies in cell lines, diseased individuals, and normal human population [176–183]. Interestingly, some benign CNVs provide a protective effect against disease in “normal” (or unaffected) individuals [184]. Thus the hidden functionality of such normal variation only becomes apparent when challenged by environmental factors. Recently, two landmark studies reported genome-wide analyses of benign CNVs in normal individuals and represent the beginning of an understanding of this type of large-scale variation [136, 137]. In these reports, Sebat et al. [137] and Iafrate et al. [136] identified 76 and 255 loci, respectively, that display benign CNVs in the human genome. Both studies demonstrate the presence of genomic imbalances among normal individuals, which overlap with genes, often coincide with segmental duplications in the genome and can contribute to phenotypic variation and disease susceptibility. Strikingly, the most common variations observed by each study were at distinct loci. The differences can be due to restricted sample size of the studies and also due to the different array platforms used in both investigations. Iafrate et al. [136] used commercially available BAC arrays, whereas Sebat et al. [137] developed and applied a custom-made oligonucleotide array for the assessment of benign CNV. Future CMA-based benign CNV analyses should include standard criteria on a common microarray platform. Since the human genome contains many gene families and duplications that can promote insertion/deletion rearrangements [185–187], many more benign germline CNVs are likely to be revealed by widespread application of CMA.

Thus, when interpreting CMA data, it is important to bear in mind that certain copy-number changes may be polymorphisms that do not affect phenotype and that some clones may be sensitive to copy-number changes occurring at more than one location in the genome [46]. Given the frequency and significance of the benign CNVs, it is important to include and address the variation within the normal population, even in CMA studies that focus on tumor-associated gains or deletions (indicating the presence of activated oncogenes or inactivated tumor-suppressor genes). As suggested by Buckley et al. [188] in a recent review on genetic polymorphisms, it is only when parallel analyses of benign CNVs and SNPs are performed in an integrated format and their phenotypic effects are understood that we will obtain a global picture of our genetic diversity.

18.2.5 Evolutionary characterization with CMA

Genomic microarray technology has been employed for use in interspecies comparisons. In two recent studies, human genome has been compared with great apes. Several common and unique features of the human genome have been noted. A comparison of five hominoid species (human, bonobo, chimpanzee, gorilla, and orangutan) using a cDNA CMA approach led to the identification of 4800 genes that gave genetic signatures unique to a specific hominoid lineage [189]. Moreover, there was a more pronounced difference between copy-number increases and decreases in humans and a number of genes amplified are thought to be involved in the structure and function of the brain. In another investigation which compared the human genome against four great ape genomes by using an LIC (“large insert clones”) array of 2460 BACs, 63 sites of DNA copy-number variation between the human and great apes were identified. A significant number of these sites existed in interstitial euchromatin [190]. These studies illustrate the potential use of CMA in interspecies comparisons.

18.2.6 Limitations

Coverage and resolution of CMA are dependent on the design and density of the array used. Although superficially appealing, an array covering the entire genome at very high resolution would have potential disadvantages in clinical use; more array probes are likely to generate a higher number of false positives. Large arrays are more expensive to fabricate, quality control, and interrogate. Large and very high-resolution arrays are likely to generate data that may be difficult to interpret. Alterations in regions of the genome that do not have established clinical relevance will be burdensome to the clinical cytogeneticists for useful interpretation.

Low-level mosaicism, balanced chromosomal translocations, inversions, and whole-genome ploidy changes may not be detected by CMA studies. The technique, therefore, should be used in conjunction with other cytogenetic techniques that are capable of determining these aberrations.

Before applying CMA in a diagnostic setting, a better knowledge of genomic polymorphisms and variations present in general populations is required. An online update on genetic variations is available at the Database of Genomic Variants, which was first developed from the results generated by Iafrate et al. [136] and is being continually updated with published results from other investigators [191–195]. The database is hosted by the Center for Applied Genomics and is available for review online [196]. The database also includes recent results for deletion polymorphisms that may have an important role in the genetics of complex traits. Deletion polymorphisms are not directly observed in most current gene mapping studies. A new method that uses SNP genotype data from parent-offspring trios to identify polymorphic deletions has been applied to data from the International HapMap Project to produce the first high-resolution population survey of deletion polymorphism [197, 198].

The DECIPHER database of submicroscopic chromosomal imbalance, initiated by the Sanger Institute, provides a list of polymorphic clones. DECIPHER, which is an acronym for DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources, allows the investigator to visualize the chromosomal location of clones which are found to be deleted or duplicated in a CMA analysis and to see whether they coincide with a recognized polymorphism or known micro-deletion/duplication syndrome. In addition, it permits the user to visualize known genes and putative genes as well as expressed-sequence tags (ESTs) within the deleted/duplicated region. All details are available on the website [199].

Other links for various polymorphism studies are:

HapMap Project [200]: a multi-country effort to identify and catalog genetic similarities and differences in human beings.
dbRIP (200): a database of human Retrotransposon Insertion Polymorphisms (RIPs).

Human Structural Variation Database [202]: a catalogue of human genomic polymorphisms ascertained by experimental and computational analyses.

Chromosome Anomaly Collection [203]: a collection that contains examples of unbalanced chromosome abnormalities (UBCAs) without phenotypic effect. In addition to sequence copy-number polymorphisms, euchromatic variants have been described. All large microscopically visible genomic variations of chromosomal regions as well as subtelomeric polymorphisms that have been previously reported and reviewed by Barber [204] are included in this collection.

18.3 Genomic microarray in a cytogenetics laboratory

The identification of unbalanced numeric and structural chromosome abnormalities can be achieved by CMA studies. This technique will not detect balanced rearrangements and has limitations in detecting low-level mosaicism for unbalanced numeric or structural rearrangements. Each laboratory must evaluate and validate the performance of the CMA method used in their laboratory for detection of mosaicism.

18.3.1 General considerations

The capacity of CMA in detecting microscopic and submicroscopic chromosomal aberrations is dependent on the resolution, which is limited by the size of the cloned DNA targets and the distance between those sequences naturally located on the chromosome. Genomic microarray should be part of an algorithm integrating all methods presently available for cytogenetic studies, and the results should be interpreted in the context of other clinical and laboratory findings. A normal microarray result does not exclude mutations in any gene represented on the microarray. Therefore, additional testing may be appropriate for certain syndromes or conditions when the microarray analysis is normal. It is at the discretion of the laboratory director whether microarray analysis is appropriate for any particular patient and whether additional testing (chromosome analysis, FISH, molecular testing) is necessary or appropriate. Analytical variables and turn-around times should be determined by the laboratory and should be based on the indication for referral.

All gains and losses identified on the CMA should be confirmed by FISH or other molecular studies. Parental FISH or microarray studies may also be indicated when an abnormality is identified by CMA. In some cases, the parental testing is performed to exclude a balanced parental rearrangement, or to exclude a parent with a deletion/duplication and subclinical presentation. In other cases, gains or losses may be detected by CMA in regions of the genome with unclear clinical significance. In these cases, parental studies may be indicated to distinguish between a familial variant and population polymorphism or a de novo, clinically relevant alteration. The laboratory director and staff should be familiar with polymorphisms and should interpret the microarray analysis in this context. Nonpaternity may be considered as a possibility in cases of apparent de novo alterations.

Monitoring pre-analytical variables, analytical variables, and post-analytical variables should be part of the laboratory's quality assurance (QA) and quality improvement (QI) programs. Such variables may include quality of the specimen received, number of hybridization failures, and number of cases requiring parental studies.

18.3.2 Specimens

Genomic microarray may be performed after DNA extraction of any specimen that yields DNA (peripheral blood, cord blood, skin fibroblasts, amniotic fluid or cultured amniocytes, chorionic villus specimens). The laboratory should establish the specimen requirements for CMA for each tissue type and the minimum DNA requirements to perform the test. Considerations include whether the test is performed once per specimen or performed twice in a "dye-swap" or "dye-reversal" strategy. The use of the dye-reversal strategy allows the laboratory two opportunities to observe and compare the patient results to ensure an accurate diagnosis. During the processing of the patient DNA specimen (extraction and labeling), the laboratory must establish their methods for DNA quantification (fluorometer, spectrophotometer), methods to ensure good-quality DNA (examination by gel electrophoresis), methods to ensure proper fragmentation (via sonication or digestion) prior to fluorescent labeling (examination by gel electrophoresis), and methods to ensure adequate labeling (gel electrophoresis, visual inspection, UV/visible spectroscopy).

In CMA, the patient DNA is compared to a control sample. It is recommended that the laboratory establish both male and female control samples to be used for comparisons. Appropriate male and female controls with known karyotypes should be established in the laboratory. In most instances, controls with normal karyotypes are used. However, there may be instances when a particular abnormal karyotype might be used if sex chromosome or ploidy aberrations are suspected.

18.3.3 Types of microarray used in the clinical laboratory

Genomic microarrays are used to detect DNA copy-number changes. They typically consist of pieces of DNA spotted onto a solid support (e.g., glass microscope slide). The microarray targets may include large insert clones (BACs, YACs, PACs), smaller insert clones (cosmids, fosmids, plasmids), or smaller DNA segments (oligonucleotides, cDNA). More recently, many laboratories use microarray platforms which include SNP probes. These arrays consist of nonpolymorphic copy-number probes (CNPs) for assessing both genotype and copy-number changes. The SNP array technology allows the detection of both copy-number changes and copy neutral changes, thus allowing for the detection of uniparental disomy. The SNP array technology has not been discussed in detail in this section.

Different types of microarrays may be used for diagnosis of chromosome abnormalities in a clinical laboratory: (1) those that are FDA approved and available through a commercial source; (2) those that are developed by the laboratory (home-brewed) with the intention of using them as a clinical device; and (3) IVD (in vitro device) microarrays which are commercially available and used in separate diagnostic laboratories. All microarrays must be validated by the testing laboratory to assure that positive and negative results can be confirmed by some alternative method. Home-brew and IVD microarrays should be subjected to the same rigor of validation. Each clone or region on the microarray should be validated using a reference DNA with a known abnormality, when/if available. Each clone should be printed multiple times on the microarray (minimum of 2–4) and in more than one location on the microarray to increase test reliability and reduce noninformative or false positive/negative results due to technical artifact. The array should contain a set of normalization clones for which a normal ratio is established after each CMA experiment.

18.3.4 Microarray data analysis

The laboratory's procedure manual should describe the statistical methods to assess the ratios of control DNA specimen to patient DNA specimen. The ratios can be averaged between multiple spots of the same clone. Normalization clones should be used to establish the baseline ratio of control DNA specimen to the patient DNA specimen prior to the analysis of the diagnostic clones contained on the device. In SNP array analysis a concurrent control DNA is not used. The patient DNA fluorochrome intensity is compared with reference spotted DNA and the ratio intensity is measured to determine both the copy-number changes and single nucleotide polymorphisms. The laboratory must establish the normal and abnormal values (deletion, duplication, and triplication) for each clone on the microarray using normal control DNA specimens for validation. The abnormal ranges should be tested using known DNA from chromosomally abnormal individuals or cell lines who carry aberrations for representative deletions, duplications, and anticipated aneuploidies. Array data are often expressed as numerical values or graphically represented.

18.3.5 Aspects specific to the validation of home-brew microarrays

It is very important that the clones used for the array be selected from the available public databases and obtained through a public or private source. Clones should be verified that they are the correct clone through end-sequencing, FISH localization or some other comparable method, and this information should be documented within the laboratory quality control plan. Clones not mapping to the proper/intended locus should be discarded, as should clones showing cross hybridization and multiple hybridization to various sites and multiple chromosomes, since neither is suitable for diagnostics. FISH verification allows for the assessment of cross hybridization, in addition to correct localization, and also provides validation that the clone is a known, chromosomally abnormal cell line.

18.3.6 Validation of FDA-approved commercial, IVD commercial, or home-brew microarrays

Each new lot of a device should be validated on two chromosomally normal control individuals (one may be the control sample) to identify problems in the printing (poor spots), problems in reagents (poor DNA quality, suboptimal reagents), or problems in the analysis. Each new lot of a device should have documentation that printing of each clone was successful (visualization of spots using a DNA-specific stain).

For home-brew microarrays, validation of each clone is achieved through end-sequencing or FISH. The laboratory director and staff should be aware of any discrepancies in array data that might represent an incorrect placement of a clone on a commercial device. Discrepancies can be addressed through FISH with the discrepant clone to metaphase cells of the patient showing the potentially anomalous result. Further, all probes destined for microarray should work equally well under the same

experimental FISH conditions, because the microarray is essentially a simultaneous FISH experiment with several hundred or thousand probes. When using a new or different type of microarray in the laboratory, a minimum of 5 different chromosomally abnormal specimens should be tested. In addition, the laboratory should make an effort to exchange samples with another reference laboratory providing microarray testing.

The laboratory should establish internal and external proficiency testing for CMA studies. The proficiency testing includes examination of normal and abnormal cell lines for CMA as part of the quality assurance program and ongoing quality improvement program. Known normal and abnormal DNA specimens should be exchanged with another reference laboratory performing microarray testing. Alternatively, DNA specimens could be received from an outside cytogenetics laboratory and correlation between cytogenetic and microarray results may be sufficient to provide on-going proficiency. These samples should include a number of normal and abnormal specimens. Specimens should be de-identified prior to submission to the testing laboratory. Alternatively, a peer-reviewed proficiency-testing program for CMA, such as that anticipated through the College of American Pathology/American College of Medical Genetics Cytogenetics Resource Committee should be subscribed to, when available.

18.3.7 Confirmation of abnormal CMA results

The laboratory should establish a protocol that allows for confirmation of abnormal or ambiguous CMA results. This may include cytogenetic analysis using banding techniques, FISH, high density arrays, PCR, or other comparable methods. Most cytogenetic laboratories may find the use of FISH, with the abnormal clones identified from CMA, as the method of choice. The laboratory director must ensure that confirmation can be established and, if by FISH, the clones are available to the laboratory in a timely manner to maintain established turn-around times.

Parental studies may be indicated after the identification of some chromosome abnormalities. The laboratory director must evaluate the CMA results and confirmatory test and determine the best method for parental studies (chromosome analysis, FISH, or CMA).

18.3.8 Genomic polymorphisms and variations

The laboratory must have a procedure to determine whether any “abnormal” clones represent “polymorphic” sites, or known regions of large benign CNV within the genome. This may include documentation of established polymorphic databases from the laboratory and/or other institutions. It is important that all abnormal clones be characterized by FISH, parental studies, or one of the methods noted above, and that the laboratory director is familiar with the rapidly changing literature on the location and frequency of these polymorphic sites. Reporting any polymorphic or questionable polymorphic clones should include a comment noting that polymorphic sites exist, and interpretation of clinical significance should be made with caution.

18.3.9 Reporting CMA results

The laboratory must determine normal and abnormal ranges for reporting CMA results. The CMA results must be expressed using ISCN-approved nomenclature (ISCN 2016) [205]. Any confirmation studies should be included in the report. Recommendations for parental testing and genetic counseling should be provided in the report. When the CMA results are normal, the laboratory director may suggest further testing, including a routine karyotype if not performed previously, when appropriate. Information about the microarray being used (company name, number of clones, number of loci represented, and lot number) should be included in the report. Additionally, the report should provide the limitations of the test in a disclaimer.

18.3.10 Examples

The algorithm for CMA testing used in our laboratory is provided in Figure 18.2. Figures 18.3–15 illustrate different cases that were studied using CMA technology in our laboratory. The figure legends describe the clinical indications and order in which various cytogenetic techniques were applied in each case. Genomic microarray was performed either concurrently or as part of an algorithm with other conventional cytogenetics and FISH investigations in each of the cases. These examples demonstrate that the incorporation of CMA in cytogenetics laboratories serves as a powerful supplement to existing cytogenetic and molecular cytogenetic methodologies and increases the detection of clinically relevant abnormalities, thus providing an improved diagnostic potential.

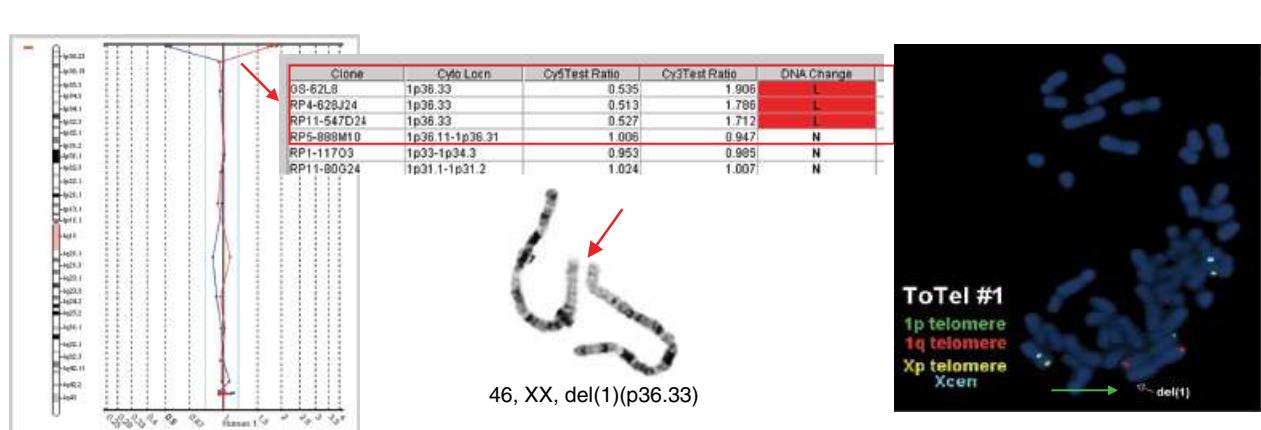
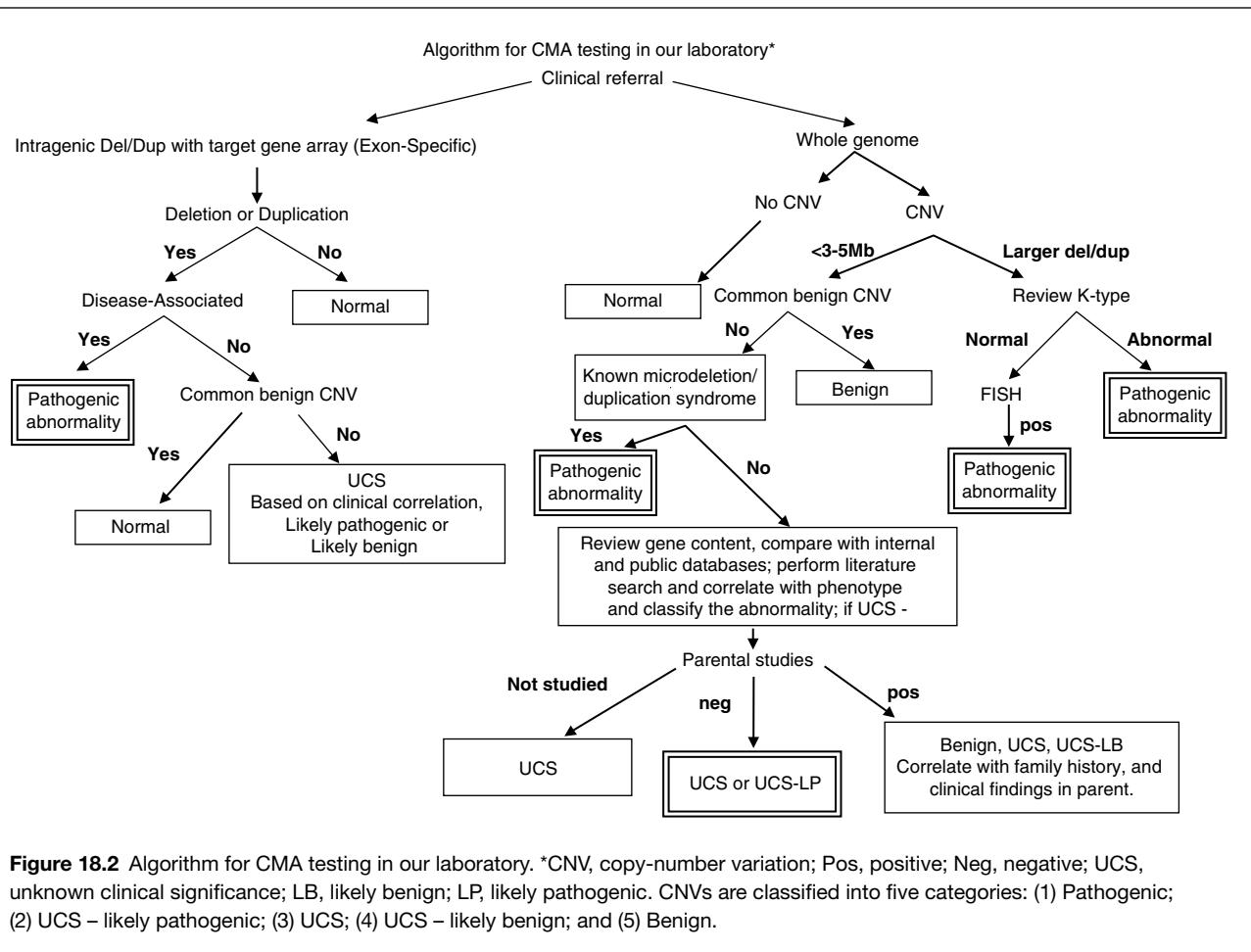


Figure 18.3 Case study 1. An 18-month-old female with developmental delay was referred for high resolution chromosomes, chromosome 15 methylation studies, and CMA. A terminal deletion of the distal light-staining region at 1p36.33 was first observed in CMA investigation and then in focused high resolution G-band studies. Results were further confirmed by 1p subtelomere FISH studies. See insert for color representation of this figure.

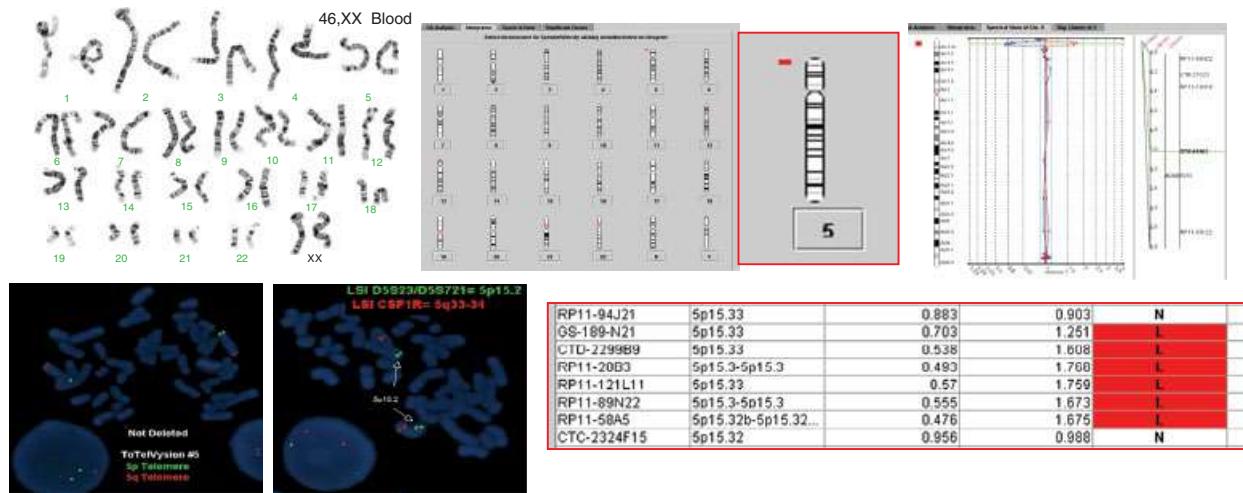


Figure 18.4 Case study 2. A four-year-old female with dysmorphic facial features, microcephaly, failure to thrive, and speech delay had normal high resolution chromosome studies and negative FISH results for 22q11.2 and 7q11.23 microdeletions. Subsequent CMA investigation using a targeted array with 606 clones/loci representing microdeletion and subtelomeric regions revealed an interstitial deletion involving six clones in 5p15.3. FISH studies for subtelomeres and other flanking regions confirmed the interstitial deletion. Follow-up FISH with a home-brew locus specific probe (not shown in picture) also confirmed the deletion. See insert for color representation of this figure.

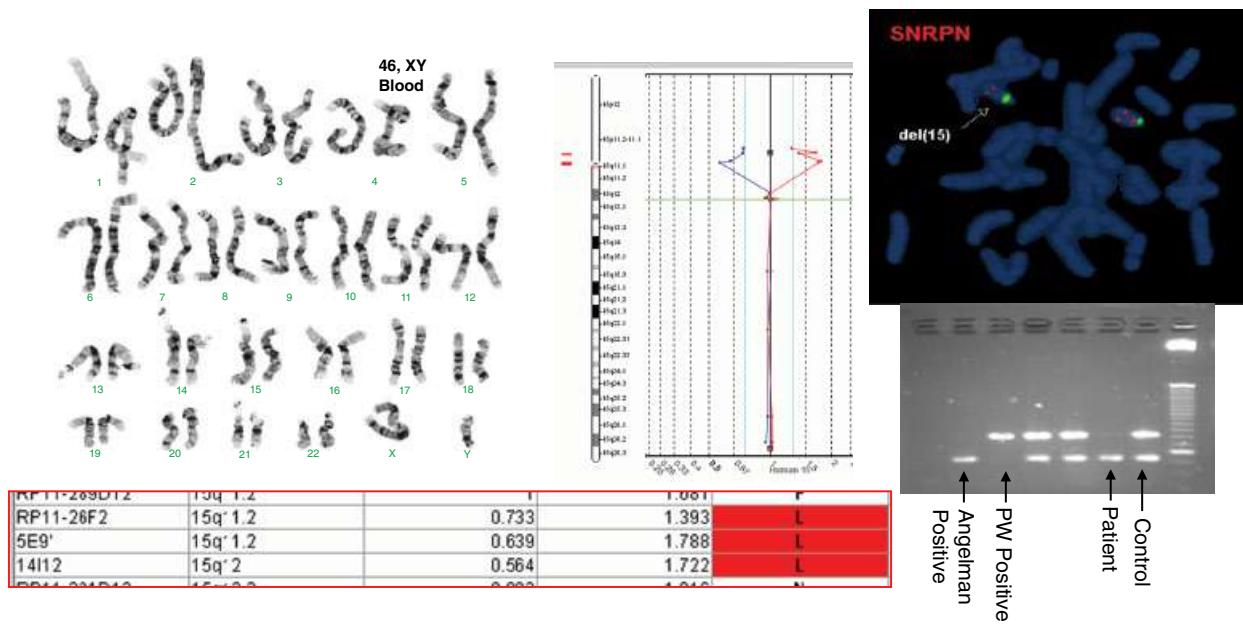


Figure 18.5 Case study 3. A 3-month-old male with respiratory difficulties, failure to thrive, and dysmorphic features, including relative microcephaly and macroglossia, was referred for high resolution chromosome and concurrent CMA studies. The karyotype was normal; however, an interstitial deletion within the Prader-Willi/Angelman (PWS/AS) region in 15q11.2-q12 was noted in CMA. The deletion was confirmed using a micro-deletion locus specific FISH probe for the SNRPN gene region. Methylation studies determined that the deletion was maternally derived, thus confirming a diagnosis of Angelman syndrome at three months of age. See insert for color representation of this figure.

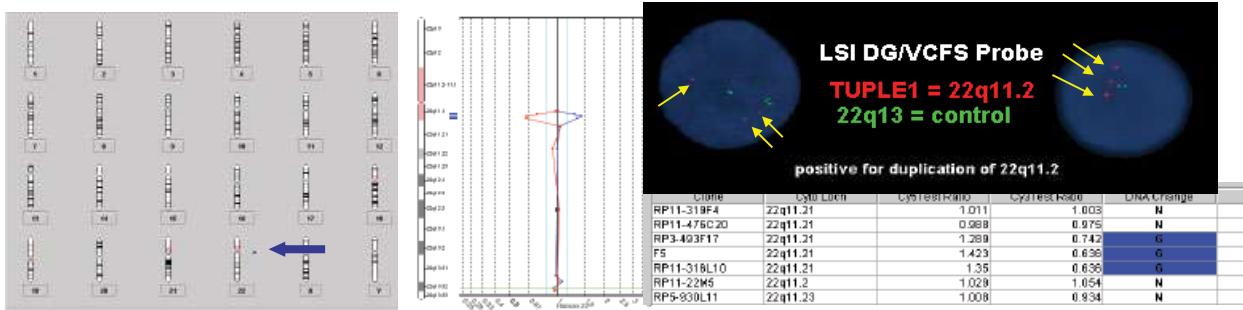


Figure 18.6 Case study 4. A 6-year-old female with global developmental delay and muscle spasms had normal cytogenetic findings and negative metaphase FISH results for microdeletion of 22q11.2 region. CMA investigation revealed a gain of three clones within the 22q11.2 region. FISH studies on interphase cells confirmed a duplication of this commonly deleted region. See insert for color representation of this figure.

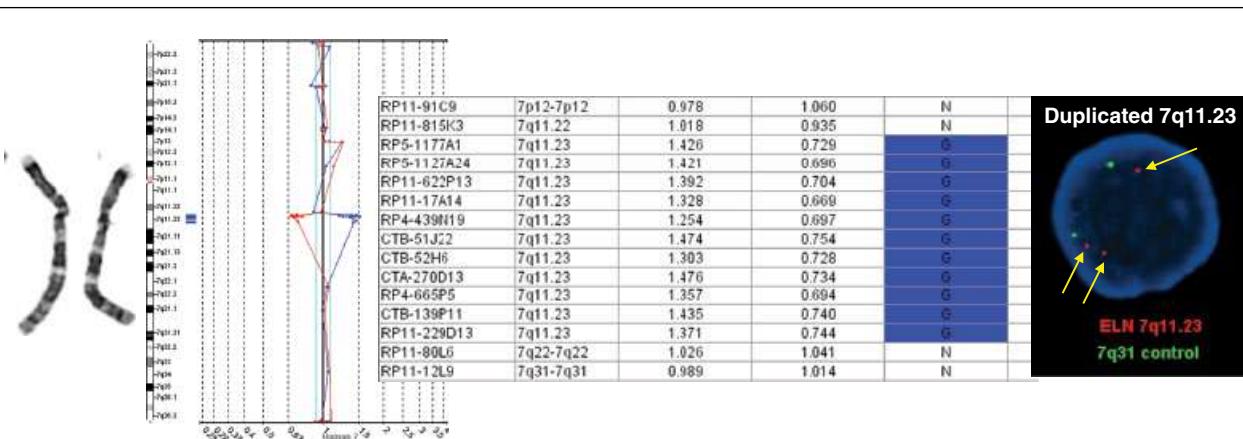


Figure 18.7 Case study 5. High-resolution chromosomes, chromosome 15 methylation studies, and CMA investigations were requested by a pediatric neurologist for a six-year-old male with central hypotonia, developmental delay, and speech delay. Results revealed a normal 46,XY karyotype, normal results on methylation studies for the Prader-Willi/Angelman region, and a gain of 11 clones by CMA within the 7q11.23 region, which is typically deleted in Elastin-Williams syndrome. Interphase FISH studies with the ELN probe, which targets the gene that codes for elastin production, exhibited three copies of the ELN region and confirmed duplication of this region. See insert for color representation of this figure.

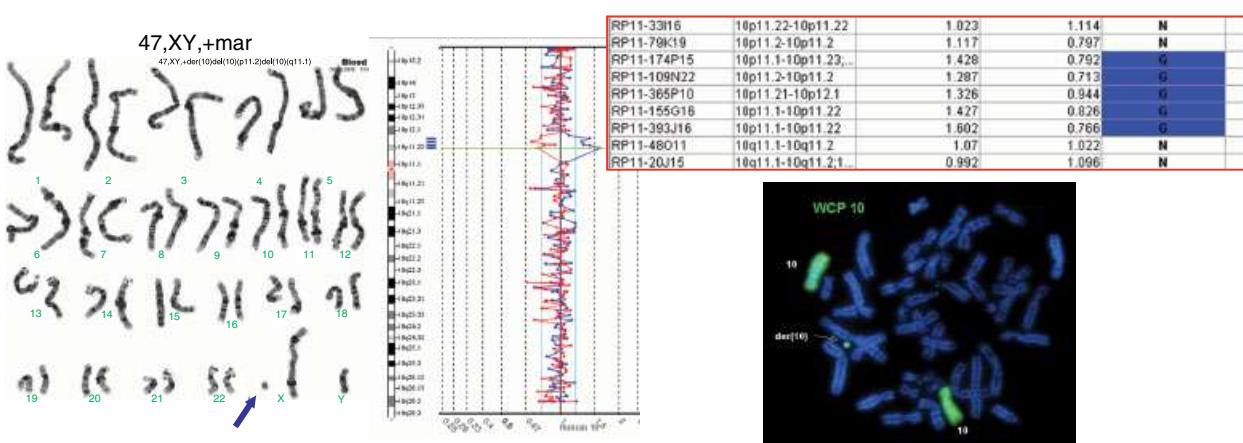


Figure 18.8 Case study 6. Complex congenital heart disease, dysmorphic facial features, and renal failure were the referral reasons for cytogenetic studies in a 15-day-old male. High resolution chromosome studies revealed a small marker chromosome which could not be accurately identified by classic G-band studies, and the karyotype was defined as 47,XY,+mar. Subsequent CMA investigation was performed which revealed a gain of five clones within 10p11.2 to 10p12 region. Follow-up confirmatory FISH studies with whole chromosome painting probes helped define the marker chromosome. The marker was redefined as der(10)del(10)(p11.2)del(10)(q11.1). Thus, CMA technology curtailed multiple FISH hybridizations, allowed for rapid and efficient characterization of this supernumerary marker chromosome, and detected the specific chromosomal region that defined the marker chromosome. See insert for color representation of this figure.

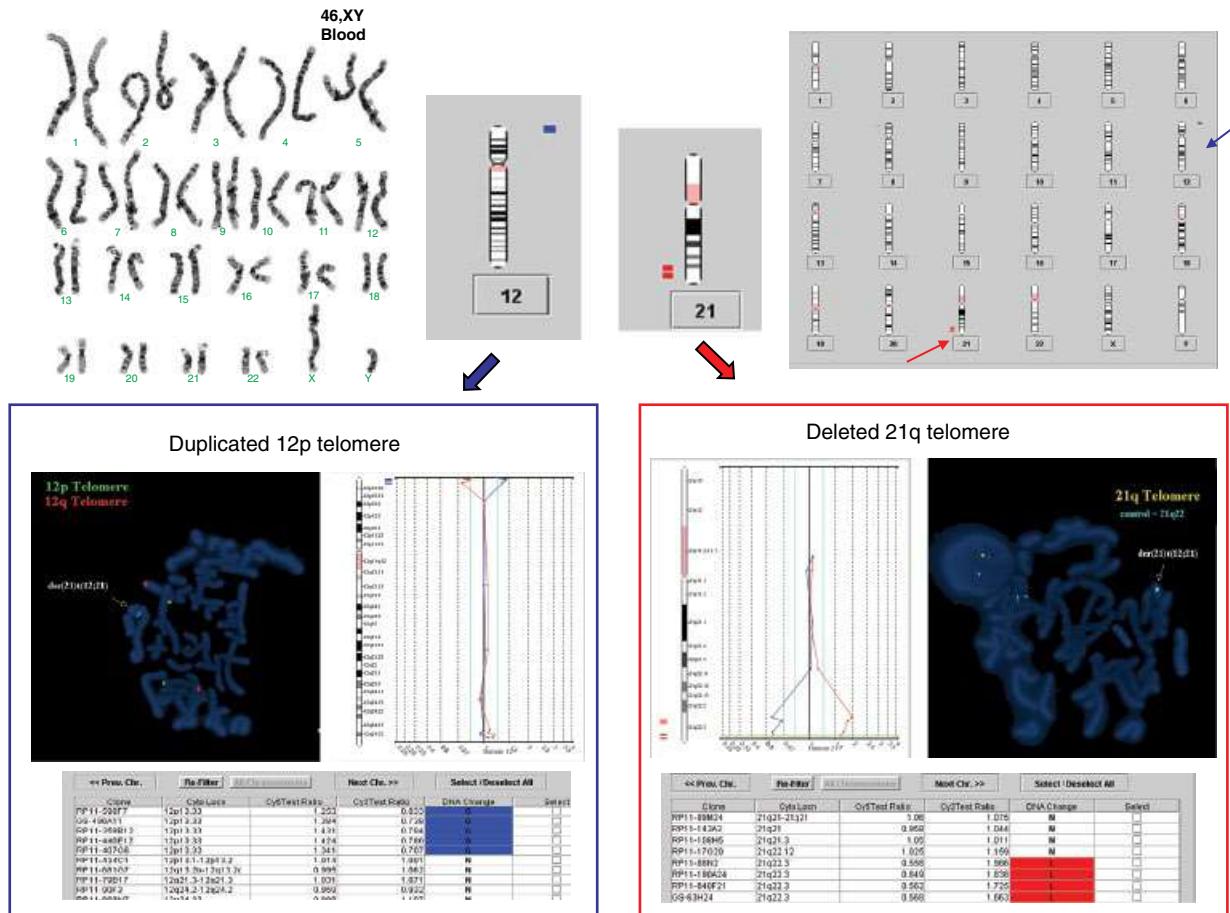


Figure 18.9 Case study 7. A 2-month-old male with multiple congenital anomalies was referred for high resolution chromosome studies and CMA. Conventional G-band studies revealed a normal 46,XY karyotype; however, CMA showed gain of five clones within 12p13.33 and loss of four clones within 21q22.3 regions. Subsequent FISH studies with subtelomere probes for 12p, 12q, and 21q confirmed the terminal duplication of 12p and deletion of 21q. The karyotype was redefined as 46,XY,der(21)t(12;21) (p13.33;q22.3). See insert for color representation of this figure.

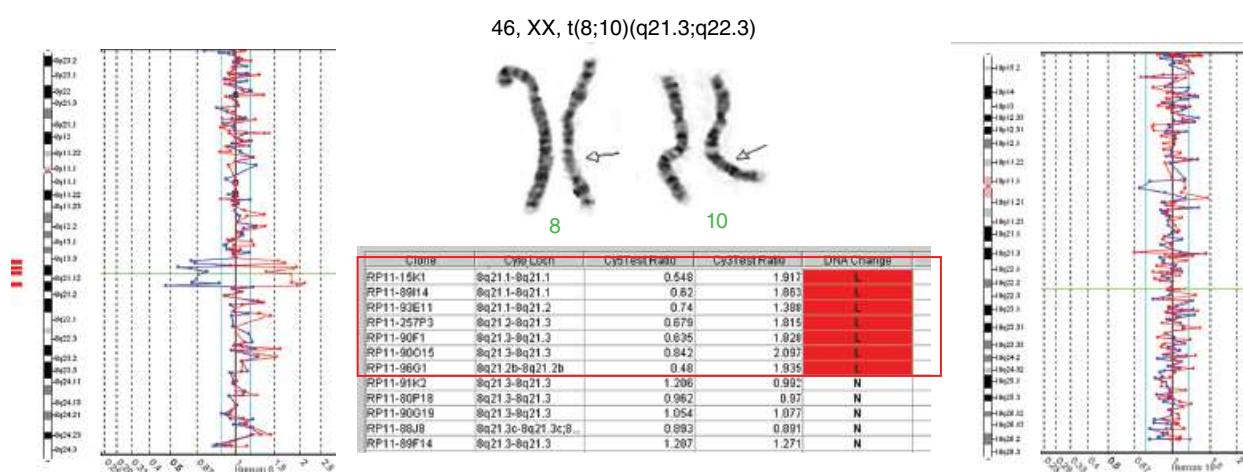


Figure 18.10 Case study 8. High-density CMA is used to interrogate regions not represented on lower density arrays and to further delineate or confirm abnormalities observed in lower density genomic arrays. A 2-year-old female with multiple congenital anomalies was referred for high resolution chromosome studies. Cytogenetic analysis revealed a balanced translocation with a karyotype: 46,XX,t(8;10)(q21.3;q22.3). Subsequent CMA studies with a high density array at 1 Mb resolution revealed a deletion of seven clones between the 8q21.1 to 8q21.3 regions. See insert for color representation of this figure.

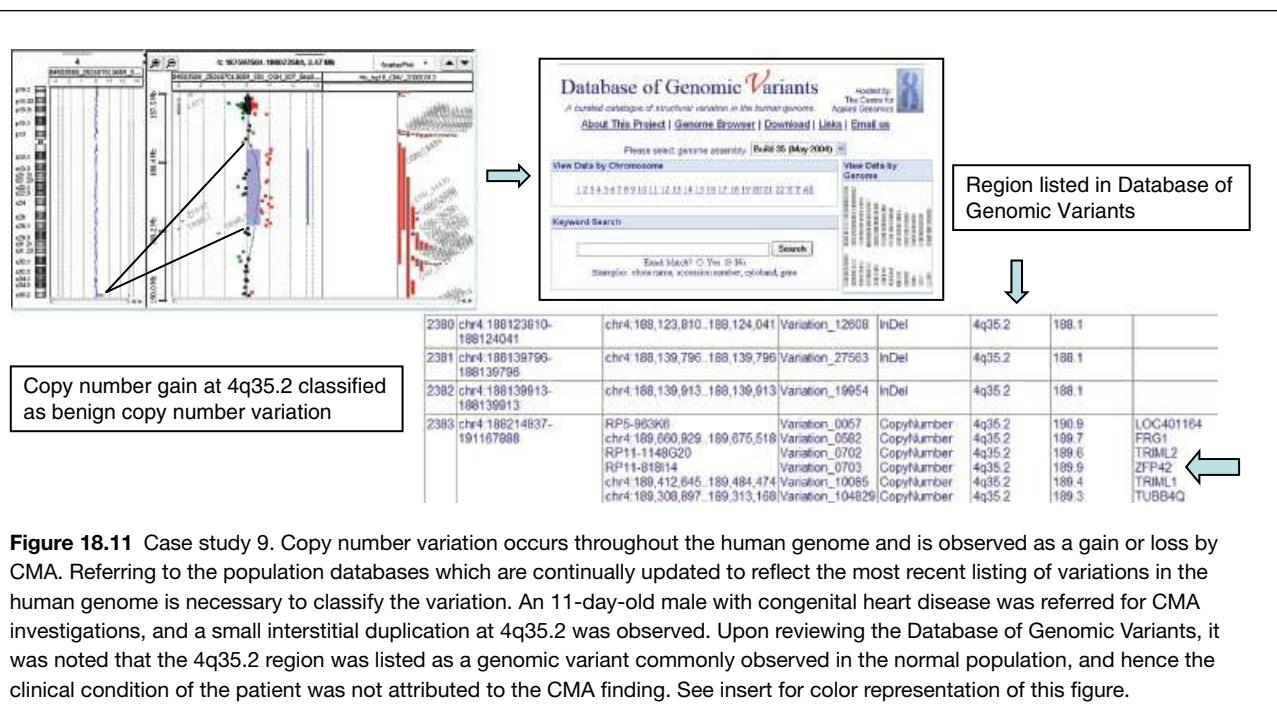


Figure 18.11 Case study 9. Copy number variation occurs throughout the human genome and is observed as a gain or loss by CMA. Referring to the population databases which are continually updated to reflect the most recent listing of variations in the human genome is necessary to classify the variation. An 11-day-old male with congenital heart disease was referred for CMA investigations, and a small interstitial duplication at 4q35.2 was observed. Upon reviewing the Database of Genomic Variants, it was noted that the 4q35.2 region was listed as a genomic variant commonly observed in the normal population, and hence the clinical condition of the patient was not attributed to the CMA finding. See insert for color representation of this figure.

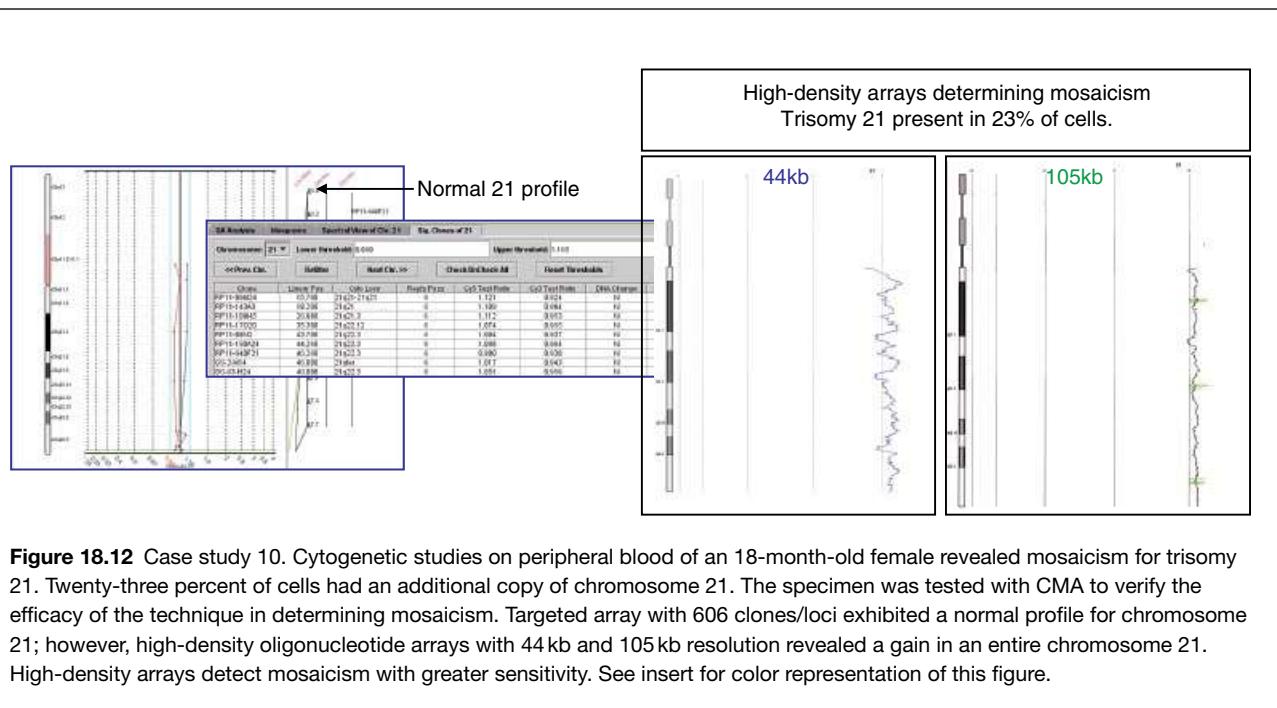


Figure 18.12 Case study 10. Cytogenetic studies on peripheral blood of an 18-month-old female revealed mosaicism for trisomy 21. Twenty-three percent of cells had an additional copy of chromosome 21. The specimen was tested with CMA to verify the efficacy of the technique in determining mosaicism. Targeted array with 606 clones/loci exhibited a normal profile for chromosome 21; however, high-density oligonucleotide arrays with 44 kb and 105 kb resolution revealed a gain in an entire chromosome 21. High-density arrays detect mosaicism with greater sensitivity. See insert for color representation of this figure.

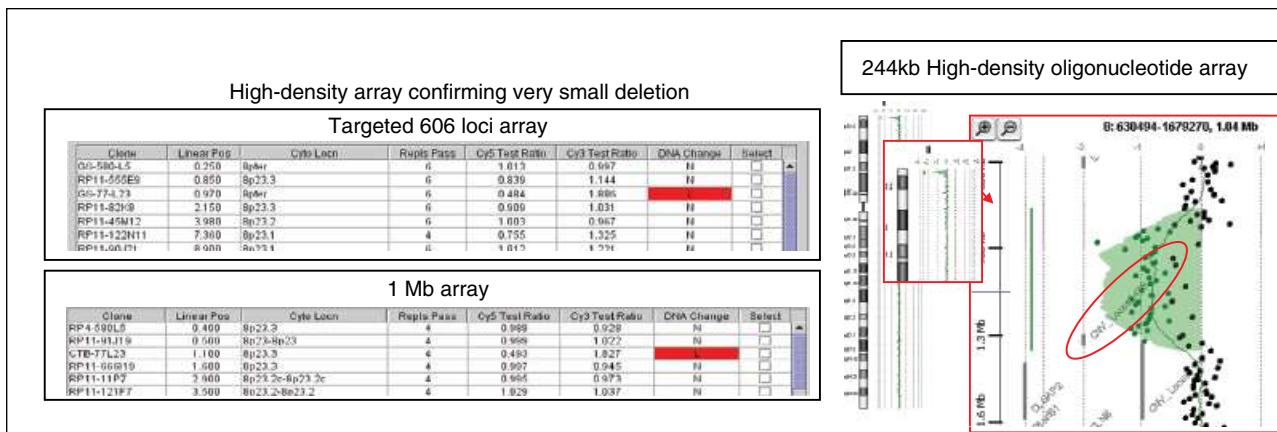


Figure 18.13 Case study 11. Genomic microarray investigations with a 606 loci array focusing on commonly microdeleted regions and subtelomere regions on peripheral blood from a 4-year-old male with developmental delay revealed loss of one clone within 8p23.3 region. The clone was not listed in the genomic variants list. Subsequent studies with a 1 Mb resolution again revealed the loss of only one clone. Further investigation with a higher density oligonucleotide array with 244 kb resolution exhibited the loss of 40 oligonucleotides in this region. See insert for color representation of this figure.

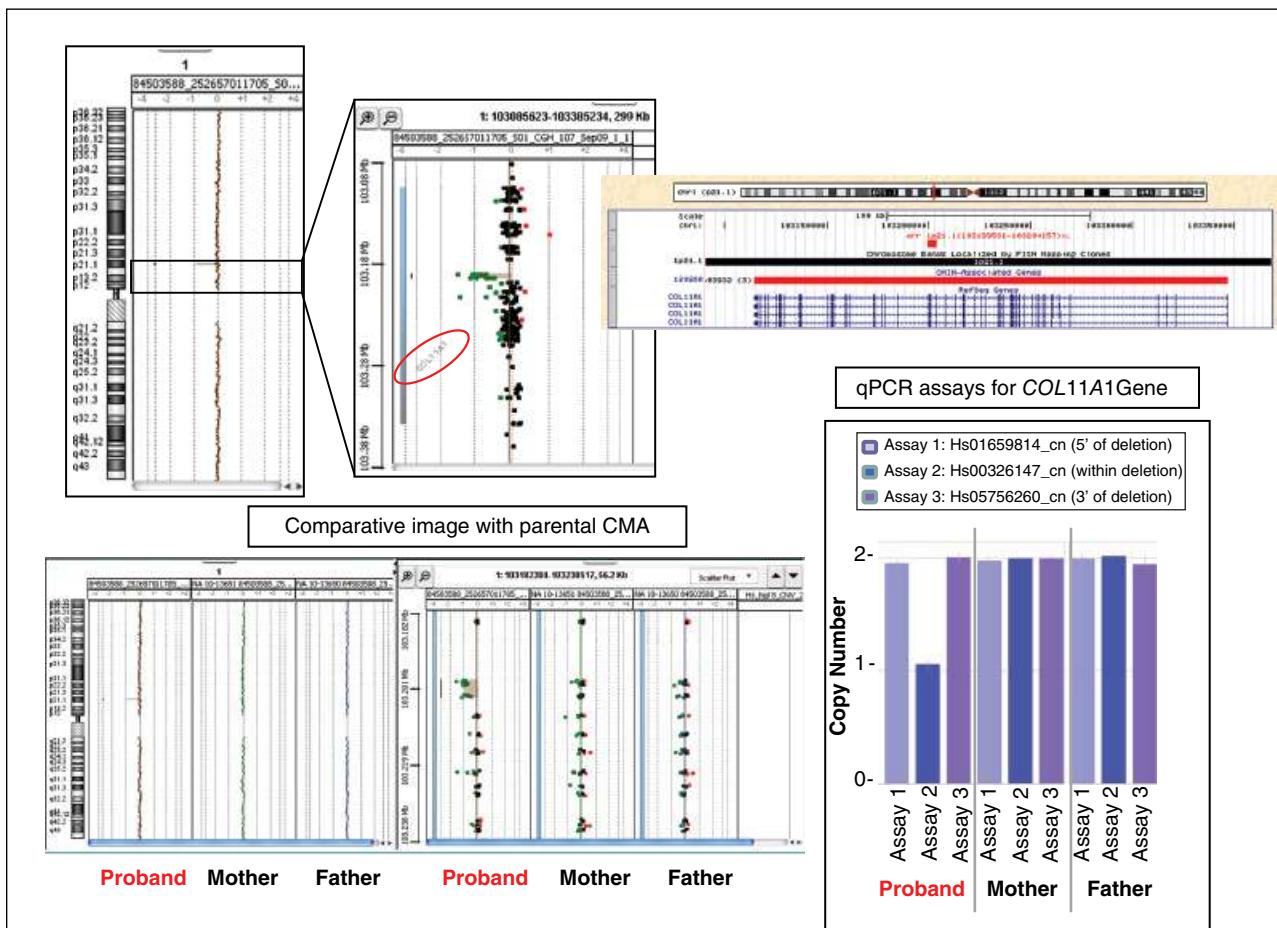


Figure 18.14 Case study 12. A 21-month-old female with dysmorphic features, multiple congenital anomalies, normal 46,XX karyotype, and normal aneuploidy FISH results at birth was referred by the clinical geneticist for CMA studies. The patient had continued ear and eye problems, including strabismus, and cataracts (treated with lens implants). Chromosomal microarray revealed a 5-kb deletion within the target gene *COL11A1* which is implicated in Stickler and Marshall syndromes. Parental CMA determined that the deletion was de novo and not inherited. Owing to the small size of the deletion, confirmatory studies were performed utilizing quantitative PCR (qPCR). Three pre-designed copy number assays for the *COL11A1* gene were performed, including one inside the minimum/maximum deletion interval and two assays outside the maximum deletion region. The qPCR assay confirmed the deletion within the gene region. Genotype-phenotype correlation suggested that the patient had Marshall syndrome. See insert for color representation of this figure.

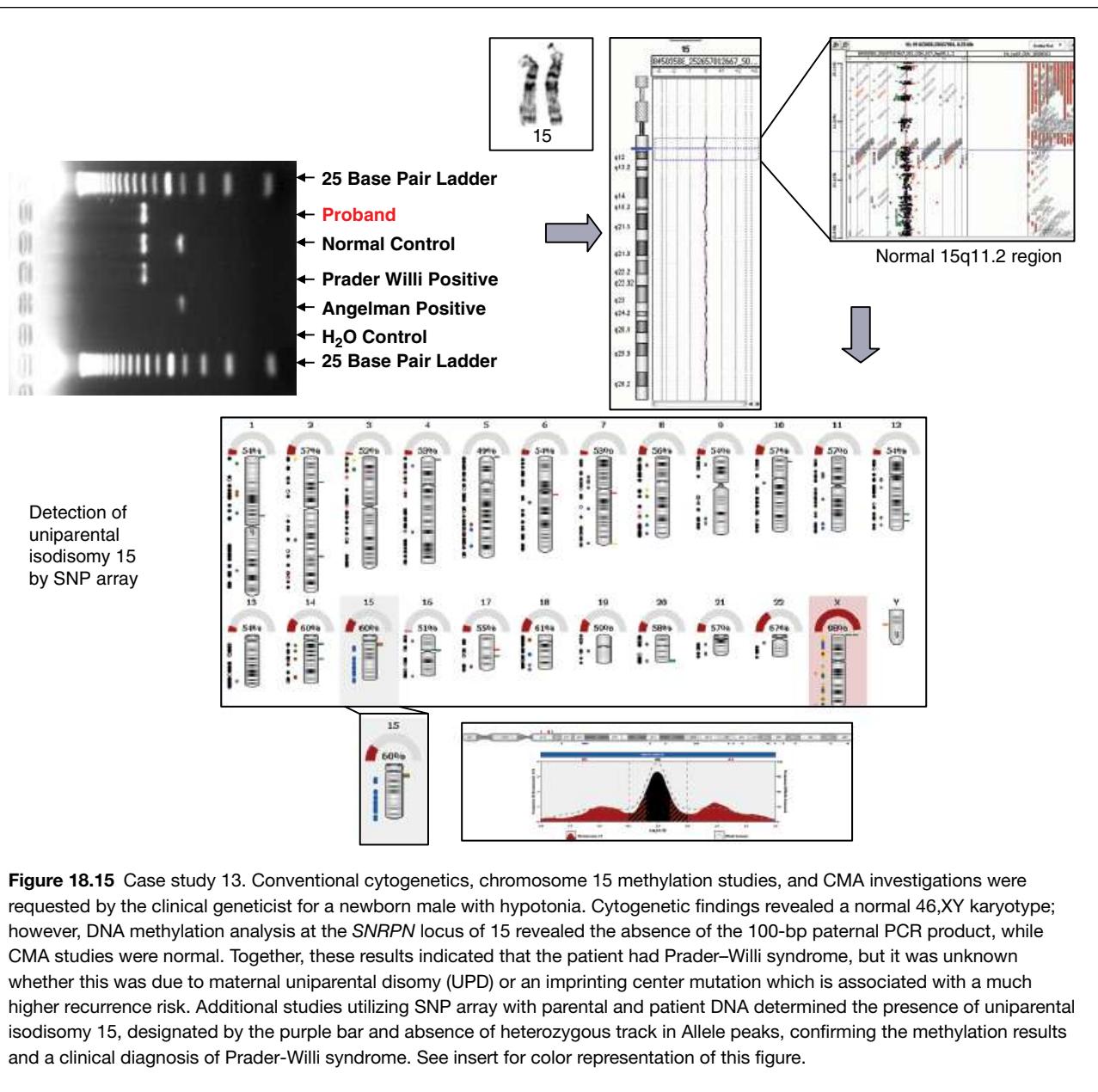


Figure 18.15 Case study 13. Conventional cytogenetics, chromosome 15 methylation studies, and CMA investigations were requested by the clinical geneticist for a newborn male with hypotonia. Cytogenetic findings revealed a normal 46,XY karyotype; however, DNA methylation analysis at the *SNRPN* locus of 15 revealed the absence of the 100-bp paternal PCR product, while CMA studies were normal. Together, these results indicated that the patient had Prader-Willi syndrome, but it was unknown whether this was due to maternal uniparental disomy (UPD) or an imprinting center mutation which is associated with a much higher recurrence risk. Additional studies utilizing SNP array with parental and patient DNA determined the presence of uniparental isodisomy 15, designated by the purple bar and absence of heterozygous track in Allele peaks, confirming the methylation results and a clinical diagnosis of Prader-Willi syndrome. See insert for color representation of this figure.

18.4 Conclusion

Array-based technology is rapidly being integrated into diagnostic laboratories and is altering the testing algorithm for many genetic conditions. Recent reviews and reports [206–214] regarding the use of CMA in clinical and research setting, its ethical implications, its clinical impact on diagnosing structural variations in the human genome, and the necessity to establish stringent validation procedures and guidelines for interpreting the data, all emphasize that a rationalized testing approach and appropriate data interpretations are imperative for all laboratories using CMA in clinical and research contexts.

Acknowledgment

We are thankful to Diane Pickering, M.S., CG(ASCP)^{CM}, Laboratory Supervisor-Microarray Section, currently Laboratory Manager-Human Genetics laboratory, University of Nebraska Medical Center, for her help in the preparation of this manuscript.

Authors' note

Because CMA has rapidly developed into a major and unquestionably valuable component in genetic testing, some of the newer advances in the field may not have been available at the time this chapter was written. Two representative examples (Figures 18.14 and 18.15) demonstrating some newer application of advances in the technology were incorporated in an update; however, the illustrations may not represent some of the most recent advances. The reader may benefit from referring to additional reports and reviews on the topic [215–238]. Further, the rapid integration and the clinical utility of the technique in diagnostic laboratories have led to the development and update of various standards, recommendations, and guidelines for application of the technique, quality control, and interpretation of the results. It will be advantageous for the reader to refer to some more recent guidelines [239–243], technical appraisals [244–248] and reviews on the application of CMA in hematologic diseases [249–252], and in prenatal [253–255], stillbirth [256], and postnatal [257–262] genetic studies.

References

1. Solinas-Toldo S, Lampel S, Stilgenbauer S, Nickolenko J, Benner A, Dohner H, Cremer T, Lichter P. Matrix-based comparative genomic hybridization: biochips to screen for genomic imbalances. *Genes Chromosomes Cancer* 1997; 20: 399–407.
2. Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y, Dairkee SH, Ljung BM, Gray JW, Albertson DG. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. *Nat Genet* 1998; 20: 207–211.
3. Snijders AM, Nowak N, Segraves R, Blackwood S, Brown N, Conroy J, Hamilton G, Hindle AK, Huey B, Kimura K, Law S, Myambo K, Palmer J, Ylstra B, Yue JP, Gray JW, Jain AN, Pinkel D, Albertson DG. Assembly of microarrays for genome-wide measurement of DNA copy number. *Nat Genet* 2001; 29: 263–264.
4. Buckley PG, Mantripragada KK, Benetkiewicz M, Tapia-Paez I, Diaz De Stahl T, Rosenquist M, Ali H, Jarbo C, De Bustos C, Hirvela C, Sinder Wilen B, Fransson I, Thyr C, Johnsson BI, Bruder CE, Menzel U, Hergersberg M, Mandahl N, Blennow E, Wedell A, Beare DM, Collins JE, Dunham I, Albertson D, Pinkel D, Bastian BC, Faruqi AF, Lasken RS, Ichimura K, Collins VP, Dumanski JP. A full-coverage, high-resolution human chromosome 22 genomic microarray for clinical and research applications. *Hum Mol Genet* 2002; 11: 3221–3229.
5. Rauch A, Ruschendorf F, Huang J, Trautmann U, Becker C, Thiel C, Jones KW, Reis A, Nurnberg P. Molecular karyotyping using an SNP array for genomewide genotyping. *J Med Genet* 2004; 41: 916–922.
6. Vermeesch JR, Melotte C, Froyen G, Van Vooren S, Dutta B, Maas N, Vermeulen S, Menten B, Speleman F, De Moor B, Van Hummelen P, Marynen P, Fryns JP, Devriendt K. Molecular karyotyping: array CGH quality criteria for constitutional genetic diagnosis. *J Histochem Cytochem* 2005; 53: 413–422.
7. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 1992; 258: 818–821.
8. Kirchhoff M, Rose H, Lundsteen C. High resolution comparative genomic hybridisation in clinical cytogenetics. *J Med Genet* 2001; 38: 740–744.
9. Kirchhoff M, Gerdes T, Maahr J, Rose H, Bentz M, Dohner H, Lundsteen C. Deletions below 10 megabase pairs are detected in comparative genomic hybridization by standard reference intervals. *Genes Chromosomes Cancer* 1999; 25: 410–413.
10. Fiegler H, Carr P, Douglas EJ, Burford DC, Hunt S, Scott CE, Smith J, Vetrici D, Gorman P, Tomlinson IP, Carter NP. DNA microarrays for comparative genomic hybridization based on DOP-PCR amplification of BAC and PAC clones. *Genes Chromosomes Cancer* 2003; 36: 361–374. Erratum in: *Genes Chromosomes Cancer* 2003; 37: 223.
11. Veltman JA, Fridlyand J, Pejavar S, Olshen AB, Korkola JE, DeVries S, Carroll P, Kuo WL, Pinkel D, Albertson D, Cordon-Cardo C, Jain AN, Waldman FM. Array-based comparative genomic hybridization for genome-wide screening of DNA copy number in bladder tumors. *Cancer Res* 2003; 63: 2872–2880.
12. Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, Jeffrey SS, Botstein D, Brown PO. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nat Genet* 1999; 23: 41–46.
13. Schoumans J, Anderlid BM, Blennow E, Teh BT, Nordenskjold M. The performance of CGH array for the detection of cryptic constitutional chromosome imbalances. *J Med Genet* 2004; 41: 198–202.

14. Jobanputra V, Sebat J, Troge J, Chung W, Anyane-Yeboa K, Wigler M, Warburton D. Application of ROMA (representational oligonucleotide microarray analysis) to patients with cytogenetic rearrangements. *Genet Med* 2005; 7: 111–118.
15. Lai Y, Zhao H. A statistical method to detect chromosomal regions with DNA copy number alterations using SNP-array-based CGH data. *Comput Biol Chem* 2005; 29: 47–54. Erratum in: *Comput Biol Chem* 2005; 29: 258.
16. Dhami P, Coffey AJ, Abbs S, Vermeesch JR, Dumanski JP, Woodward KJ, Andrews RM, Langford C, Vetrie D. Exon array CGH: detection of copy-number changes at the resolution of individual exons in the human genome. *Am J Hum Genet* 2005; 76: 750–762.
17. Albertson DG, Ylstra B, Segraves R, Collins C, Dairkee SH, Kowbel D, Kuo WL, Gray JW, Pinkel D. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. *Nat Genet* 2000; 25: 144–146.
18. Bruder CE, Hirvela C, Tapia-Paez I, Fransson I, Segraves R, Hamilton G, Zhang XX, Evans DG, Wallace AJ, Baser ME, Zucman-Rossi J, Hergersberg M, Boltshauser E, Papi L, Rouleau GA, Poptodorov G, Jordanova A, Rask-Andersen H, Kluwe L, Mautner V, Sainio M, Hung G, Mathiesen T, Moller C, Pulst SM, Harder H, Heiberg A, Honda M, Niimura M, Sahlen S, Blennow E, Albertson DG, Pinkel D, Dumanski JP. High resolution deletion analysis of constitutional DNA from neurofibromatosis type 2 (NF2) patients using microarray-CGH. *Hum Mol Genet* 2001; 10: 271–282.
19. Mantripragada KK, Buckley PG, Jarbo C, Menzel U, Dumanski JP. Development of NF2 gene specific, strictly sequence defined diagnostic microarray for deletion detection. *J Mol Med* 2003; 81: 443–451.
20. Mantripragada KK, Buckley PG, Benetkiewicz M, De Bustos C, Hirvela C, Jarbo C, Bruder CE, Wensman H, Mathiesen T, Nyberg G, Papi L, Collins VP, Ichimura K, Evans G, Dumanski JP. High-resolution profiling of an 11 Mb segment of human chromosome 22 in sporadic schwannoma using array-CGH. *Int J Oncol* 2003; 22: 615–622.
21. Van Buggenhout G, Melotte C, Dutta B, Froyen G, Van Hummelen P, Marynen P, Matthijs G, de Ravel T, Devriendt K, Fryns JP, Vermeesch JR. Mild Wolf-Hirschhorn syndrome: micro-array CGH analysis of atypical 4p16.3 deletions enables refinement of the genotype-phenotype map. *J Med Genet* 2004; 41: 691–698.
22. Yu W, Ballif BC, Kashork CD, Heilstedt HA, Howard LA, Cai WW, White LD, Liu W, Beaudet AL, Bejjani BA, Shaw CA, Shaffer LG. Development of a comparative genomic hybridization microarray and demonstration of its utility with 25 well-characterized 1p36 deletions. *Hum Mol Genet* 2003; 12: 2145–2152.
23. Ekong R, Jeremiah S, Judah D, Lehmann O, Mirzayans F, Hung YC, Walter MA, Bhattacharya S, Gant TW, Povey S, Wolfe J. Chromosomal anomalies on 6p25 in iris hypoplasia and Axenfeld-Rieger syndrome patients defined on a purpose-built genomic microarray. *Hum Mutat* 2004; 24: 76–85.
24. Redon R, Rio M, Gregory SG, Cooper RA, Fiegler H, Sanlaville D, Banerjee R, Scott C, Carr P, Langford C, Cormier-Daire V, Munnoch A, Carter NP, Colleaux L. Tiling path resolution mapping of constitutional 1p36 deletions by array-CGH: contiguous gene deletion or “deletion with positional effect” syndrome? *J Med Genet* 2005; 42: 166–171.
25. Veltman JA, Schoenmakers EF, Eussen BH, Janssen I, Merkx G, van Cleef B, van Ravenswaaij CM, Brunner HG, Smeets D, van Kessel AG. High-throughput analysis of subtelomeric chromosome rearrangements by use of array-based comparative genomic hybridization. *Am J Hum Genet* 2002; 70: 1269–1276.
26. Ishkanian AS, Malloff CA, Watson SK, DeLeeuw RJ, Chi B, Coe BP, Snijders A, Albertson DG, Pinkel D, Marra MA, Ling V, MacAulay C, Lam WL. A tiling resolution DNA microarray with complete coverage of the human genome. *Nat Genet* 2004; 36: 299–303.
27. Lucito R, Healy J, Alexander J, Reiner A, Esposito D, Chi M, Rodgers L, Brady A, Sebat J, Troge J, West JA, Rostan S, Nguyen KC, Powers S, Ye KQ, Olshen A, Venkatraman E, Norton L, Wigler M. Representational oligonucleotide microarray analysis: a high-resolution method to detect genome copy number variation. *Genome Res* 2003; 13: 2291–2305.
28. Sanlaville D, Lapierre JM, Turleau C, Coquin A, Borck G, Colleaux L, Vekemans M, Romana SP. Molecular karyotyping in human constitutional cytogenetics. *Eur J Med Genet* 2005; 48: 214–231. Review.
29. Lockwood WW, Chari R, Chi B, Lam WL. Recent advances in array comparative genomic hybridization technologies and their applications in human genetics. *Eur J Hum Genet* 2006; 14: 139–148.
30. Vissers LE, Veltman JA, van Kessel AG, Brunner HG. Identification of disease genes by whole genome CGH arrays. *Hum Mol Genet* 2005; 15 Spec No. 2: R215–223. Review.

31. Oostlander AE, Meijer GA, Ylstra B. Microarray-based comparative genomic hybridization and its applications in human genetics. *Clin Genet* 2004; 66: 488–495. Review.
32. Inazawa J, Inoue J, Imoto I. Comparative genomic hybridization (CGH)-arrays pave the way for identification of novel cancer-related genes. *Cancer Sci* 2004; 95: 559–563. Review.
33. Jong K, Marchiori E, Meijer G, Vaart AV, Ylstra B. Breakpoint identification and smoothing of array comparative genomic hybridization data. *Bioinformatics* 2004; 20: 3636–3637.
34. Kim SY, Nam SW, Lee SH et al: ArrayCyGHt: a web application for analysis and visualization of array-CGH data. *Bioinformatics* 2005; 21: 2554–2555.
35. Awad IA, Rees CA, Hernandez-Boussard T, Ball CA, Sherlock G. Caryoscope: an Open Source Java application for viewing microarray data in a genomic context. *BMC Bioinformatics* 2004; 5: 151.
36. Margolin AA, Greshock J, Naylor TL, Mosse Y, Maris JM, Bignell G, Saeed AI, Quackenbush J, Weber BL. CGHAnalyzer: a standalone software package for cancer genome analysis using array based DNA copy number data. *Bioinformatics* 2005; 21: 3308–3311.
37. Chen W, Erdogan F, Ropers HH, Lenzner S, Ullmann R. CGHPRO – a comprehensive data analysis tool for array CGH. *BMC Bioinformatics* 2005; 6: 85.
38. Lingjaerde OC, Baumbusch LO, Liestol K, Glad IK, Borresen-Dale AL. CGH-Explorer: a program for analysis of array-CGH data. *Bioinformatics* 2005; 21: 821–822.
39. Wang P, Kim Y, Pollack J, Narasimhan B, Tibshirani R. A method for calling gains and losses in array CGH data. *Biostatistics* 2005; 6: 45–58.
40. Autio R, Hautaniemi S, Kauraniemi P, Yli-Harja O, Astola J, Wolf M, Kallioniemi A. CGH-Plotter: MATLAB toolbox for CGH-data analysis. *Bioinformatics* 2003; 19: 1714–1715.
41. Myers CL, Dunham MJ, Kung SY, Troyanskaya OG. Accurate detection of aneuploidies in array CGH and gene expression microarray data. *Bioinformatics* 2004; 20: 3533–3543.
42. Yi Y, Mirosevich J, Shyr Y, Matusik R, George Jr AL. Coupled analysis of gene expression and chromosomal location. *Genomics* 2005; 85: 401–412.
43. Wang J, Meza-Zepeda LA, Kresse SH, Myklebost O. M-CGH: analysing microarray-based CGH experiments. *BMC Bioinformatics* 2004; 5: 74.
44. Beheshti B, Braude I, Marrano P, Thorner P, Zielenska M, Squire JA. Chromosomal localization of DNA amplifications in neuroblastoma tumors using cDNA microarray comparative genomic hybridization. *Neoplasia* 2003; 5: 53–62.
45. Chi B, DeLeeuw RJ, Coe BP, MacAulay C, Lam WL. SeeGH -a software tool for visualization of whole genome array comparative genomic hybridization data. *BMC Bioinformatics* 2004; 5: 13.
46. Albertson DG, Pinkel D. Genomic microarrays in human genetic disease and cancer. *Hum Mol Genet* 2003; 12(Spec No 2): R145–152. Review.
47. Mockler TC, Chan S, Sundaresan A, Chen H, Jacobsen SE, Ecker JR. Applications of DNA tiling arrays for whole-genome analysis. *Genomics* 2005; 85: 1–15. Erratum in: *Genomics* 2005; 85: 655.
48. Shaffer LG, Bejjani BA. A cytogeneticist's perspective on genomic microarrays. *Hum Reprod Update* 2004; 10: 221–226. Review.
49. Carter NP, Vetrici D. Applications of genomic microarrays to explore human chromosome structure and function. *Hum Mol Genet* 2004; 13 Spec No 2: R297–302.
50. Davies JJ, Wilson IM, Lam WL. Array CGH technologies and their applications to cancer genomes. *Chromosome Res* 2005; 13: 237–248. Review. Erratum in: *Chromosome Res* 2005; 13: 423.
51. Ciccone R, Giorda R, Gregato G, Guerrini R, Giglio S, Carrozzo R, Bonaglia MC, Priolo E, Lagana C, Tenconi R, Rocchi M, Pramparo T, Zuffardi O, Rossi E. Reciprocal translocations: a trap for cytogeneticists? *Hum Genet* 2005; 117: 571–582.
52. Prescott K, Woodfine K, Stubbs P, Super M, Kerr B, Palmer R, Carter NP, Scambler P. A novel 5q11.2 deletion detected by microarray comparative genomic hybridisation in a child referred as a case of suspected 22q11 deletion syndrome. *Hum Genet* 2005; 116: 83–90.

53. Gunn SR, Mohammed M, Reveles XT, Viskochil DH, Palumbos JC, Johnson-Pais TL, Hale DE, Lancaster JL, Hardies LJ, Boespflug-Tanguy O, Cody JD, Leach RJ. Molecular characterization of a patient with central nervous system dysmyelination and cryptic unbalanced translocation between chromosomes 4q and 18q. *Am J Med Genet A* 2003; 120: 127–135.
54. Vissers LE, de Vries BB, Osoegawa K, Janssen IM, Feuth T, Choy CO, Straatman H, van der Vliet W, Huys EH, van Rijk A, Smeets D, van Ravenswaaij-Arts CM, Knoers NV, van der Burgt I, de Jong PJ, Brunner HG, van Kessel AG, Schoenmakers EF, Veltman JA. Array-based comparative genomic hybridization for the genomewide detection of submicroscopic chromosomal abnormalities. *Am J Hum Genet* 2003; 73: 1261–1270.
55. Shaw-Smith C, Redon R, Rickman L, Rio M, Willatt L, Fiegler H, Firth H, Sanlaville D, Winter R, Colleaux L, Bobrow M, Carter NP. Microarray based comparative genomic hybridisation (array-CGH) detects submicroscopic chromosomal deletions and duplications in patients with learning disability/mental retardation and dysmorphic features. *J Med Genet* 2004; 41: 241–248.
56. de Vries BB, Pfundt R, Leisink M, Koolen DA, Vissers LE, Janssen IM, Reijmersdal S, Nillesen WM, Huys EH, Leeuw N, Smeets D, Sistermans EA, Feuth T, van Ravenswaaij-Arts CM, van Kessel AG, Schoenmakers EF, Brunner HG, Veltman JA. Diagnostic genome profiling in mental retardation. *Am J Hum Genet* 2005; 77: 606–616.
57. Bisgaard AM, Kirchhoff M, Turner Z, Jepsen B, Brondum-Nielsen K, Cohen M, Hamborg-Petersen B, Bryndorf T, Tommerup N, Skovby F. Additional chromosomal abnormalities in patients with a previously detected abnormal karyotype, mental retardation, and dysmorphic features. *Am J Med Genet A* 2006; 140: 2180–2187.
58. Shaffer LG, Kashork CD, Saleki R, Rorem E, Sundin K, Ballif BC, Bejjani BA. Targeted genomic microarray analysis for identification of chromosome abnormalities in 1500 consecutive clinical cases. *J Pediatr* 2006; 149: 98–102.
59. Flint J, Wilkie AO, Buckle VJ, Winter RM, Holland AJ, McDermid HE. The detection of subtelomeric chromosomal rearrangements in idiopathic mental retardation. *Nat Genet* 1995; 9: 132–140.
60. Knight SJ, Regan R, Nicod A, Horsley SW, Kearney L, Homfray T, Winter RM, Bolton P, Flint J. Subtle chromosomal rearrangements in children with unexplained mental retardation. *Lancet* 1999; 354: 1676–1681.
61. Biesecker LG. The end of the beginning of chromosome ends. *Am J Med Genet* 2002; 107: 263–266. Review.
62. De Vries BB, Winter R, Schinzel A, van Ravenswaaij-Arts C. Telomeres: a diagnosis at the end of the chromosomes. *J Med Genet* 2003; 40: 385–398. Review.
63. Flint J, Knight S. The use of telomere probes to investigate submicroscopic rearrangements associated with mental retardation. *Curr Opin Genet Dev* 2003; 13: 310–316.
64. Harada N, Hatchwell E, Okamoto N, Tsukahara M, Kurosawa K, Kawame H, Kondoh T, Ohashi H, Tsukino R, Kondoh Y, Shimokawa O, Ida T, Nagai T, Fukushima Y, Yoshiura K, Niikawa N, Matsumoto N. Subtelomere specific microarray based comparative genomic hybridisation: a rapid detection system for cryptic rearrangements in idiopathic mental retardation. *J Med Genet* 2004; 41: 130–136.
65. Knight SJ, Horsley SW, Regan R, Lawrie NM, Maher EJ, Cardy DL, Flint J, Kearney L. Development and clinical application of an innovative fluorescence in situ hybridization technique which detects submicroscopic rearrangements involving telomeres. *Eur J Hum Genet* 1997; 5: 1–8.
66. Koolen DA, Nillesen WM, Versteeg MH, Merkx GF, Knoers NV, Kets M, Vermeer S, van Ravenswaaij CM, de Kovel CG, Brunner HG, Smeets D, de Vries BB, Sistermans EA. Screening for subtelomeric rearrangements in 210 patients with unexplained mental retardation using multiplex ligation dependent probe amplification (MLPA). *J Med Genet* 2004; 41: 892–899.
67. Rosenberg C, Knijnenburg J, Bakker E, Vianna-Morgante AM, Sloos W, Otto PA, Kriek M, Hansson K, Krepischi-Santos AC, Fiegler H, Carter NP, Bijlsma EK, van Haeringen A, Szuhai K, Tanke HJ. Array-CGH detection of micro rearrangements in mentally retarded individuals: clinical significance of imbalances present both in affected children and normal parents. *J Med Genet* 2006; 43: 180–186.
68. Schoumans J, Ruivenkamp C, Holmberg E, Kyllerman M, Anderlid BM, Nordenskjold M. Detection of chromosomal imbalances in children with idiopathic mental retardation by array based comparative genomic hybridisation (array-CGH). *J Med Genet* 2005; 42: 699–705.
69. Menten B, Maas N, Thienpont B, Buysse K, Vandesompele J, Melotte C, de Ravel T, Van Vooren S, Balikova I, Backx L, Janssens S, De Paepe A, De Moor B, Moreau Y, Marynen P, Fryns JP, Mortier G, Devriendt K, Speleman F.

- Vermeesch JR. Emerging patterns of cryptic chromosomal imbalances in patients with idiopathic mental retardation and multiple congenital anomalies: a new series of 140 patients and review of the literature. *J Med Genet* 2006; 43: 625–633.
70. Batista DA, Pai GS, Stetten G. Molecular analysis of a complex chromosomal rearrangement and a review of familial cases. *Am J Med Genet* 1994; 53: 255–263. Review.
 71. Phelan MC, Blackburn W, Rogers RC, Crawford EC, Cooley NR Jr, Schrock E, Ning Y, Ried T. FISH analysis of a complex chromosome rearrangement involving nine breakpoints on chromosomes 6, 12, 14 and 16. *Prenat Diagn* 1998; 18: 1174–1180.
 72. Astbury C, Christ LA, Aughton DJ, Cassidy SB, Kumar A, Eichler EE, Schwartz S. Detection of deletions in de novo “balanced” chromosome rearrangements: further evidence for their role in phenotypic abnormalities. *Genet Med* 2004; 6: 81–89.
 73. Astbury C, Christ LA, Aughton DJ, Cassidy SB, Fujimoto A, Pletcher BA, Schafer IA, Schwartz S. Delineation of complex chromosomal rearrangements: evidence for increased complexity. *Hum Genet* 2004; 114: 448–457.
 74. Borg I, Squire M, Menzel C, Stout K, Morgan D, Willatt L, O’Brien PC, Ferguson-Smith MA, Ropers HH, Tommerup N, Kalscheuer VM, Sargent DR. A cryptic deletion of 2q35 including part of the PAX3 gene detected by breakpoint mapping in a child with autism and a de novo 2;8 translocation. *J Med Genet* 2002; 39: 391–399.
 75. Kirchhoff M, Rose H, Maahr J, Gerdes T, Bugge M, Tommerup N, Turner Z, Lespinasse J, Jensen PK, Wirth J, Lundsteen C. High resolution comparative genomic hybridisation analysis reveals imbalances in dyschromosomal patients with normal or apparently balanced conventional karyotypes. *Eur J Hum Genet* 2000; 8: 661–668.
 76. Gribble SM, Prigmore E, Burford DC, Porter KM, Ng BL, Douglas EJ, Fiegler H, Carr P, Kalaitzopoulos D, Clegg S, Sandstrom R, Temple IK, Youings SA, Thomas NS, Dennis NR, Jacobs PA, Crolla JA, Carter NP. The complex nature of constitutional de novo apparently balanced translocations in patients presenting with abnormal phenotypes. *J Med Genet* 2005; 42: 8–16.
 77. Veltman JA, Yntema HG, Lugtenberg D, Arts H, Briault S, Huys EH, Osoegawa K, de Jong P, Brunner HG, Geurts van Kessel A, van Bokhoven H, Schoenmakers EF. High resolution profiling of X chromosomal aberrations by array comparative genomic hybridisation. *J Med Genet* 2004; 41: 425–432.
 78. Tyson C, McGillivray B, Chijiwa C, Rajcan-Separovic E. Elucidation of a cryptic interstitial 7q31.3 deletion in a patient with a language disorder and mild mental retardation by array-CGH. *Am J Med Genet A* 2004; 129: 254–260.
 79. Rosenberg C, Knijnenburg J, Chauffaille Mde L, Brunoni D, Catelani AL, Sloos W, Szuhai K, Tanke HJ. Array CGH detection of a cryptic deletion in a complex chromosome rearrangement. *Hum Genet* 2005; 116: 390–394.
 80. Harvard C, Malenfant P, Koochek M, Creighton S, Mickelson EC, Holden JJ, Lewis ME, Rajcan-Separovic E. A variant Cri du Chat phenotype and autism spectrum disorder in a subject with de novo cryptic microdeletions involving 5p15.2 and 3p24.3-25 detected using whole genomic array CGH. *Clin Genet* 2005; 67: 341–351.
 81. Veltman JA, Jonkers Y, Nuijten I, Janssen I, van der Vliet W, Huys E, Vermeesch J, Van Buggenhout G, Fryns JP, Admiraal R, Terhal P, Lacombe D, van Kessel AG, Smeets D, Schoenmakers EF, van Ravenswaaij-Arts CM. Definition of a critical region on chromosome 18 for congenital aural atresia by arrayCGH. *Am J Hum Genet* 2003; 72: 1578–1584.
 82. Kline AD, White ME, Wapner R, Rojas K, Biesecker LG, Kamholz J, Zackai EH, Muenke M, Scott CI Jr, Overhauser J. Molecular analysis of the 18q- syndrome – and correlation with phenotype. *Am J Hum Genet* 1993; 52: 895–906. Review.
 83. Rauen KA, Albertson DG, Pinkel D, Cotter PD. Additional patient with del(12)(q21.2q22): further evidence for a candidate region for cardio-facio-cutaneous syndrome? *Am J Med Genet* 2002; 110: 51–56.
 84. Slager RE, Newton TL, Vlangos CN, Finucane B, Elsea SH. Mutations in RAI1 associated with Smith-Magenis syndrome. *Nat Genet* 2003; 33: 466–468.
 85. Kishino T, Lalonde M, Wagstaff J. UBE3A/E6-AP mutations cause Angelman syndrome. *Nat Genet* 1997; 15: 70–73. Erratum in: *Nat Genet* 1997; 15: 411.
 86. Lindsay EA, Vitelli F, Su H, Morishima M, Huynh T, Prampano T, Jurecic V, Ogunrinu G, Sutherland HF, Scambler PJ, Bradley A, Baldini A. Tbx1 haploinsufficiency in the DiGeorge syndrome region causes aortic arch defects in mice. *Nature* 2001; 410: 97–101.
 87. Klaassens M, van Dooren M, Eussen HJ, Douben H, den Dekker AT, Lee C, Donahoe PK, Galjaard RJ, Goemaere N, de Krijger RR, Wouters C, Wauters J, Oostra BA, Tibboel D, de Klein A. Congenital diaphragmatic hernia and chromosome 15q26: determination of a candidate region by use of fluorescence in situ hybridization and array-based comparative genomic hybridization. *Am J Hum Genet* 2005; 76: 877–882.

88. Klein OD, Cotter PD, Albertson DG, Pinkel D, Tidyman WE, Moore MW, Rauen KA. Prader-Willi syndrome resulting from an unbalanced translocation: characterization by array comparative genomic hybridization. *Clin Genet* 2004; 65: 477–482.
89. Zhang X, Snijders A, Segraves R, Zhang X, Niebuhr A, Albertson D, Yang H, Gray J, Niebuhr E, Bolund L, Pinkel D. High-resolution mapping of genotype-phenotype relationships in cri du chat syndrome using array comparative genomic hybridization. *Am J Hum Genet* 2005; 76: 312–326.
90. Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, de Vries BB, Janssen IM, van der Vliet WA, Huys EH, de Jong PJ, Hamel BC, Schoenmakers EF, Brunner HG, Veltman JA, van Kessel AG. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004; 36: 955–957.
91. Shaw CJ, Stankiewicz P, Bien-Willner G, Bello SC, Shaw CA, Carrera M, Perez Jurado L, Estivill X, Lupski JR. Small marker chromosomes in two patients with segmental aneusomy for proximal 17p. *Hum Genet* 2004; 115: 1–7.
92. Sahoo T, Shaw CA, Young AS, Whitehouse NL, Schroer RJ, Stevenson RE, Beaudet AL. Array-based comparative genomic hybridization analysis of recurrent chromosome 15q rearrangements. *Am J Med Genet A* 2005; 139: 106–113.
93. Drazinic CM, Ercan-Sencicek AG, Gault LM, Hisama FM, Qumsiyeh MB, Nowak NJ, Cubells JF, State MW. Rapid array-based genomic characterization of a subtle structural abnormality: a patient with psychosis and der(18)t(5;18) (p14.1;p11.23). *Am J Med Genet A* 2005; 134: 282–289.
94. Ki A, Rauen KA, Black LD, Kostiner DR, Sandberg PL, Pinkel D, Albertson DG, Norton ME, Cotter PD. Ring 21 chromosome and a satellites 1p in the same patient: novel origin for an ectopic NOR. *Am J Med Genet A* 2003; 120: 365–369.
95. Koolen DA, Veltman JA, Renier WO, Droog RP, van Kessel AG, de Vries BB. Chromosome 22q11 deletion and pachygyria characterized by array-based comparative genomic hybridization. *Am J Med Genet A* 2004; 131: 322–324.
96. Shaw CJ, Shaw CA, Yu W, Stankiewicz P, White LD, Beaudet AL, Lupski JR. Comparative genomic hybridisation using a proximal 17p BAC/PAC array detects rearrangements responsible for four genomic disorders. *J Med Genet* 2004; 41: 113–119.
97. Giampietro PF, Babu D, Zabel CA, Silberman T, Zador I, DeBauche D, Ravnan JB, Dave BJ. Novel clinical features in a child with partial deletion of chromosome 11 [del(11)(q24.2)]: further evidence for phenotypic heterogeneity. *Am J Med Genet A* 2006; 140: 385–387.
98. de Pater JM, Poot M, Beemer FA, Bijlsma JB, Hack WW, Van Dam WM, Eleveld MJ, Loneus WH, Engelen JJ. Virilization of the external genitalia and severe mental retardation in a girl with an unbalanced translocation 1;18. *Eur J Med Genet* 2006; 49: 19–27.
99. Glass IA, Rauen KA, Chen E, Parkes J, Alberston DG, Pinkel D, Cotter PD. Ring chromosome 15: characterization by array CGH. *Hum Genet* 2006; 118: 611–617.
100. Le Caignec C, De Mas P, Vincent MC, Boceno M, Bourrouillou G, Rival JM, David A. Subtelomeric 6p deletion: clinical, FISH, and array CGH characterization of two cases. *Am J Med Genet A* 2005; 132: 175–180. Review.
101. Le Caignec C, Swillen A, Van Asche E, Fryns JP, Vermesch JR. Interstitial 6q deletion: clinical and array CGH characterisation of a new patient. *Eur J Med Genet* 2005; 48: 339–345.
102. Sahoo T, Peters SU, Madduri NS, Glaze DG, German JR, Bird LM, Barbieri-Welge R, Bichell TJ, Beaudet AL, Bacino CA. Microarray based comparative genomic hybridization testing in deletion bearing patients with Angelman syndrome: genotype-phenotype correlations. *J Med Genet* 2006; 43: 512–516.
103. Albertson DG, Ylstra B, Segraves R, Collins C, Dairkee SH, Kowbel D, Kuo WL, Gray JW, Pinkel D. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. *Nat Genet* 2000; 25: 144–146.
104. Mission PP, Kuo WL, Stokoe D, Olshen AB, Treseler PA, Chin K, Chen C, Polikoff D, Jain AN, Pinkel D, Albertson DG, Jablons DM, Gray JW. Genomic copy number analysis of non-small cell lung cancer using array comparative genomic hybridization: implications of the phosphatidylinositol 3-kinase pathway. *Cancer Res* 2002; 62: 3636–3640.
105. Greshock J, Naylor TL, Margolin A, Diskin S, Cleaver SH, Futreal PA, deJong PJ, Zhao S, Liebman M, Weber BL. 1-Mb resolution array-based comparative genomic hybridization using a BAC clone set optimized for cancer gene analysis. *Genome Res* 2004; 14: 179–187.

106. Kohlhammer H, Schwaenen C, Wessendorf S, Holzmann K, Kestler HA, Kienle D, Barth TF, Moller P, Ott G, Kalla J, Radlwimmer B, Pscherer A, Stilgenbauer S, Dohner H, Lichter P, Bentz M. Genomic DNA-chip hybridization in t(11;14)-positive mantle cell lymphomas shows a high frequency of aberrations and allows a refined characterization of consensus regions. *Blood* 2004; 104: 795–801.
107. Holzmann K, Kohlhammer H, Schwaenen C, Wessendorf S, Kestler HA, Schwoerer A, Rau B, Radlwimmer B, Dohner H, Lichter P, Gress T, Bentz M. Genomic DNA-chip hybridization reveals a higher incidence of genomic amplifications in pancreatic cancer than conventional comparative genomic hybridization and leads to the identification of novel candidate genes. *Cancer Res* 2004; 64: 4428–4433. Erratum in: *Cancer Res* 2004; 64: 6358.
108. Garnis C, Campbell J, Zhang L, Rosin MP, Lam WL. OCGR array: an oral cancer genomic regional array for comparative genomic hybridization analysis. *Oral Oncol* 2004; 40: 511–519.
109. Schwaenen C, Nessling M, Wessendorf S, Salvi T, Wrobel G, Radlwimmer B, Kestler HA, Haslinger C, Stilgenbauer S, Dohner H, Bentz M, Lichter P. Automated array-based genomic profiling in chronic lymphocytic leukemia: development of a clinical tool and discovery of recurrent genomic alterations. *Proc Natl Acad Sci USA* 2004; 101: 1039–1044.
110. Wessendorf S, Schwaenen C, Kohlhammer H, Kienle D, Wrobel G, Barth TF, Nessling M, Moller P, Dohner H, Lichter P, Bentz M. Hidden gene amplifications in aggressive B-cell non-Hodgkin lymphomas detected by microarray-based comparative genomic hybridization. *Oncogene* 2003; 22: 1425–1429.
111. Monni O, Barlund M, Mousses S, Kononen J, Sauter G, Heiskanen M, Paavola P, Avela K, Chen Y, Bittner ML, Kallioniemi A. Comprehensive copy number and gene expression profiling of the 17q23 amplicon in human breast cancer. *Proc Natl Acad Sci USA* 2001; 98: 5711–5716.
112. Redon R, Husseinet T, Bour G, Caulee K, Jost B, Muller D, Abecassis J, du Manoir S. Amplicon mapping and transcriptional analysis pinpoint cyclin L as a candidate oncogene in head and neck cancer. *Cancer Res* 2002; 62: 6211–6217.
113. Goldstein M, Rennert H, Bar-Shira A, Burstein Y, Yaron Y, Orr-Urtreger A. Combined cytogenetic and array-based comparative genomic hybridization analyses of Wilms tumors: amplification and overexpression of the multidrug resistance associated protein 1 gene (MRP1) in a metachronous tumor. *Cancer Genet Cytogenet* 2003; 141: 120–127.
114. Ishizuka T, Tanabe C, Sakamoto H, Aoyagi K, Maekawa M, Matsukura N, Tokunaga A, Tajiri T, Yoshida T, Terada M, Sasaki H. Gene amplification profiling of esophageal squamous cell carcinomas by DNA array CGH. *Biochem Biophys Res Commun* 2002; 296: 152–155.
115. Zhao J, Roth J, Bode-Lesniewska B, Pfaltz M, Heitz PU, Komminoth P. Combined comparative genomic hybridization and genomic microarray for detection of gene amplifications in pulmonary artery intimal sarcomas and adrenocortical tumors. *Genes Chromosomes Cancer* 2002; 34: 48–57.
116. Mao X, Orchard G, Lillington DM, Russell-Jones R, Young BD, Whittaker S. Genetic alterations in primary cutaneous CD30+ anaplastic large cell lymphoma. *Genes Chromosomes Cancer* 2003; 37: 176–185.
117. Mao X, Orchard G, Lillington DM, Russell-Jones R, Young BD, Whittaker SJ. Amplification and overexpression of JUNB is associated with primary cutaneous T-cell lymphomas. *Blood* 2003; 101: 1513–1519.
118. Pollack JR, Sorlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE, Tibshirani R, Botstein D, Borresen-Dale AL, Brown PO. Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. *Proc Natl Acad Sci U.S.A.* 2002; 99: 12963–12968.
119. Sanchez-Izquierdo D, Buchonnet G, Siebert R, Gascogne RD, Climent J, Karran L, Marin M, Blesa D, Horsman D, Rosenwald A, Staudt LM, Albertson DG, Du MQ, Ye H, Marynen P, Garcia-Conde J, Pinkel D, Dyer MJ, Martinez-Climent JA. MALT1 is deregulated by both chromosomal translocation and amplification in B-cell non-Hodgkin lymphoma. *Blood* 2003; 101: 4539–4546.
120. Martinez-Climent JA, Alizadeh AA, Segraves R, Blesa D, Rubio-Moscardo F, Albertson DG, Garcia-Conde J, Dyer MJ, Levy R, Pinkel D, Lossos IS. Transformation of follicular lymphoma to diffuse large cell lymphoma is associated with a heterogeneous set of DNA copy number and gene expression alterations. *Blood* 2003; 101: 3109–3117.
121. Wilhelm M, Veltman JA, Olshen AB, Jain AN, Moore DH, Presti JC Jr, Kovacs G, Waldman FM. Array-based comparative genomic hybridization for the differential diagnosis of renal cell cancer. *Cancer Res* 2002; 62: 957–960.
122. Weiss MM, Kuipers EJ, Postma C, Snijders AM, Siccama I, Pinkel D, Westerga J, Meuwissen SG, Albertson DG, Meijer GA. Genomic profiling of gastric cancer predicts lymph node status and survival. *Oncogene* 2003; 22: 1872–1879.

123. Snijders AM, Nowee ME, Fridlyand J, Piek JM, Dorsman JC, Jain AN, Pinkel D, van Diest PJ, Verheijen RH, Albertson DG. Genome-wide-array-based comparative genomic hybridization reveals genetic homogeneity and frequent copy number increases encompassing CCNE1 in fallopian tube carcinoma. *Oncogene* 2003; 22: 4281–4286.
124. Garnis C, Buys TP, Lam WL. Genetic alteration and gene expression modulation during cancer progression. *Mol Cancer* 2004; 3: 9,1–23. Review
125. Garnis C, Coe BP, Ishkanian A, Zhang L, Rosin MP, Lam WL. Novel regions of amplification on 8q distinct from the MYC locus and frequently altered in oral dysplasia and cancer. *Genes Chromosomes Cancer* 2004; 39: 93–98.
126. Garnis C, Baldwin C, Zhang L, Rosin MP, Lam WL. Use of complete coverage array comparative genomic hybridization to define copy number alterations on chromosome 3p in oral squamous cell carcinomas. *Cancer Res* 2003; 63: 8582–8585.
127. Coe BP, Henderson LJ, Garnis C, Tsao MS, Gazdar AF, Minna J, Lam S, Macaulay C, Lam WL. High-resolution chromosome arm 5p array CGH analysis of small cell lung carcinoma cell lines. *Genes Chromosomes Cancer* 2005; 42: 308–313.
128. Henderson LJ, Coe BP, Lee EH, Girard L, Gazdar AF, Minna JD, Lam S, Macaulay C, Lam WL. Genomic and gene expression profiling of minute alterations of chromosome arm 1p in small-cell lung carcinoma cells. *Br J Cancer* 2005; 92: 1553–1560.
129. Garnis C, Campbell J, Davies JJ, Macaulay C, Lam S, Lam WL. Involvement of multiple developmental genes on chromosome 1p in lung tumorigenesis. *Hum Mol Genet* 2005; 14: 475–482.
130. Mantripragada KK, Tapia-Paez I, Blennow E, Nilsson P, Wedell A, Dumanski JP. DNA copy-number analysis of the 22q11 deletion-syndrome region using array-CGH with genomic and PCR-based targets. *Int J Mol Med* 2004; 13: 273–239.
131. Bignell GR, Huang J, Greshock J, Watt S, Butler A, West S, Grigorova M, Jones KW, Wei W, Stratton MR, Futreal PA, Weber B, Shapero MH, Wooster R. High-resolution analysis of DNA copy number using oligonucleotide microarrays. *Genome Res* 2004; 14: 287–295.
132. Zhao X, Li C, Paez JG, Chin K, Janne PA, Chen TH, Girard L, Minna J, Christiani D, Leo C, Gray JW, Sellers WR, Meyerson M. An integrated view of copy number and allelic alterations in the cancer genome using single nucleotide polymorphism arrays. *Cancer Res* 2004; 64: 3060–3071.
133. Kennedy GC, Matsuzaki H, Dong S, Liu WM, Huang J, Liu G, Su X, Cao M, Chen W, Zhang J, Liu W, Yang G, Di X, Ryder T, He Z, Surti U, Phillips MS, Boyce-Jacino MT, Fodor SP, Jones KW. Large-scale genotyping of complex DNA. *Nat Biotechnol* 2003; 21: 1233–1237.
134. Matsuzaki H, Dong S, Loi H, Di X, Liu G, Hubbell E, Law J, Berntsen T, Chadha M, Hui H, Yang G, Kennedy GC, Webster TA, Cawley S, Walsh PS, Jones KW, Fodor SP, Mei R. Genotyping over 100,000 SNPs on a pair of oligonucleotide arrays. *Nat Methods* 2004; 1: 109–111.
135. Brennan C, Zhang Y, Leo C, Feng B, Cauwels C, Aguirre AJ, Kim M, Protopopov A, Chin L. High-resolution global profiling of genomic alterations with long oligonucleotide microarray. *Cancer Res* 2004; 64: 4744–4748.
136. Iafrate AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y, Scherer SW, Lee C. Detection of large-scale variation in the human genome. *Nat Genet* 2004; 36: 949–951.
137. Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P, Maner S, Massa H, Walker M, Chi M, Navin N, Lucito R, Healy J, Hicks J, Ye K, Reiner A, Gilliam TC, Trask B, Patterson N, Zetterberg A, Wigler M. Large-scale copy number polymorphism in the human genome. *Science* 2004; 305: 525–528.
138. Bertone P, Stolc V, Royce TE, Rozowsky JS, Urban AE, Zhu X, Rinn JL, Tongprasit W, Samanta M, Weissman S, Gerstein M, Snyder M. Global identification of human transcribed sequences with genome tiling arrays. *Science* 2004; 306: 2242–2246.
139. Daigo Y, Chin SF, Gorringe KL, Bobrow LG, Ponder BA, Pharoah PD, Caldas C. Degenerate oligonucleotide primed-polymerase chain reaction-based array comparative genomic hybridization for extensive amplicon profiling of breast cancers : a new approach for the molecular analysis of paraffin-embedded cancer tissue. *Am J Pathol* 2001; 158: 1623–1631.
140. Albertson DG. Profiling breast cancer by array CGH. *Breast Cancer Res Treat* 2003; 78: 289–298. Review.
141. van Beers EH, Nederlof PM. Array-CGH and breast cancer. *Breast Cancer Res* 2006; 8: 210 Review.

142. Hui AB, Lo KW, Yin XL, Poon WS, Ng HK. Detection of multiple gene amplifications in glioblastoma multiforme using array-based comparative genomic hybridization. *Lab Invest* 2001; 81: 717–723.
143. Pandita A, Zielinska M, Thorner P, Bayani J, Godbout R, Greenberg M, Squire JA. Application of comparative genomic hybridization, spectral karyotyping, and microarray analysis in the identification of subtype-specific patterns of genomic changes in rhabdomyosarcoma. *Neoplasia* 1999; 1: 262–275.
144. Hui AB, Lo KW, Teo PM, To KF, Huang DP. Genome wide detection of oncogene amplifications in nasopharyngeal carcinoma by array based comparative genomic hybridization. *Int J Oncol* 2002; 20: 467–473. Erratum in: *Int J Oncol* 2002; 21: 451.
145. Schraml P, Schwerdtfeger G, Burkhalter F, Raggi A, Schmidt D, Ruffalo T, King W, Wilber K, Mihatsch MJ, Moch H. Combined array comparative genomic hybridization and tissue microarray analysis suggest PAK1 at 11q13.5-q14 as a critical oncogene target in ovarian carcinoma. *Am J Pathol* 2003; 163: 985–992.
146. Tay ST, Leong SH, Yu K, Aggarwal A, Tan SY, Lee CH, Wong K, Visvanathan J, Lim D, Wong WK, Soo KC, Kon OL, Tan P. A combined comparative genomic hybridization and expression microarray analysis of gastric cancer reveals novel molecular subtypes. *Cancer Res* 2003; 63: 3309–3316.
147. Weiss MM, Snijders AM, Kuipers EJ, Ylstra B, Pinkel D, Meuwissen SG, van Diest PJ, Albertson DG, Meijer GA. Determination of amplicon boundaries at 20q13.2 in tissue samples of human gastric adenocarcinomas by high-resolution microarray comparative genomic hybridization. *J Pathol* 2003; 200: 320–326.
148. Dijkman R, Tensen CP, Jordanova ES, Knijnenburg J, Hoefnagel JJ, Mulder AA, Rosenberg C, Raap AK, Willemze R, Szuhai K, Vermeer MH. Array-based comparative genomic hybridization analysis reveals recurrent chromosomal alterations and prognostic parameters in primary cutaneous large B-cell lymphoma. *J Clin Oncol* 2006; 24: 296–305.
149. de Leeuw RJ, Davies JJ, Rosenwald A, Bebb G, Gascoyne RD, Dyer MJ, Staudt LM, Martinez-Climent JA, Lam WL. Comprehensive whole genome array CGH profiling of mantle cell lymphoma model genomes. *Hum Mol Genet* 2004; 13: 1827–1837.
150. Schraders M, Pfundt R, Straatman HM, Janssen IM, van Kessel AG, Schoenmakers EF, van Krieken JH, Groenen PJ. Novel chromosomal imbalances in mantle cell lymphoma detected by genome-wide array-based comparative genomic hybridization. *Blood* 2005; 105: 1686–1693.
151. Fritz B, Schubert F, Wrobel G, Schwaenen C, Wessendorf S, Nessling M, Korz C, Rieker RJ, Montgomery K, Kucherlapati R, Mechtersheimer G, Eils R, Joos S, Lichter P. Microarray-based copy number and expression profiling in dedifferentiated and pleomorphic liposarcoma. *Cancer Res* 2002; 62: 2993–2998.
152. Akervall J. Genomic screening of head and neck cancer and its implications for therapy planning. *Eur Arch Otorhinolaryngol* 2006; 263: 297–304.
153. Thorgeirsson SS, Lee JS, Grisham JW. Functional genomics of hepatocellular carcinoma. *Hepatology* 2006; 43: S145–S150. Review.
154. Buchholz M, Boeck W, Fensterer H, Muller F, Wenger C, Michl P, Adler G, Gress TM. Use of DNA arrays/microarrays in pancreatic research. *Pancreatology* 2001; 1: 581–586. Review.
155. Mosse YP, Greshock J, Weber BL, Maris JM. Measurement and relevance of neuroblastoma DNA copy number changes in the post-genome era. *Cancer Lett* 2005; 228: 83–90. Review.
156. Chen QR, Bilke S, Khan J. High-resolution cDNA microarray-based comparative genomic hybridization analysis in neuroblastoma. *Cancer Lett* 2005; 228: 71–81. Review.
157. Ohira M, Oba S, Nakamura Y, Hirata T, Ishii S, Nakagawara A. A review of DNA microarray analysis of human neuroblastomas. *Cancer Lett* 2005; 18: 228: 5–11. Review.
158. Scaruffi P, Valent A, Schramm A, Astrahanseff K, Eggert A, Tonini GP. Application of microarray-based technology to neuroblastoma. *Cancer Lett* 2005; 228: 13–20. Review.
159. Lossos IS, Alizadeh AA, Diehn M, Warnke R, Thorstenson Y, Oefner PJ, Brown PO, Botstein D, Levy R. Transformation of follicular lymphoma to diffuse large-cell lymphoma: alternative patterns with increased or decreased expression of c-myc and its regulated genes. *Proc Natl Acad Sci U S A* 2002; 99: 8886–8891.
160. Nyante SJ, Devries S, Chen YY, Hwang ES. Array-based comparative genomic hybridization of ductal carcinoma *in situ* and synchronous invasive lobular cancer. *Hum Pathol* 2004; 35: 759–763.

161. Van den Veyver IB, Beaudet AL. Comparative genomic hybridization and prenatal diagnosis. *Curr Opin Obstet Gynecol* 2006; 18: 185–191.
162. Lapierre JM, Tachdjian G. Detection of chromosomal abnormalities by comparative genomic hybridization. *Curr Opin Obstet Gynecol* 2005; 17: 171–177. Review.
163. Larrabee PB, Johnson KL, Pestova E, Lucas M, Wilber K, LeShane ES, Tantravahi U, Cowan JM, Bianchi DW. Microarray analysis of cell-free fetal DNA in amniotic fluid: a prenatal molecular karyotype. *Am J Hum Genet* 2004; 75: 485–491.
164. Wells D, Levy B. Cytogenetics in reproductive medicine: the contribution of comparative genomic hybridization (CGH). *Bioessays* 2003; 25: 289–300. Review.
165. Brisset S, Kasakyan S, L’Hermine AC, Mairovitz V, Gautier E, Aubry MC, Benkhaliha M, Tachdjian G. De novo monosomy 9p24.3-pter and trisomy 17q24.3-qter characterised by microarray comparative genomic hybridisation in a fetus with an increased nuchal translucency. *Prenat Diagn* 2006; 26: 206–213.
166. Glass IA, Rauen KA, Chen E, Parkes J, Alberston DG, Pinkel D, Cotter PD. Ring chromosome 15: characterization by array CGH. *Hum Genet* 2006; 118: 611–617.
167. Iqbal MA, Ramadan S, Ali FA, Kurdi W. Complex de novo cryptic subtelomeric rearrangements in a fetus with multiple ultrasonographic abnormalities and a normal karyotype at amniocentesis. *Prenat Diagn* 2005; 25: 1142–1149.
168. de Pater JM, Nikkels PG, Poot M, Eleveld MJ, Stigter RH, van der Sijjs-Bos CJ, Loneus WH, Engelen JJ. Striking facial dysmorphisms and restricted thymic development in a fetus with a 6-megabase deletion of chromosome 14q. *Pediatr Dev Pathol* 2005; 8: 497–503.
169. Le Caignec C, Boceno M, Saugier-Veber P, Jacquemont S, Joubert M, David A, Frebourg T, Rival JM. Detection of genomic imbalances by array based comparative genomic hybridisation in fetuses with multiple malformations. *J Med Genet* 2005; 42: 121–128.
170. Schaeffer AJ, Chung J, Heretis K, Wong A, Ledbetter DH, Lese Martin C. Comparative genomic hybridization-array analysis enhances the detection of aneuploidies and submicroscopic imbalances in spontaneous miscarriages. *Am J Hum Genet* 2004; 74: 1168–1174.
171. Ballif BC, Kashork CD, Saleki R, Rorem E, Sundin K, Bejjani BA, Shaffer LG. Detecting sex chromosome anomalies and common triploidies in products of conception by array-based comparative genomic hybridization. *Prenat Diagn* 2006; 26: 333–339.
172. Rickman L, Fiegler H, Shaw-Smith C, Nash R, Cirigliano V, Voglino G, Ng BL, Scott C, Whittaker J, Adinolfi M, Carter NP, Bobrow M. Prenatal detection of unbalanced chromosomal rearrangements by array CGH. *J Med Genet* 2006; 43: 353–361.
173. Le Caignec C, Spits C, Sermon K, De Rycke M, Thienpont B, Debrock S, Staessen C, Moreau Y, Fryns JP, Van Steirteghem A, Liebaers I, Vermeesch JR. Single-cell chromosomal imbalances detection by array CGH. *Nucleic Acids Res* 2006; 34: e68.
174. Rickman L, Fiegler H, Carter NP, Bobrow M. Prenatal diagnosis by array-CGH. *Eur J Med Genet* 2005; 48: 232–240. Review.
175. Siniscalco M, Robledo R, Orru S, Contu L, Yadav P, Ren Q, Lai H, Roe B. A plea to search for deletion polymorphism through genome scans in populations. *Trends Genet* 2000; 16: 435–437.
176. Barber JC, Joyce CA, Collinson MN, Nicholson JC, Willatt LR, Dyson HM, Bateman MS, Green AJ, Yates JR, Dennis NR. Duplication of 8p23.1: a cytogenetic anomaly with no established clinical significance. *J Med Genet* 1998; 35: 491–496.
177. Ghanem N, Uring-Lambert B, Abbal M, Hauptmann G, Lefranc MP, Lefranc G. Polymorphism of MHC class III genes: definition of restriction fragment linkage groups and evidence for frequent deletions and duplications. *Hum Genet* 1988; 79: 209–218.
178. Groot PC, Mager WH, Frants RR. Interpretation of polymorphic DNA patterns in the human alpha-amylase multigene family. *Genomics* 1991; 10: 779–785.
179. Engelen JJ, Moog U, Evers JL, Dassen H, Albrechts JC, Hamers AJ. Duplication of chromosome region 8p23.1-->p23.3: a benign variant? *Am J Med Genet* 2000; 91: 18–21.

180. Lin H, Pizer ES, Morin PJ. A frequent deletion polymorphism on chromosome 22q13 identified by representational difference analysis of ovarian cancer. *Genomics* 2000; 69: 391–394.
181. Buckland PR. Polymorphically duplicated genes: their relevance to phenotypic variation in humans. *Ann Med* 2003; 35: 308–315. Review.
182. Ciccoira M, Rossi A, Bonapace S, Zanolla L, Perrot A, Francis DP, Golia G, Franceschini L, Osterziel KJ, Zardini P. Effects of ACE gene insertion/deletion polymorphism on response to spironolactone in patients with chronic heart failure. *Am J Med* 2004; 116: 657–661.
183. Ryu B, Song J, Sohn T, Hruban RH, Kern SE. Frequent germline deletion polymorphism of chromosomal region 8p12-p21 identified as a recurrent homozygous deletion in human tumors. *Genomics* 2001; 72: 108–112.
184. Carrington M, Dean M, Martin MP, O'Brien SJ. Genetics of HIV-1 infection: chemokine receptor CCR5 polymorphism and its consequences. *Hum Mol Genet* 1999; 8: 1939–1945. Review.
185. Ji Y, Eichler EE, Schwartz S, Nicholls RD. Structure of chromosomal duplicons and their role in mediating human genomic disorders. *Genome Res* 2000; 10: 597–610. Review.
186. Cheung J, Estivill X, Khaja R, MacDonald JR, Lau K, Tsui LC, Scherer SW. Genome-wide detection of segmental duplications and potential assembly errors in the human genome sequence. *Genome Biol* 2003; 4: R25.
187. Lupski JR. Genomic disorders: structural features of the genome can lead to DNA rearrangements and human disease traits. *Trends Genet* 1998; 14: 417–22. Review.
188. Buckley PG, Mantripragada KK, Piotrowski A, Diaz de Stahl T, Dumanski JP. Copy-number polymorphisms: mining the tip of an iceberg. *Trends Genet* 2005; 21: 315–317. Review.
189. Locke DP, Segraves R, Carbone L, Archidiacono N, Albertson DG, Pinkel D, Eichler EE. Large-scale variation among human and great ape genomes determined by array comparative genomic hybridization. *Genome Res* 2003; 13: 347–357.
190. Fortna A, Kim Y, McLaren E, Marshall K, Hahn G, Meltesen L, Brenton M, Hink R, Burgers S, Hernandez-Boussard T, Karimpour-Fard A, Glueck D, McGavran L, Berry R, Pollack J, Sikela JM. Lineage-specific gene duplication and loss in human and great ape evolution. *PLoS Biol* 2004; 2: E207.
191. de Vries BB, Pfundt R, Leisink M, Koolen DA, Vissers LE, Janssen IM, Reijmersdal S, Nillesen WM, Huys EH, Leeuw N, Smeets D, Sistermans EA, Feurth T, van Ravenswaaij-Arts CM, van Kessel AG, Schoenmakers EF, Brunner HG, Veltman JA. Diagnostic genome profiling in mental retardation. *Am J Hum Genet* 2005; 77: 606–616.
192. Schoumans J, Ruivenkamp C, Holmberg E, Kyllerman M, Anderlid BM, Nordenskjold M. Detection of chromosomal imbalances in children with idiopathic mental retardation by array based comparative genomic hybridisation (array-CGH). *J Med Genet* 2005; 42: 699–705.
193. Feuk L, MacDonald JR, Tang T, Carson AR, Li M, Rao G, Khaja R, Scherer SW. Discovery of human inversion polymorphisms by comparative analysis of human and chimpanzee DNA sequence assemblies. *PLoS Genet* 2005; 1: e56.
194. Tuzun E, Sharp AJ, Bailey JA, Kaul R, Morrison VA, Pertz LM, Haugen E, Hayden H, Albertson D, Pinkel D, Olson MV, Eichler EE. Fine-scale structural variation of the human genome. *Nat Genet* 2005; 37: 727–732.
195. Sharp AJ, Locke DP, McGrath SD, Cheng Z, Bailey JA, Vallente RU, Pertz LM, Clark RA, Schwartz S, Segraves R, Oseroff VV, Albertson DG, Pinkel D, Eichler EE. Segmental duplications and copy-number variation in the human genome. *Am J Hum Genet* 2005; 77: 78–88.
196. <http://projects.tcag.ca/variation/>
197. Conrad DF, Andrews TD, Carter NP, Hurles ME, Pritchard JK. A high-resolution survey of deletion polymorphism in the human genome. *Nat Genet* 2006; 38: 75–81.
198. Hinds DA, Kloek AP, Jen M, Chen X, Frazer KA. Common deletions and SNPs are in linkage disequilibrium in the human genome. *Nat Genet* 2006; 38: 82–85.
199. <http://www.sanger.ac.uk/PostGenomics/decipher/>
200. HapMap Project: <http://www.hapmap.org/>
201. dbRIP: <http://falcon.roswellpark.org:9090/>
202. Human Structural Variation Database: <http://humanparalogy.gs.washington.edu/structuralvariation/>
203. The Chromosome Anomaly Collection: <http://www.som.soton.ac.uk/research/geneticsdiv/Anomaly%20Register/>

204. Barber JC. Directly transmitted unbalanced chromosome abnormalities and euchromatic variants. *J Med Genet* 2005; 42: 609–629. Review.
205. ISCN (2016): *An International System for Human Cytogenomic Nomenclature*. McGowan-Jordan J, Simons A, Schmid M (eds). Basel: S Karger, 2016.
206. Dave BJ, Sanger WG. Role of Cytogenetics and Molecular Cytogenetics in the diagnosis of genetic imbalances. *Semin Pediatr Neurol* 2007; 14: 2–6. Review.
207. Aradhya S, Cherry AM. Array-based comparative genomic hybridization: clinical contexts for targeted and whole-genome designs. *Genet Med* 2007; 9: 553–559. Review.
208. Tabor HK, Cho MK. Ethical implications of array comparative genomic hybridization in complex phenotypes: points to consider in research. *Genet Med* 2007; 9: 626–631. Review.
209. Michels E, De Preter K, Van Roy N, Speleman F. Detection of DNA copy number alterations in cancer by array comparative genomic hybridization. *Genet Med* 2007; 9: 574–584. Review.
210. Lee C, Iafrate AJ, Brothman AR. Copy number variations and clinical cytogenetic diagnosis of constitutional disorders. *Nat Genet* 2007; 39(Suppl.): S48–S54. Review.
211. Rodriguez-Revenga L, Mila M, Rosenberg C, Lamb A, Lee C. Structural variation in the human genome: the impact of copy number variants on clinical diagnosis. *Genet Med* 2007; 9: 600–606. Review.
212. Thorland EC, Gonzales PR, Gliem TJ, Wiktor AE, Ketterling RP. Comprehensive validation of array comparative genomic hybridization platforms: how much is enough? *Genet Med* 2007; 9: 632–641. Review.
213. Manning M, Hudgins L. Use of array-based technology in the practice of medical genetics. *Genet Med* 2007; 9: 650–653.
214. Shaffer LG, Beaudet AL, Brothman AR, Hirsch B, Levy B, Martin CL, Mascarello JT, Rao KW; Working Group of the Laboratory Quality Assurance Committee of the American College of Medical Genetics. Microarray analysis for constitutional cytogenetic abnormalities. *Genet Med* 2007; 9: 654–662.
215. Darilek S, Ward P, Pursley A, Plunkett K, Furman P, Magoulas P, Patel A, Cheung SW, Eng CM. Pre- and postnatal genetic testing by array-comparative genomic hybridization: genetic counseling perspectives. *Genet Med* 2008; 10: 13–18. Review.
216. Kallioniemi A. CGH microarrays and cancer. *Curr Opin Biotechnol* 2008; 19: 36–40. Review.
217. Costa JL, Meijer G, Ylstra B, Caldas C. Array comparative genomic hybridization copy number profiling: a new tool for translational research in solid malignancies. *Semin Radiat Oncol* 2008; 18: 98–104. Review.
218. Zenz T, Mertens D, Döhner H, Stilgenbauer S. Molecular diagnostics in chronic lymphocytic leukemia - pathogenetic and clinical implications. *Leuk Lymphoma* 2008; 49: 864–873. Review.
219. Baldwin EL, Lee JY, Blake DM, Bunke BP, Alexander CR, Kogan AL, Ledbetter DH, Martin CL. Enhanced detection of clinically relevant genomic imbalances using a targeted plus whole genome oligonucleotide microarray. *Genet Med* 2008; 10: 415–429.
220. Slavotinek AM. Novel microdeletion syndromes detected by chromosome microarrays. *Hum Genet* 2008; 124: 1–17. Review.
221. Van den Veyver IB, Patel A, Shaw CA, Pursley AN, Kang SH, Simovich MJ, Ward PA, Darilek S, Johnson A, Neill SE, Bi W, White LD, Eng CM, Lupski JR, Cheung SW, Beaudet AL. Clinical use of array comparative genomic hybridization (CMA) for prenatal diagnosis in 300 cases. *Prenat Diagn* 2009; 29: 29–39.
222. Sagoo GS, Butterworth AS, Sanderson S, Shaw-Smith C, Higgins JP, Burton H. Array CGH in patients with learning disability (mental retardation) and congenital anomalies: updated systematic review and meta-analysis of 19 studies and 13,926 subjects. *Genet Med* 2009; 11: 139–146. Review.
223. Chen X, Jorgenson E, Cheung ST. New tools for functional genomic analysis. *Drug Discov Today* 2009; 14(15–16): 754–760. Review.
224. Maciejewski JP, Tiu RV, O'Keefe C. Application of array-based whole genome scanning technologies as a cytogenetic tool in haematological malignancies. *Br J Haematol* 2009; 146: 479–488. Review.
225. Miller DT, Adam MP, Aradhya S, Biesecker LG, Brothman AR, Carter NP, Church DM, Crolla JA, Eichler EE, Epstein CJ, Fauchet WA, Feuk L, Friedman JM, Hamosh A, Jackson L, Kaminsky EB, Kok K, Krantz ID, Kuhn RM, Lee C, Ostell JM, Rosenberg C, Scherer SW, Spinner NB, Stavropoulos DJ, Tepperberg JH, Thorland EC, Vermeesch JR,

- Waggoner DJ, Watson MS, Martin CL, Ledbetter DH. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. *Am J Hum Genet* 2010; 86: 749–764. Review.
226. Simpson JL. Preimplantation genetic diagnosis at 20 years. *Prenat Diagn* 2010; 30: 682–695. Review.
 227. Seto M. Genomic profiles in B cell lymphoma. *Int J Hematol* 2010; 92: 238–245. Review.
 228. Morrow EM. Genomic copy number variation in disorders of cognitive development. *J Am Acad Child Adolesc Psychiatry* 2010; 49: 1091–1104. Review.
 229. Rosenfeld JA, Ballif BC, Torchia BS, Sahoo T, Ravnan JB, Schultz R, Lamb A, Bejjani BA, Shaffer LG. Copy number variations associated with autism spectrum disorders contribute to a spectrum of neurodevelopmental disorders. *Genet Med* 2010; 12: 694–702.
 230. Manning M, Hudgins L. Professional Practice and Guidelines Committee. Array-based technology and recommendations for utilization in medical genetics practice for detection of chromosomal abnormalities. *Genet Med* 2010; 12: 742–745.
 231. Boone PM, Bacino CA, Shaw CA, Eng PA, Hixson PM, Pursley AN, Kang SH, Yang Y, Wiszniewska J, Nowakowska BA, del Gaudio D, Xia Z, Simpson-Patel G, Immken LL, Gibson JB, Tsai AC, Bowers JA, Reimschisel TE, Schaaf CP, Potocki L, Scaglia F, Gambin T, Sykulski M, Bartnik M, Derwinska K, Wisniewiecka-Kowalnik B, Lalani SR, Probst FJ, Bi W, Beaudet AL, Patel A, Lupski JR, Cheung SW, Stankiewicz P. Detection of clinically relevant exonic copy-number changes by array CGH. *Hum Mutat* 2010; 31: 1326–1342.
 232. Yang JY, Niemierko A, Bajcsy R, Xu D, Athey BD, Zhang A, Ersoy OK, Li GZ, Borodovsky M, Zhang JC, Arabnia HR, Deng Y, Dunker AK, Liu Y, Ghafoor A. 2K09 and thereafter: the coming era of integrative bioinformatics, systems biology and intelligent computing for functional genomics and personalized medicine research. *BMC Genomics* 2010; 11(Suppl. 3): I1.
 233. Hillman SC, Pretlove S, Coomarasamy A, McMullan DJ, Davison EV, Maher ER, Kilby MD. Additional information from array comparative genomic hybridization technology over conventional karyotyping in prenatal diagnosis: a systematic review and meta-analysis. *Ultrasound Obstet Gynecol* 2011; 37: 6–14. Review.
 234. Tuefferd M, de Bondt A, Van den Wyngaert I, Talloen W, Göhlmann H. Microarray profiling of DNA extracted from FFPE tissues using SNP 6.0 Affymetrix platform. *Methods Mol Biol* 2011; 724: 147–160.
 235. Mullighan CG. Single nucleotide polymorphism microarray analysis of genetic alterations in cancer. *Methods Mol Biol* 2011; 730: 235–258.
 236. Thiel A, Beier M, Ingenhag D, Servan K, Hein M, Moeller V, Betz B, Hildebrandt B, Evers C, Germing U, Royer-Pokora B. Comprehensive array CGH of normal karyotype myelodysplastic syndromes reveals hidden recurrent and individual genomic copy number alterations with prognostic relevance. *Leukemia* 2011; 25: 387–399.
 237. Papenhausen P, Schwartz S, Risheg H, Keitges E, Gadi I, Burnside RD, Jaswaney V, Pappas J, Pasion R, Friedman K, Tepperberg J. UPD detection using homozygosity profiling with a SNP genotyping microarray. *Am J Med Genet A* 2011; 155: 757–768.
 238. Pinto D, Darvishi K, Shi X, Rajan D, Rigler D, Fitzgerald T, Lionel AC, Thiruvahindrapuram B, Macdonald JR, Mills R, Prasad A, Noonan K, Gribble S, Prigmore E, Donahoe PK, Smith RS, Park JH, Hurles ME, Carter NP, Lee C, Scherer SW, Feuk L. Comprehensive assessment of array-based platforms and calling algorithms for detection of copy number variants. *Nat Biotechnol*. 2011; 29: 512–520.
 239. Kearney HM, South ST, Wolff DJ, Lamb A, Hamosh A, Rao KW; Working Group of the American College of Medical Genetics. American College of Medical Genetics. Recommendations for the design and performance expectations for clinical genomic copy number microarrays intended for use in the postnatal setting for detection of constitutional abnormalities. *Genet Med* 2011; 13: 676–679.
 240. Kearney HM, Thorland EC, Brown KK, Quintero-Rivera F, South ST; Working Group of the American College of Medical Genetics Laboratory Quality Assurance Committee. American College of Medical Genetics standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants. *Genet Med* 2011; 13: 680–685.
 241. Rehder CW, David KL, Hirsch B, Toriello HV, Wilson CM, Kearney HM. American College of Medical Genetics and Genomics: Standards and guidelines for documenting suspected consanguinity as an incidental finding of genomic testing. *Genet Med* 2013; 15: 150–152.

242. Cooley LD, Lebo M, Li MM, Slovak ML, Wolff DJ; Working Group of the American College of Medical Genetics and Genomics (ACMG) Laboratory Quality Assurance Committee. American College of Medical Genetics and Genomics technical standards and guidelines: microarray analysis for chromosome abnormalities in neoplastic disorders. *Genet Med* 2013; 15: 484–494.
243. South ST, Lee C, Lamb AN, Higgins AW, Kearney HM. Working Group for the American College of Medical Genetics and Genomics Laboratory Quality Assurance Committee. ACMG Standards and Guidelines for Constitutional Cytogenomic microarray analysis, including postnatal and prenatal applications: revision 2013. *Genet Med* 2013; 15: 901–909.
244. Pyatt RE, Astbury C. Interpretation of copy number alterations identified through clinical microarray-comparative genomic hybridization. *Clin Lab Med* 2011;31:565–580. Review.
245. Sato-Otsubo A, Sanada M, Ogawa S. Single-nucleotide polymorphism array karyotyping in clinical practice: where, when, and how? *Semin Oncol* 2012; 39: 13–25. Review.
246. Brady PD, Vermeesch JR. Genomic microarrays: a technology overview. *Prenat Diagn* 2012; 32: 336–343. Review.
247. McDonnell SK, Riska SM, Klee EW, Thorland EC, Kay NE, Thibodeau SN, Parker AS, Eckel-Passow JE. Experimental designs for array comparative genomic hybridization technology. *Cytogenet Genome Res* 2013; 139: 250–257. Review.
248. Schaaf CP, Wiszniewska J, Beaudet AL. Copy number and SNP arrays in clinical diagnostics. *Annu Rev Genomics Hum Genet*. 2011; 12: 25–51. Review.
249. Bench AJ. The role of molecular genetic analysis within the diagnostic haemato-oncology laboratory. *Int J Lab Hematol* 2012; 34: 21–34. Review.
250. Simons A, Sikkema-Raddatz B, de Leeuw N, Konrad NC, Hastings RJ, Schoumans J. Genome-wide arrays in routine diagnostics of hematological malignancies. *Hum Mutat* 2012; 33: 941–948.
251. Wheeler HE, Maitland ML, Dolan ME, Cox NJ, Ratain MJ. Cancer pharmacogenomics: strategies and challenges. *Nat Rev Genet* 2013; 14: 23–34. Review.
252. Das K, Tan P. Molecular cytogenetics: recent developments and applications in cancer. *Clin Genet*. 2013; 84: 315–325.
253. Lamb AN. Laboratory aspects of prenatal microarray analysis. *Clin Lab Med*. 2011; 31: 615–630, ix. Review.
254. Wapner RJ, Martin CL, Levy B, Ballif BC, Eng CM, Zachary JM, Savage M, Platt LD, Saltzman D, Grobman WA, Klugman S, Scholl T, Simpson JL, McCall K, Aggarwal VS, Bunke B, Nahum O, Patel A, Lamb AN, Thom EA, Beaudet AL, Ledbetter DH, Shaffer LG, Jackson L. Chromosomal microarray versus karyotyping for prenatal diagnosis. *N Engl J Med* 2012; 367: 2175–2184.
255. Wei Y, Xu F, Li P. Technology-driven and evidence-based genomic analysis for integrated pediatric and prenatal genetics evaluation. *J Genet Genomics* 2013; 40: 1–14. Review.
256. Reddy UM, Page GP, Saade GR, Silver RM, Thorsten VR, Parker CB, Pinar H, Willinger M, Stoll BJ, Heim-Hall J, Varner MW, Goldenberg RL, Bukowski R, Wapner RJ, Drews-Botsch CD, O'Brien BM, Dudley DJ, Levy B; NICHD Stillbirth Collaborative Research Network. Karyotype versus microarray testing for genetic abnormalities after stillbirth. *N Engl J Med* 2012; 367: 2185–2193.
257. de Leeuw N, Hehir-Kwa JY, Simons A, Geurts van Kessel A, Smeets DF, Faas BH, Pfundt R. SNP array analysis in constitutional and cancer genome diagnostics—copy number variants, genotyping and quality control. *Cytogenet Genome Res* 2011; 135: 212–221. Review.
258. Southard AE, Edelmann LJ, Gelb BD. Role of copy number variants in structural birth defects. *Pediatrics* 2012; 129: 755–763. Review.
259. Bochud M. Genetics for clinicians: from candidate genes to whole genome scans (technological advances). *Best Pract Res Clin Endocrinol Metab* 2012; 26: 119–132. Review.
260. Sund KL, Zimmerman SL, Thomas C, Mitchell AL, Prada CE, Grote L, Bao L, Martin LJ, Smolarek TA. Regions of homozygosity identified by SNP microarray analysis aid in the diagnosis of autosomal recessive disease and incidentally detect parental blood relationships. *Genet Med* 2013; 15: 70–78.
261. Katsanis SH, Katsanis N. Molecular genetic testing and the future of clinical genomics. *Nat Rev Genet* 2013; 14: 415–426. Review.
262. Ellison JW, Rosenfeld JA, Shaffer LG. Genetic basis of intellectual disability. *Annu Rev Med* 2013; 64: 441–450. Review.

CHAPTER 19

Mathematics for the cytogenetic technologist

Patricia K. Dowling

Department/Cytogenetics, Pathline Emerge Pathology Services, Ramsey, NJ, USA

19.1 General concepts

Many aspects of life in the cytogenetics laboratory involve the use of mathematics. One of the most important tasks is the accurate preparation of reagents. This requires knowledge about how to prepare solutions and dilutions. In other areas of the laboratory it may be important to understand how to approximate cell concentration using a hemacytometer, how to set up a dose response experiment when testing a new reagent, how to determine the quantity of DNA in a sample for use in a chromosome microarray, or how to determine the normal cut-off for a new FISH probe. All these tasks require the use of numbers and formulas.

Not all technologists perform these tasks with enough frequency to have committed the necessary steps to memory. Some technologists, not unlike the population at large, have an aversion to anything mathematical. This chapter will serve as a resource to explain the mathematical manipulations that are likely to be encountered in a cytogenetics laboratory.

19.1.1 Scientific notation

Before revealing how to make the perfect solution, it is important to review some basic information about scientific notation (how numbers are expressed and manipulated in the laboratory). Except for “46” and thereabouts, numbers used in genetics tend to be very large or very small. There are approximately 6,000,000,000 base pairs of DNA in the 46 human chromosomes. The weight of these base pairs from one diploid cell is approximately 0.000000000070 grams. Some reagents are used at concentrations of 0.000006 M. It is customary to initiate bone marrow cultures with approximately 1,000,000 cells per milliliter of culture medium.

Scientific notation is based on the powers of 10 (like the metric system). The two parts of scientific notation are the **mantissa**, the number remaining after factoring out 10 (dividing by 10) as many times as possible, and the **exponent**, the number of times 10 was factored out. The number 1,230,000 expressed in scientific notation is 1.23×10^6 ; 1.23 is the mantissa and 6 is the exponent (10 raised to a power of 6). The number 0.0000123 expressed in scientific notation is 1.23×10^{-5} . It is useful to appreciate the simplicity and symmetry of the powers of 10 (see Table 19.1).

It is simple to convert a conventionally written number to scientific notation. For numbers greater than 1, place a decimal point at the end of the number. Move the decimal point leftward until one or two nonzero numbers are positioned to the left of the decimal point. This becomes the mantissa. Count the times the decimal point was shifted leftward. This becomes the exponent of 10. When the decimal point moves to the left the resulting exponent is positive.

Table 19.1 The powers of 10. Understanding the symbolic representation for macro and micro weights and measures is critical to all scientific fields

1×10^6	1,000,000	$\frac{1,000,000}{1}$	$\frac{10^6}{1}$	M	mega-
1×10^5	100,000	$\frac{100,000}{1}$	$\frac{10^5}{1}$		
1×10^4	10,000	$\frac{10,000}{1}$	$\frac{10^4}{1}$		
1×10^3	1,000	$\frac{1,000}{1}$	$\frac{10^3}{1}$	k	kilo-
1×10^2	100	$\frac{100}{1}$	$\frac{10^2}{1}$	h	hecto-
1×10^1	10	$\frac{10}{1}$	$\frac{10^1}{1}$	da	deca-
1×10^0	1	$\frac{1}{1}$	$\frac{10^0}{1}$		
1×10^{-1}	0.1	$\frac{1}{10}$	$\frac{10^{-1}}{1}$	d	deci-
1×10^{-2}	0.01	$\frac{1}{100}$	$\frac{10^{-2}}{1}$	c	centi-
1×10^{-3}	0.001	$\frac{1}{1000}$	$\frac{10^{-3}}{1}$	m	milli-
1×10^{-4}	0.0001	$\frac{1}{10,000}$	$\frac{10^{-4}}{1}$		
1×10^{-5}	0.00001	$\frac{1}{100,000}$	$\frac{10^{-5}}{1}$		
1×10^{-6}	0.000001	$\frac{1}{1,000,000}$	$\frac{10^{-6}}{1}$	μ	micro-

EXAMPLE 19.1 SCIENTIFIC NOTATION FOR NUMBERS GREATER THAN 10

How is 156,347 written in scientific notation?

156347. Place a decimal point at the end.

15634.7 → 1563.47 → 156.347 → 15.6347 → 1.56347

Count the times the decimal point was shifted leftward.

1.56347×10^5

Alternatively, using a calculator, enter the number and divide by 10. Continue to press the <Enter> [or <= >] key. Count the number of times the decimal point moves to the left until one or two nonzero numbers remain to the left of the decimal point. This number is the exponent to which 10 is raised.

For numbers less than 1, move the decimal point to the right until one or two nonzero numbers are positioned to the left of the decimal point. Count the times the decimal point was shifted rightward. This becomes the exponent of 10. When the decimal point moves to the right, the resulting exponent is negative.

EXAMPLE 19.2 SCIENTIFIC NOTATION FOR NUMBERS LESS THAN 1

How is 0.00001563 written in scientific notation?

0.00001563

$0.00001563 \rightarrow 000.001563 \rightarrow 0000.01563 \rightarrow 00000.1563 \rightarrow 000001.563$

Count the times the decimal point was shifted rightward.

1.563×10^{-5}

19.1.2 Manipulating numbers written in scientific notation**Addition and subtraction**

When adding or subtracting numbers written in scientific notation, if the exponents are not identical, convert all numbers to the same power of 10, then add or subtract the mantissas. The exponent will not change.

EXAMPLE 19.3 ADDING AND SUBTRACTING IN SCIENTIFIC NOTATION

Example 19.3 (a) Solve the following: $(6.5 \times 10^4) + (3.8 \times 10^2)$

Convert 3.8×10^2 to 0.038×10^4 by moving the decimal point leftward two times (leftward movement results in a positive exponent change from 2 to 4).

Add: $(6.5 \times 10^4) + (0.038 \times 10^4)$

Result: 6.538×10^4

To solve with a calculator, perform the following steps:

$6.5 \text{ EXP } 4 + 3.8 \text{ EXP } 2 =$ [If necessary, convert 65380 to 6.538×10^4]

Example 19.3 (b) Solve the following: $(6.4 \times 10^{-2}) - (2.8 \times 10^{-3})$

Convert 6.4×10^{-2} to 64×10^{-3} by moving the decimal point rightward one time (rightward movement results in a negative exponent change from -2 to -3).

Subtract: $(64 \times 10^{-3}) - (2.8 \times 10^{-3})$

Result: $61.2 \times 10^{-3} = 6.1 \times 10^{-2}$

To solve with a calculator, perform the following steps:

$6.4 \text{ EXP } 2 [+/-] - 2.8 \text{ EXP } 3 [+/-] =$ [If necessary, convert 0.0612 to 6.1×10^{-2}]

Multiplication and division

The following rules apply when multiplying and dividing numbers written in scientific notation:

Multiply the mantissas, add the exponents.

Divide the mantissas (numerator divided by the denominator), subtract the exponents (numerator minus denominator).

EXAMPLE 19.4 MULTIPLYING AND DIVIDING IN SCIENTIFIC NOTATION

Example 19.4 (a) Solve the following: $(3 \times 10^4) \times (4 \times 10^2)$

Multiply the mantissas: $3 \times 4 = 12$

Add the exponents: $4 + 2 = 6$

Result: $12 \times 10^6 = 1.2 \times 10^7$

To solve with a calculator, perform the following steps:

$3 \text{ EXP } 4 \times 4 \text{ EXP } 2 =$ [If necessary, convert 12000000 to 1.2×10^7]

Example 19.4 (b) Solve the following: $(6 \times 10^{-3}) \times (6 \times 10^4)$

Multiple the mantissas: $6 \times 6 = 36$

Add the exponents: $-3 + 4 = 1$

Result: $36 \times 10^1 = 3.6 \times 10^2$

To solve with a calculator, perform the following steps:

$6 \text{ EXP } 3 [+/-] \times 6 \text{ EXP } 4 [+/-]$ = [If necessary, convert 360 to 3.6×10^2]

Example 19.4 (c) Solve the following: $(5 \times 10^{-3}) \times (8 \times 10^{-2})$

Multiple the mantissas: $5 \times 8 = 40$

Add the exponents: $-3 + -2 = -5$

Result: $40 \times 10^{-5} = 4.0 \times 10^{-4}$

To solve with a calculator, perform the following steps:

$5 \text{ EXP } 3 [+/-] \times 8 \text{ EXP } 2 [+/-]$ = [If necessary, convert 0.0004 to 4×10^{-4}]

Example 19.4 (d) Solve the following: $(9 \times 10^4) / (3 \times 10^2)$

Divide the mantissas: $9 / 3 = 3$

Subtract the exponents: $4 - 2 = 2$

Result: 3×10^2

To solve with a calculator, perform the following steps:

$9 \text{ EXP } 4 / 3 \text{ EXP } 2$ = [If necessary, convert 300 to 3×10^2]

Example 19.4 (e) Solve the following: $(6 \times 10^3) / (3 \times 10^{-2})$

Divide the mantissas: $6 / 3 = 2$

Subtract the exponents: $3 - (-2) = 5$

Result: 2×10^5

To solve with a calculator, perform the following steps:

$6 \text{ EXP } 3 / 3 \text{ EXP } 2$ = [If necessary, convert 200000 to 2×10^5]

Example 19.4 (f) Solve the following: $(5 \times 10^{-5}) / (2.5 \times 10^{-2})$

Divide the mantissas: $5 / 2.5 = 2$

Subtract the exponents: $-5 - (-2) = -3$

Result: 2×10^{-3}

To solve with a calculator, perform the following steps:

$5 \text{ EXP } 5 [+/-] / 2.5 \text{ EXP } 2 [+/-]$ = [If necessary, convert 0.002 to 2×10^{-3}]

19.1.3 Unit conversion

It is not unusual to find that the mass of a reagent (how much the reagent weighs) is expressed in units different from those of the desired solution concentration. In these instances, converting to identical units is recommended. The units of mass that are likely to be encountered in a cytogenetics laboratory and the conversion factors are as follows:

$$1\text{kg} = 1000\text{g} = 1 \times 10^3\text{g}$$

$$1\text{g} = 1000\text{mg} = 1 \times 10^3\text{mg}$$

$$1\text{g} = 1,000,000\text{\mu g} = 1 \times 10^6\text{\mu g}$$

$$1\text{mg} = 0.001\text{g} = 1 \times 10^{-3}\text{g}$$

$$1\text{\mu g} = 0.000001\text{g} = 1 \times 10^{-6}\text{g}$$

EXAMPLE 19.5 CONVERTING MILLIGRAMS (mg) TO MICROGRAMS (μg)

Example 19.5 (a) The mass of a reagent is 100 mg. The final concentration of the solution to be prepared is expressed in μg . How many μg are present in 100 mg?

Write the equation:

$$\begin{aligned} 100\text{mg} &= n \mu\text{g} \\ 1 \times 10^2 \text{mg} &= n \mu\text{g} \end{aligned}$$

Any quantity multiplied or divided by 1 does not change (these are referred to as unit fractions). A unit divided by the same unit is eliminated from the equation (cancelled out). There are two units included in the equation, so two conversions must be made. The only unit remaining after multiplying by unit fractions and canceling out unwanted units will be the required unit (the one following n).

Write the equation with the required conversion factors (unit fractions):

$$1 \times 10^2 \text{mg} \times \frac{1\text{g}}{1 \times 10^3 \text{mg}} \times \frac{1 \times 10^6 \mu\text{g}}{1\text{g}} = n \mu\text{g}$$

Cancel the appropriate units.

$$1 \times 10^2 \text{mg} \times \frac{1\text{g}}{1 \times 10^3 \text{mg}} \times \frac{1 \times 10^6 \mu\text{g}}{1\text{g}} = n \mu\text{g}$$

Remembering the rules for multiplication and division in scientific notation, the equation becomes:

$$\begin{aligned} \frac{(1 \times 1 \times 1) \times 10^{(2+6)}}{(1 \times 1) \times 10^3} \mu\text{g} &= n \mu\text{g} \\ \frac{1 \times 10^8}{1 \times 10^3} \mu\text{g} &= n \mu\text{g} \\ 1 \times 10^{(8-3)} \mu\text{g} &= n \mu\text{g} \\ 1 \times 10^5 \mu\text{g} &= n \mu\text{g} \\ \text{Result : } 100\text{mg} &= 1 \times 10^5 \mu\text{g} \end{aligned}$$

Example 19.5 (b) How is 2 g/L expressed in $\mu\text{g}/\text{mL}$?

$$\text{Write the equation : } 2 \frac{\text{g}}{\text{L}} = n \frac{\mu\text{g}}{\text{mL}}$$

Write the equation with the required conversion factors (unit fractions):

$$2 \frac{\text{g}}{\text{L}} \times \frac{1\text{L}}{1 \times 10^3 \text{mL}} \times \frac{1 \mu\text{g}}{1 \times 10^{-6} \text{g}} = n \frac{\mu\text{g}}{\text{mL}}$$

Cancel the appropriate units.

$$2 \frac{\text{g}}{\text{L}} \times \frac{1\text{L}}{1 \times 10^3 \text{mL}} \times \frac{1 \mu\text{g}}{1 \times 10^{-6} \text{g}} = n \frac{\mu\text{g}}{\text{mL}}$$

Remembering the rules for multiplication and division in scientific notation, the equation becomes:

$$\frac{(2 \times 1 \times 1) \times 10^0 \mu\text{g}}{(1 \times 1 \times 1) \times 10^{(3+6)} \text{mL}} = n \frac{\mu\text{g}}{\text{mL}}$$

This becomes:

$$\frac{2 \times 10^0 \mu\text{g}}{1 \times 10^{-3} \text{mL}} = n \frac{\mu\text{g}}{\text{mL}}$$

The result is:

$$\begin{aligned} 2 \times 10^{(0-(-3))} \frac{\mu\text{g}}{\text{mL}} &= 2 \times 10^3 \frac{\mu\text{g}}{\text{mL}} \\ 2 \frac{\text{g}}{\text{L}} &= 2 \times 10^3 \frac{\mu\text{g}}{\text{mL}} \end{aligned}$$

19.2 Solutions

Solutions are found in abundance in the cytogenetics laboratory. The correct preparation of solutions is crucial to obtaining consistently high quality results. Not all solutions are made frequently enough for the technologist to remember the exact amount of ingredients to use, and unless this skill is practiced often, the various formulas may not be easily recalled. While the process is not exceedingly complicated, even the thought of having to use mathematical formulas and to figure out equations can be a daunting task to math-phobic cytogenetic technologists. This section will provide assistance for all facets of solution-making. An especially excellent resource for this purpose is included in the book *Lab Math: A Handbook of Measurements, Calculations, and Other Quantitative Skills for Use at the Bench*, by Dany Spencer Adams [1]. This section will include aspects of Chapter 4 of *Lab Math* that are germane and beyond improvement.

19.2.1 Definitions

The meaning of several terms should be understood by any technologist charged with solution-making.

Diluent – the medium of solvent that is added to a concentrated solution to dilute it

Diluting 1 : X – diluting according to a certain ratio. A 1 : X dilution means that the concentrated solution is diluted to 1/Xth of its full-strength concentration. Add 1 volume of concentrate to (X – 1) volumes of diluent to create a total volume equal to X. In other words, what was diluted will be 1/Xth of the total volume in the final solution. For example, 1 : 100 means 1 part concentrate and 99 parts diluent, 1 : 15 means 1 part concentrate and 14 parts diluent; 1 : 2 means 1 part concentrate and 1 part diluent, 1 : 1 means straight concentrate.

Molality (m) – the moles of solute per kilogram of solvent

Molar – adjective describing molarity

Molarity (M) – the moles of solute per liter of solution

NOTE: Molarity is not analogous to molality. Molarity is moles per liter of solution and molality is moles per kilogram of solvent.

Mole (mol) – an amount equal to 6.022×10^{23} molecules of a substance. One mole of a substance has a mass equal to its molecular weight in grams.

Percent (%) – per one hundred; for example, 48% of N = $48/100 \times N = 0.48N$.

Percent volume per volume (v/v) or weight per weight (w/w) – the amounts of ingredients in a solution described as a percentage of the total solution; for example, 100% = 1 g/1 g, 1% = 10 mL/liter.

Percent weight per volume (w/v) – based on the mass/volume of pure water: 1 mL of water has a mass of 1 gram; hence, 1 g/mL = 100%, 10% = 100 mg/mL, 1% = 10 mg/mL.

NOTE: When % is given without mention of units, it is assumed to mean w/v.

Q.S. – “quantity sufficient”; add enough solvent to bring the total volume to the desired amount

Reagent – a compound or solution

Solute – the dissolved, or dispersed, phase of a solution (as opposed to the liquid phase)

Solution – a homogeneous mixture (usually liquid) of two or more substances; one or more solutes dissolved in solvent. The solutes can be solid, liquid, or gas; the solvent can be liquid or gas.

Solvent – the dispersing (i.e., liquid) phase of a solution; the substance into which a solute is dissolved

19.2.2 Moles and molarity

An especially satisfying discussion of moles and molarity is given in *Lab Math*, excerpts of which appear below.

Moles and molarity are both units. Mole is the SI [International System of Units] unit for the dimension of an amount of a substance, and molarity is the SI unit for concentration, where concentration is the amount of a substance per liter of solution. So, the number of molecules of a chemical is counted in moles, and the concentration of molecules in a solution is quantified as the molarity.

The word “mole” is like the word “dozen” in that it is a special name for a particular number. Dozen is a name for the quantity 12; mole is a name for the quantity 6.022×10^{23} . 6.022×10^{23} goes one better than 12 in that it actually has *two* special names. One of the names, at the risk of being repetitive, is “mole” and the other is “Avogadro’s number” (in honor of Amedeo Avogadro, who in 1811 was the first to distinguish between atoms and molecules, and who stated that equal volumes of all gases at the same temperature and pressure contain the same number of molecules, now known as Avogadro’s principle). When you talk about moles of a chemical, you are talking about the number of smallest indivisible units of that chemical, i.e., the number of molecules of that chemical. A mole of a chemical is 6.022×10^{23} molecules, just like a dozen eggs are 12 “smallest indivisible units of eggness” (1).

One mole of a chemical has a mass equal to the molecular weight (MW). This information is readily available, along with the chemical formula, on the manufacturer’s label found on the bottle containing the chemical. In most cases the mass in grams per mole is given as the formula weight (FW), a term that is used interchangeably with molecular weight for biological applications. If a compound includes water molecules (is hydrated), the FW includes the mass of the water.

To determine how many moles of a chemical are present, the chemical is weighed using a balance and the mass is divided by the formula weight (FW). For example, HEPES has an FW of 238.3. If a quantity of HEPES weighs 327.4g, then there are $327.4 \text{ g} \div 238.3 \text{ g/mole} = 1.37$ moles of HEPES in that quantity.

19.2.3 Preparing a molar solution

Typical solutions used in the cytogenetics laboratory are made by adding dry chemical solutes to liquid solvents. Several pieces of information are required when preparing a solution:

- the final concentration (molarity) required
- the total amount of solution to be made
- the FW of the solute.

As a general rule, a solution should be prepared by adding the required mass of chemical or chemicals (determined by “weighing” with a balance) to approximately 80% of the final volume of solvent in a suitable mixing vessel (beaker). Once all the chemicals are added, the solution should be transferred to a graduated cylinder and the remaining solvent added to the desired final volume. Adopting this method as a routine part of solution-making will prevent inadvertent dilution of the target concentration if the mass of the added chemicals overshoots the target volume.

EXAMPLE 19.6 PREPARING A 0.075 MOLAR (M) SOLUTION OF POTASSIUM CHLORIDE (KCl).

Prepare 500 mL of 0.075 M KCl. The FW of KCl is 74.55.

Relate each component to the preparation of 1 liter of solution.

$$0.075\text{M} = 0.075 \text{ moles/liter}$$

$$0.075 \text{ moles} \times \frac{74.55 \text{ g}}{\text{mole}} = 5.59 \text{ g}$$

$$\frac{500 \text{ mL}}{1000 \text{ mL}} = 0.5$$

$$5.59 \text{ g} \times 0.5 = 2.79 \text{ g}$$

To prepare 500 mL of 0.075 M KCl, add 2.79 g of KCl to 400 mL ($500 \times 0.8 = 400$) of water. Transfer the partially-made solution to a graduated cylinder. Add additional water (Q.S.) until the meniscus reaches 500 mL. This expression can be rewritten as the following formula:

$$0.075 \text{ moles} \times \frac{74.55 \text{ g}}{\text{mole}} \times \frac{5 \times 10^{-3} \text{ liter}}{\text{liter}} = 2.79 \text{ g}$$

A fast and easy method to calculate the grams of solute needed to prepare a solution of a particular molarity can be deduced by simplifying this formula. In the above example:

$$0.075 \text{ moles} \times \frac{74.55 \text{ g}}{\text{mole}} \times \frac{5 \times 10^{-1} \text{ liter}}{\text{liter}} = 2.79 \text{ g}$$

The second term (74.55 g/mole) is the formula weight of the chemical, so substitute FW for this term. Since multiplication is commutative, the “liter” in the denominator of the third term can be moved to the first term, 0.075 moles/liter, which is the desired molarity. The simplified equation becomes:

$$0.075M \times FW \times 5 \times 10^{-1} \text{ liter} = 2.79 \text{ g}$$

As a general formula: **M × FW × V = g**, where
M=the final molarity of the solution [in mole/liter],
FW=the formula weight of the solute [in g/mole],
V=the desired final volume [in liters],
g=the grams of solute to add to the solvent [g].

EXAMPLE 19.7 PREPARING 400 mL OF 1.25×10^{-1} M THYMIDINE

Prepare 400 mL of 1.25×10^{-1} M thymidine, FW 242.23.

Using the general formula:

$$1.25 \times 10^{-1} M \times 242.23 \times 4 \times 10^{-1} L = 12.11 \text{ g}$$

How was this achieved? Including the appropriate units, all but g is canceled out:

$$1.25 \times 10^{-1} \frac{\text{mol}}{\text{L}} \times 242.23 \frac{\text{g}}{\text{mol}} \times 4 \times 10^{-1} \text{L} = 12.11 \text{ g}$$

Remembering the rules for multiplication in scientific notation:

$$(1.25 \times 242.23 \times 4) \times (10^{-1} \times 10^{-1}) = 1211.12 \times 10^{-2} = 12.11 \text{ g}$$

When using a calculator, perform the following steps:

$$1.25 \text{ EXP } 1[+/-] \times 242.23 \times 4 \text{ EXP } 1[+/-] = 12.1115 = 12.11 \text{ g}$$

EXAMPLE 19.8 PREPARING 1×10^{-2} M STOCK SOLUTION FUdR

How much 1×10^{-2} M stock solution can be made from 50 mg of FUdR, FW 246.19?

In this case the final molarity and starting quantity of solute are known and the general formula becomes:

$$V = \frac{g}{M \times FW}$$

Until this process becomes familiar, it is instructive to include the appropriate units.

$$L = \frac{g}{\frac{\text{mol}}{\text{L}} \times \frac{\text{g}}{\text{mol}}}$$

or

$$L = g \times \frac{\text{L}}{\text{mol}} \times \frac{\text{mol}}{\text{g}}$$

Cancelling units leaves L, or the volume of solution that can be made.

$$L = g \times \frac{L}{\text{mol}} \times \frac{\text{mol}}{g}$$

Now that confidence in the general formula has risen, the numbers can be plugged in:

$$L = \frac{5 \times 10^{-2}}{1 \times 10^{-2} \times 246.19} = 0.02 L = 20 \text{ mL}$$

Did you get the same answer?

To solve using a calculator, perform the following steps:

$$10 \text{EXP} 2[\pm] \times 246.19[\text{M}+]$$

$$5 \text{EXP} 2[\pm] / [\text{RM}] = 0.02L$$

By manipulating the general formula, any molar solution should be easily prepared as long as three of the four variables are known.

19.2.4 Making molar solutions using hydrated compounds

Many chemicals are available with water molecules included (in hydrated form). If this is the case, the FW including the water is used in the general formula for making molar solutions. It must be remembered that hydrated compounds not only add solute, but also water to the final solution. The volume of water added by the chemical must be considered before the final volume of solvent is added or the final concentration may be more dilute than required or may even overflow the mixing vessel.

Working with hydrated compounds is not complicated if it is remembered that the volume of one mole of water is 18.015 mL (the FW is 18.015).

EXAMPLE 19.9 PREPARING 2L OF 0.5M NaCO₃

Prepare 2L of 0.5 M NaCO₃, FW 105.99. The usual anhydrous reagent has been replaced by the monohydrated version, NaCO₃ · H₂O, FW 124.00.

The familiar, anhydrous formula is:

$$M \times \text{FW} \times V = g$$

where M=the final molarity of the solution [in mole/liter],

FW=the formula weight of the solute [in g/mole],

V=the desired final volume [in liters],

g=the grams of solute to add to the solvent [g]

$$0.5 \times 105.99 \times 2 = 105.99 \text{ g of NaCO}_3, \text{Q.S. to 2 liters}$$

The new, monohydrated formula is:

$$0.5 \times 124.00 \times 2 = 124.00 \text{ g of NaCO}_3, \text{Q.S. to 2 liters}$$

When using hydrated compounds, it is important to remember that the water included in the compound will contribute to the final volume. The volume that will be added can be easily determined by the following formula:

$$M \times V \times \# \text{H}_2\text{O} \times 18.015 = \text{mL H}_2\text{O}$$

For this solution:

$0.5 \times 2 \times 1 \times 18.015 = 18.015 \text{ mL}$ of water will be contributed by the solute.

The 80% rule should be modified when using hydrated compounds. Determine the number of milliliters of water that will be added by using the above formula. Subtract this number from the final volume. Place 80% of the resulting volume into a mixing vessel. Add the solute. Transfer the solution to a graduated cylinder and Q.S. to the total volume.

A quick formula for determining the number of grams of hydrated compound to add when the number of grams of anhydrous compound is known is as follows:

$$g(\text{anhydrous}) \times \frac{\text{FW(hydrated)}}{\text{FW(anhydrous)}} = g(\text{hydrated})$$

EXAMPLE 19.10 PREPARING 1.5 M $\text{Na}_3\text{PO}_4 \cdot 6\text{H}_2\text{O}$

Prepare 2 liters of 1.5 M $\text{Na}_3\text{PO}_4 \cdot 6\text{H}_2\text{O}$, FW 272.03.

$$1.5 \times 272.03 \times 2 = 816.09\text{g}$$

$1.5 \times 2 \times 6 \times 18.015 = 324.27\text{mL}$ of water will be added by the solute.

Place 80% of $(2000 - 324\text{mL}) = 1341\text{mL}$ of water in a mixing vessel. Add the hydrated solute. Transfer to a graduated cylinder and Q.S. to 2000 mL.

A quick formula for determining the number of grams of anhydrous compound to add when the number of grams of hydrated compound is known is as follows:

$$g(\text{hydrated}) \times \frac{\text{FW(anhydrous)}}{\text{FW(hydrated)}} = g(\text{anhydrous})$$

19.2.5 Making percent solutions

By definition, percent means “per 100.” 25% means 25 per 100 or 0.25 (25 divided by 100). When making a percent solution, a number of grams of solute (liquid or solid) are added to 100 mL of solvent (liquid). When the solute is a solid, the percent solution is referred to as weight (in grams)/volume (100 mL) (w/v). When the solute is a liquid, the percent solution is referred to as volume (in milliliters)/volume (100 mL) (v/v).

EXAMPLE 19.11 PREPARING 20% NaCl

Prepare 100 mL of 20% NaCl (w/v).

Weigh 20 g of NaCl and add to 80 mL of water. Transfer to a graduated cylinder and Q.S. to 100 mL.

EXAMPLE 19.12 PREPARING 0.8% (W/V) $\text{NaC}_6\text{H}_5\text{O}$

Prepare 1 liter of 0.8% (w/v) $\text{NaC}_6\text{H}_5\text{O}$.

Weigh 8 g of $\text{NaC}_6\text{H}_5\text{O}$ and add to 800 mL of water. Transfer to a graduated cylinder and Q.S. to 1000 mL.

$$\begin{aligned} \frac{0.8\text{g}}{100\text{mL}} &= \frac{\text{Xg}}{1000\text{mL}} \\ 100 \times \text{X} &= 0.8 \times 1000 \\ \text{X} &= 800 / 100 = 8\text{g} \end{aligned}$$

EXAMPLE 19.13 PREPARING 35% (W/V) KCl SOLUTION

Prepare 50mL of a 35% (w/v) KCl solution.

$$35\% \times 50\text{mL} = 0.35 \times 50 = 17.5\text{g}$$

Weigh 17.5g of KCl and add to 40mL of water. Transfer to a graduated cylinder and Q.S. to 50mL.

Alternatively,

$$\begin{aligned}\frac{35\text{g}}{100\text{mL}} &= \frac{X\text{g}}{50\text{mL}} \\ 100 \times X &= 35 \times 50 = 1750 \\ X &= \frac{1750}{100} = 17.5\text{g}\end{aligned}$$

EXAMPLE 19.14 PREPARING 70% (V/V) ETHANOL

Prepare 200mL of 70% (v/v) ethanol.

$$70\% \times 200 = 140$$

Place 140mL of 100% ethanol into a graduated cylinder and add 60mL of water to a final volume of 200mL.

Alternatively,

$$\begin{aligned}\frac{70\text{mL}}{100\text{mL}} &= \frac{X\text{mL}}{200\text{mL}} \\ 100 \times X &= 70 \times 200 = 14000 \\ X &= \frac{14000}{100} = 140\text{mL}\end{aligned}$$

19.2.6 Stock solutions

Most cytogenetics laboratories find it time- and space-saving to prepare solutions at concentrations higher than will be required for final use. These stock solutions need to be diluted to “working” concentration. Several types of stock solutions can be made. Some are molar solutions that are prepared at a higher molarity. Some are made at a particular multiple (x) of the final working concentration (1x) (i.e., 2x, 5x, 10x, 1000x). Some are prepared at a higher percent (%) solution.

19.2.7 Diluting molar solutions

The basic formula for making dilutions is **C₁V₁ = C₂V₂**, where

C₁ = the more concentrated (stock) solution that you have on hand

V₁ = the volume of the stock solution to be used to make the diluted solution

C₂ = the diluted concentration (working solution) that you want to make

V₂ = the final volume of the working solution that you want to make

19.2.8 Diluting a stock molar solution to a specific concentration

The structure of the basic formula may be modified to easily yield the recipe for this type of dilution. Keep in mind that conversion factors may have to be added if the units involved are not identical. It is also worthwhile to state the variables in words to ensure that the correct question is being answered. If the specific molarity of a diluted solution to be made is known and the quantity of the concentrated stock solution to be added is to be determined, the following equation can be used:

$$\frac{\text{Concentration of the working solution}}{\text{Concentration of the stock solution}} \times \text{Final volume} = \text{Volume of stock solution to add}$$

Notice how this formula relates to the basic formula, C₁V₁ = C₂V₂:

$$\frac{C_2}{C_1} \times V_2 = V_1$$

$$\frac{C_2V_2}{C_1} = V_1$$

EXAMPLE 19.15 PREPARING 100mL 0.5M CaCl₂ FROM A 5M STOCK SOLUTION

Prepare 100mL of 0.5M CaCl₂ from a 5M stock solution.

$$\frac{0.5\text{M}}{5\text{M}} \times 100\text{mL} = 10\text{mL}$$

To prepare 100mL of 0.5M CaCl₂ from a 5M stock solution, add 10mL of 5M stock solution to 80mL of water. Transfer to a graduated cylinder and Q.S. to 100mL.

EXAMPLE 19.16 PREPARING 5mL OF 50mM NaCl FROM A 2M STOCK SOLUTION

Prepare 5mL of 50mM NaCl from a 2M stock solution.

$$\frac{50\text{mM}}{2\text{M}} \times \frac{1\text{M}}{1000\text{mM}} \times 5\text{mL} = 0.125\text{mL} = 125\mu\text{L}$$

In this case, a conversion factor must be added to convert molar to millimolar. To prepare 5mL of 50mM NaCl from a 2M stock solution, add 125μL of 2M stock solution to 4mL of water. Transfer to a graduated cylinder and Q.S. to 5mL.

EXAMPLE 19.17 PREPARING HYPOTONIC WORKING SOLUTION 2.5 × 10⁻² M NaC₆H₅O AND 7.5 × 10⁻² M KCl

Prepare 250mL of a hypotonic working solution containing 2.5 × 10⁻² M NaC₆H₅O and 7.5 × 10⁻² M KCl. Available stock solutions are 0.5 M NaC₆H₅O and 1 M KCl.

The dilution of each solute contained in a solution containing multiple solutes is treated separately. Each component is combined into the final solution keeping in mind the contribution of each volume to the final volume.

For NaC₆H₅O,

$$\frac{2.5 \times 10^{-2}\text{M}}{5 \times 10^{-1}\text{M}} \times 250\text{mL} = 12.5\text{mL}$$

For KCl,

$$\frac{7.5 \times 10^{-2}\text{M}}{1\text{M}} \times 250\text{mL} = 18.8\text{mL}$$

To prepare a hypotonic working solution containing 2.5 × 10⁻² M NaC₆H₅O and 7.5 × 10⁻² M KCl, add 12.5mL of 0.5 M NaC₆H₅O and 18.8mL of 1 M KCl to 175mL of water. Transfer to a graduated cylinder and Q.S to 250mL.

EXAMPLE 19.18 PREPARING 2.5 \times WORKING SOLUTION FROM A 10 \times STOCK SOLUTION

Prepare 500 mL of 2.5 \times working solution from a 10 \times stock solution.

$$C_1V_1 = C_2V_2$$

or

$$V_1 = \frac{C_2V_2}{C_1}$$

$$V_1 = \frac{(2.5 \times)(500 \text{ mL})}{10 \times}$$

$$V_1 = 125 \text{ mL}$$

To prepare the working solution, add 125 mL of 10 \times stock solution to 275 mL of solvent. Transfer to a graduated cylinder and Q.S. to 500 mL.

19.2.9 Using dilution ratios

Another method to resolve how to dilute a solution to a specific concentration is to determine the ratio of diluted to stock solution concentration, and then to convert that ratio to a fraction. The final volume is then made up of that fraction of concentrated solute with the remainder made up of solvent.

EXAMPLE 19.19 PREPARING WORKING SOLUTION VIA DILUTION RATIO

Referring to Example 19.17 above, for NaC₆H₅O the ratio of diluted to concentrated is

$$\frac{2.5 \times 10^{-2} \text{ M}}{5 \times 10^{-1} \text{ M}} = \frac{1}{X}$$

$$2.5 \times 10^{-2} \times X = 5 \times 10^{-1} \times 1$$

$$X = \frac{5 \times 10^{-1}}{2.5 \times 10^{-2}} = 20$$

1/20th of the final volume is made up of NaC₆H₅O.

$$\frac{250}{20} \text{ mL} = 12.5 \text{ mL}$$

For KCl, the ratio of diluted to concentrated is

$$\frac{7.5 \times 10^{-2} \text{ M}}{1 \text{ M}} = \frac{1}{M}$$

$$7.5 \times 10^{-2} \times X = 1 \times 1$$

$$X = \frac{1}{7.5 \times 10^{-2}} = 13.3$$

1/13.3th of the final volume is made up of KCl.

$$\frac{250}{13.3} \text{ mL} = 18.8 \text{ mL}$$

EXAMPLE 19.20 PREPARING 1 : 3 FIXATIVE SOLUTION

Although the fixative ratio can be modified to accommodate specific problematic situations (see Chapter 2, section 2.4.7, Fixative ratio), you plan to prepare the basic formula, 1 : 3 glacial acetic acid–absolute methanol. You need 50 mL of fresh fixative for harvesting a peripheral blood sample, but you don't want to measure fractions. How would you figure out the quantity you will need?

Step 1: Determine your basic measurable unit. If each unit of measure includes 1 mL of glacial acetic acid + 3 mL of absolute methanol, then the basic whole-number unit is 4.

$$1X + 3X = 4X$$

Your initial desired quantity (50), however, is NOT divisible by 4 evenly; therefore, you need to adjust-up the desired quantity to a number that is divisible by 4 and is > your needed quantity). In this example, it is 52.

$$4X = 52$$

$$X = 13$$

Step 2: Determine how much acid and base must be combined to make 52 mL of 1 : 3 fixative solution.

$$\text{Glacial acetic acid} = 1 \times X \text{ (or 13, in this example)} = 13 \text{ mL}$$

$$\text{Absolute methanol} = 3 \times X \text{ (or 13, in this example)} = 39 \text{ mL}$$

Hence, adding 13 mL (1X) glacial acetic acid to 39 mL (3X) absolute methanol will make 52 mL of 1 : 3 modified fixative solution, which meets your initial 50 mL of fixative requirement.

Diluting percent solutions

In cytogenetics laboratories where FISH is performed, protocols may require the preparation of ethanol solution for serial dehydration steps. This can be performed easily by preparing 100 mL of alcohol for each dilution. However, if the desire is to conserve the 100% ethanol stock and only 50 mL is required for each Coplin jar, then knowing how to prepare dilutions of percent solutions other than 100% is required.

The following equation can be used to prepare dilutions of percent solutions for any purpose in the laboratory. It is easiest to convert the percent solution to a value divided by 100 (i.e., 70% is 70/100). On the left-hand side of the equation place the concentration of the stock solution that you have multiplied by the unknown amount of stock (x mL) that will be added divided by the desired final volume (50 mL). On the right side of the equation place the desired final concentration (i.e., 70/100).

EXAMPLE 19.21 PREPARING ETHANOL DILUTIONS

For a FISH procedure, three different ethanol percent solutions are required for the dehydration step, 100%, 85%, and 70%. Each Coplin jar holds 50 mL. Prepare 85% and 70% solutions from 100 mL of 100% ethanol.

Step 1: Prepare 100 mL of 85% ethanol from 100% ethanol.

Since 85% means 85 mL per 100 mL (v/v), 85 mL of 100% ethanol would be added to 15 mL of water in a graduated cylinder to bring the final volume to 100 mL.

Step 2: Prepare 50 mL of 70% ethanol from 85% ethanol.

$$\frac{85}{100} \times \frac{X \text{ mL}}{50 \text{ mL}} = \frac{70}{100}$$

$$\frac{85X}{5000} = \frac{70}{100}$$

$$8500X = 350000$$

$$X = 41.18 = 41 \text{ mL} [\text{reduce to two significant numbers}]$$

To prepare 50 mL of 70% ethanol from 85% ethanol, add 41 mL of 85% ethanol to a graduated cylinder that contains 9 mL of water. Since only 41 mL of the 100 mL of 85% ethanol was used, you still have 50 mL of the remaining 59 mL for the 85% ethanol dehydration step.

If each dilution is made from 100% ethanol, 205 mL are required (85 + 70 to make dilutions and 50 for the Coplin jar). If dilutions are made serially, 135 mL of 100% ethanol is used (85 for the initial dilution and 50 for the Coplin jar) for a net saving of 70 mL of 100% ethanol. Since alcohol dilutions for dehydration must be made fresh, the result is less discarded alcohol.

19.2.10 Converting molarity to percent

Solutions may be prepared in a number of ways. Occasionally, a solution prepared as a molar solution must be expressed as a percent solution. This may occur, for example, when a new protocol is being considered. If a reagent in a protocol under consideration is expressed as a molar solution and the existing protocol uses the same chemical, but expressed as a percent solution, how is it determined whether the protocol is different or not? Remembering that molar solutions are expressed as a concentration of grams per 1000 mL and that percent solutions are expressed as a concentration of grams per 100 mL, it is obvious that a simple conversion is possible. The only extra information that is needed is the formula weight of the reagent in question.

EXAMPLE 19.22 HOW IS 3M NaCl EXPRESSED AS A PERCENT SOLUTION?

Step 1: Determine how many grams of NaCl are contained in a 3M solution. The formula weight of NaCl is 58.44.

Set up an equation of ratios:

$$\frac{58.44 \text{ g}}{1\text{M}} = \frac{X \text{ g}}{3\text{M}}$$

$$3 \times 58.44 = 1X$$

$$175.3 = X$$

To prepare a 3M solution of NaCl, 175.3 g of NaCl was added to 800 mL of water. It was transferred to a graduated cylinder and the volume was brought to 1000 mL.

Step 2: Determine how many grams of 3M NaCl are contained in 100 mL (a percent solution). Set up another equation of ratios:

$$\frac{175.3 \text{ g}}{1000 \text{ mL}} = \frac{X \text{ g}}{100 \text{ mL}}$$

$$(175.3)(100) = 1000X$$

$$X = 175,300 / 1000 = 17.5$$

Alternatively, it is obvious that 100 is 1/10 of 1000. So, 175.3/10 is 17.5.

Since a 3M solution of NaCl contains 17.5 g of sodium chloride in 100 mL, this is equivalent to a 17.5% solution of NaCl.

19.2.11 Converting percent to molarity

A protocol in a journal article describes the use of 0.8% sodium citrate as a hypotonic solution. Although the same chemical is used in the cytogenetics laboratory, it is made as a 2.7×10^{-2} M solution. Are these two protocols the same?

EXAMPLE 19.23 HOW IS 0.8% SODIUM CITRATE CONVERTED TO A MOLAR VALUE EQUIVALENT?

Step 1: A 0.8% solution of sodium citrate contains 0.8 g in 100 mL of solution. It is easy to see that 1000 mL of solution contains 8 g of sodium citrate.

Step 2: The formula weight of sodium citrate is 294.1. Set up an equation of ratios:

$$\frac{XM}{8 \text{ g}} = \frac{1\text{M}}{294.1\text{g}}$$

$$294.1X = 8$$

$$X = \frac{8}{294.1} = 0.027 = 2.7 \times 10^{-2} \text{ M}$$

A 0.8% sodium citrate solution is equivalent to a 2.7×10^{-2} M solution.

19.2.12 Serial dilutions (application for dose-response experiments)

While making serial dilutions is not usually a common occurrence in a cytogenetics laboratory, there is at least one important application that should be familiar. This involves determining how much of a reagent should be added, say to a culture, to yield the desired effect, say to increase the length of the chromosomes. This application can be used for any experimental

design where a range of concentrations are tested to determine the optimal dose to add to obtain a desired result. Other potential applications involve optimizing reagents for staining procedures, determining how much mitogen to add to a bone marrow culture, or determining the concentration of a new hypotonic solution. In these types of experiments, all other variables are held constant (total volume, time, concentration of other reagents besides the one being tested, etc.). It is generally best to begin these types of experiments by using doses that differ by an order of magnitude (i.e., by a power of 10; 1 : 10 dilutions) and narrowing the search by using doses that differ by a lesser power (i.e., down to a power of 2; 1 : 2 dilutions). Serial dilutions may be performed by several methods. When ample reagents are available and the volumes to be prepared are fairly large (milliliters, not microliters), the process of making serial dilutions is simple but fairly imprecise. When reagents are present in small quantity or are expensive or hazardous, a more time-consuming but more precise method is available.

Method 1 serial dilution – fast and imprecise (best for 1 : 10 or 1 : 2 dilutions)

1 : 10 Serial Dilution (see Figure 19.1)

1. Determine how many dilutions are needed.
2. Determine how much of each dilution will be required. Prepare 20% more than is actually required and use vessels that are 50% larger than the final volume.
3. For a 1 : 10 serial dilution, place the vessels in a row on the bench and label each vessel with the concentration they will hold. The first vessel is the one containing the full-strength concentration of reagent to be diluted (1 : 1).
4. Place nine-tenths of the total volume (the volume required plus 20%) of solvent (i.e., water) into each empty vessel.
5. Transfer one-tenth of the required volume of the full-strength reagent (i.e., from the first vessel) into the next vessel (1 : 10 dilution). Mix.
6. Take one-tenth of the volume just made (1 : 10 dilution) and transfer it into the next vessel (1 : 100 dilution). Mix.
7. Take one-tenth of the volume just made (1 : 100 dilution) and transfer it into the next vessel (1 : 1000 dilution). Mix.
8. Continue until all dilutions are complete.

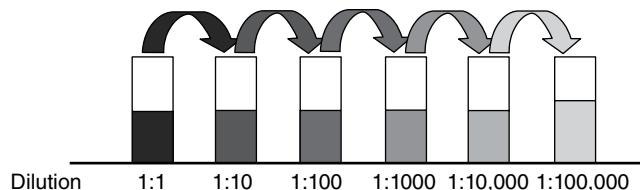


Figure 19.1 1 : 10 Serial dilution. The 1 : 10 serial dilution provides a fast but imprecise series of dilutions in dose-response experimentation where a range of concentrations are tested in order to determine the optimal dose for obtaining a desired result from a reagent.

EXAMPLE 19.24

To determine the optimal concentration of a new “hypotonic solution,” prepare an appropriate serial dilution. The required volume of each dilution is 100 mL. The concentration of the full-strength (1 : 1) reagent is 1×10^{-2} M. The solvent is water.

Step 1. Information about the starting concentration of a new reagent is generally available from a reliable source (i.e., a literature search, a journal article, a trusted colleague). For most applications in cytogenetics, the first approximation of the concentration of a new reagent (start) will be found within two 1:10 dilutions on either side of the starting concentration. If none of the first dilutions are acceptable, further dilutions may be tried.

Step 2. Prepare 120 mL (100 mL plus $20\% \times 100$) of 1×10^{-2} M “hypotonic solution”.

Step 3. Label this vessel and four other vessels as follows (see Figure 19.2):

1st vessel (1 : 1) = 1×10^{-2} M

2nd vessel (1 : 10) = 1×10^{-3} M

3rd vessel (1 : 100) = 1×10^{-4} M (recommended concentration)

4th vessel (1 : 1000) = 1×10^{-5} M

5th vessel (1 : 10,000) = 1×10^{-6} M

- Step 4. Place 108 mL of water (9/10th of 120 mL) in vessels 2–5.
- Step 5. Remove 12 mL of 1 : 1 “hypotonic solution” (1/10th of 120 mL) and transfer it into the 2nd vessel (1 : 10). Mix well.
- Step 6. Remove 12 mL of 1 : 10 “hypotonic solution” (1/10th of 120 mL) and transfer it into the 3rd vessel (1 : 100). Mix well.
- Step 7. Remove 12 mL of 1 : 100 “hypotonic solution” (1/10th of 120 mL) and transfer it into the 4th vessel (1 : 1000). Mix well.
- Step 8. Remove 12 mL of 1 : 1000 “hypotonic solution” (1/10th of 120 mL) and transfer it into the 5th vessel (1 : 10,000). Mix well.

The first vessel will have 108 mL remaining. Vessels 2 to 4 will have 108 mL ($108 + 12 - 12$). The fifth vessel will contain 120 mL of “hypotonic solution.”

1 : 2 Serial dilution (see Figure 19.3)

- Determine how many dilutions are needed.
- Determine how much of each dilution will be required. Prepare double the amount that is actually required and use vessels that are large enough to contain the final volume.
- For a 1 : 2 serial dilution, place the vessels in a row on the bench and label each vessel with the concentration they will hold. The first vessel is the one containing the full-strength concentration of reagent to be diluted (1 : 1).
- Place the double volume of solvent (i.e., water) into each empty vessel.
- Transfer half of the double volume of the full-strength reagent (i.e., from the first vessel) into the next vessel (1 : 2 dilution). Mix.
- Take half of the volume just made (1 : 2 dilution) and transfer it into the next vessel (1 : 4 dilution). Mix.
- Take half of the volume just made (1 : 4 dilution) and transfer it into the next vessel (1 : 8 dilution). Mix.
- Continue until all dilutions are complete.

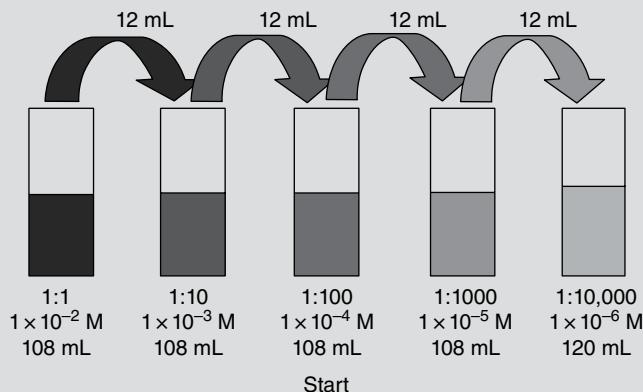


Figure 19.2 1 : 10 Serial dilution example. This figure demonstrates an example using the 1:10 series dilution for determining the optimal concentration of a new hypotonic solution (see Example 19.24). The required volume of each dilution is 100 mL. The concentration of the full-strength (1 : 1) reagent is $1 \times 10^{-2} \text{ M}$. The solvent is water.

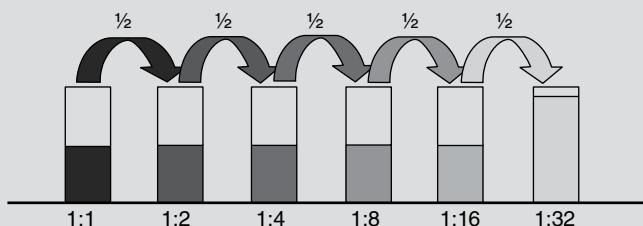


Figure 19.3 1 : 2 Serial dilution. In dose-response experimentation, it is generally best to begin with an order of magnitude that is easy to work with and that can provide a broad range (i.e., by a power of 10; 1 : 10 dilutions). The search can then be narrowed by using values that differ by a lesser power (i.e., down to 2, as in the 1 : 2 dilutions). This method further refines the dose-response experiment outcome.

EXAMPLE 19.25

The recommended starting concentration of a new “hypotonic solution” yields the best results based on the 1 : 10 dilution experiment; however, the results indicate that further optimization is required. Prepare an appropriate 1 : 2 dilution experiment. The starting concentration is 1×10^{-4} M. The required volume of “hypotonic solution” is 100 mL. The solvent is water.

Step 1. For most applications in cytogenetics, the optimal concentration of a new reagent (start) will be found within two 1:2 dilutions on either side of the starting concentration (each side will approach, but not equal, the concentration of an experimental value that was deemed to be unacceptable). To determine the 1:1 concentration that will be serially diluted, multiple the concentration selected in the 1:10 serial dilution experiment by 2 times the number of vessels with higher concentrations that will be tested. In this case there will be two vessels with higher concentrations than 1×10^{-4} M, so multiple 1×10^{-4} M by 4 (2×2). The 1:1 starting concentration is 4×10^{-4} M.

Step 2. Prepare 200 mL (2×100) of 4×10^{-4} M “hypotonic solution”.

Step 3. Label this vessel and four other vessels as follows (see Figure 19.4):

1st vessel (1:1)= 4×10^{-4} M

2nd vessel (1:2)= 2×10^{-4} M

3rd vessel (1:4)= 1×10^{-4} M (the selected 1:10 concentration)

4th vessel (1:8)= 5×10^{-5} M

5th vessel (1:16)= 2.5×10^{-5} M

Step 4. Place 100 mL of water in vessels 2 – 5.

Step 5. Remove 100 mL of 1:1 “hypotonic solution” (1/2 of 200) and transfer it into the 2nd vessel (1:2). Mix well.

Step 6. Remove 100 mL of 1:2 “hypotonic solution” (1/2 of 200) and transfer it into the 3rd vessel (1:4). Mix well.

Step 7. Remove 100 mL of 1:4 “hypotonic solution” (1/2 of 200) and transfer it into the 4th vessel (1:8). Mix well.

Step 8. Remove 100 mL of 1:8 “hypotonic solution” (1/2 of 200 mL) and transfer it into the 5th vessel (1 : 16). Mix well.

The first vessel will have 100 mL remaining. Vessels 2 to 4 will have 100 mL ($100 + 100 - 100$). The fifth vessel will contain 200 mL of “hypotonic solution.”

Method 2 serial dilution – more time-consuming, more precise

When reagents are present in small quantities, are expensive, or hazardous, a more precise method for performing serial dilutions is required.

1. Determine how much of each dilution is required. This is the final volume, V_f .
2. Determine how many different dilutions are required, N . Assemble a row of N vessels.

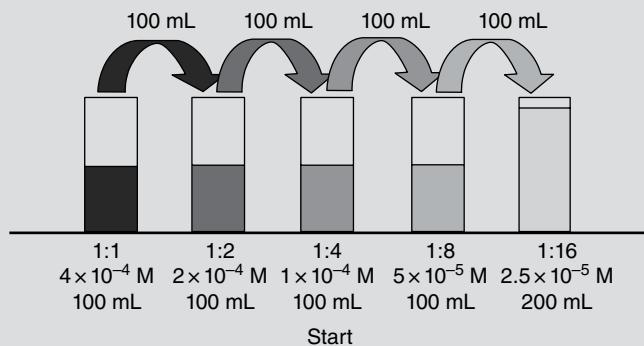


Figure 19.4 1 : 2 Serial dilution example. This figure demonstrates a 1 : 2 dose-response serial dilution that was needed for more refined optimization of a proposed reagent’s concentration. Figure 19.2 demonstrated the 1 : 10 serial dilution to evaluate a new hypotonic solution. This series further optimizes those results with a 1 : 2 dilution, starting at 1×10^{-4} M concentration (see Example 19.25). The required volume of “hypotonic solution” is 100 mL. The solvent is water.

3. Determine an appropriate dilution factor, X , that will be applied to the initial concentration, C_i . As above, a first approach may use 1:10 dilutions, while further optimization may require 1:2 dilutions.
4. Determine what the final concentration in each tube will be:

$$\text{Vessel } 1 = \frac{1}{X} \times C_i$$

$$\text{Vessel } 2 = \frac{1}{X^2} \times C_i$$

$$\text{Vessel } 3 = \frac{1}{X^3} \times C_i$$

$$\text{Vessel } N = \frac{1}{X^N} \times C_i$$

1. Label each vessel with the final concentration each will hold.
2. Place V_f solvent into each vessel.
3. Set a pipette to $\frac{V_f}{X-1} \cdot \frac{V_f}{X-1}$

With a new pipette tip, pick up $\frac{V_f}{X-1}$ of the starting concentration and place it into the first vessel. Mix well. Change the pipette tip.

Pick up $\frac{V_f}{X-1}$ of the solution in the first vessel, the one just made, and place it into the second vessel. Mix well.

Change the pipette tip.

Pick up $\frac{V_f}{X-1}$ of the solution in the second vessel, the one just made, and place it into the third vessel. Mix well.

Change the pipette tip. Continue in this fashion until all dilutions are completed.

Since the same amount of solution that is added to each vessel is also removed to make the subsequent dilution, the final volume in each tube, except the last tube, will remain constant. The last tube will have $\frac{V_f}{X-1}$ more solution, but the dilution will be correct. If equal volumes are required in all vessels, remove $\frac{V_f}{X-1}$ from the final vessel and discard.

EXAMPLE 19.26

A new mitogen is available. A literature search reveals that, of the several laboratories using this mitogen, a final concentration from 2.5×10^{-2} M to 5×10^{-5} M is reported. Determine the most effective concentration to use in bone marrow cultures at your laboratory. Since the range of doses reported in the literature spans several orders of magnitude, a good first approach is to include a wide range of concentrations in the initial experiment, including those reported.

The initial concentration (C_i) of "mitogen" is selected to be 1×10^{-2} M. Five 1 : 10 dilutions will be made. One mL of each dilution is required.

$$C_i = 1 \times 10^{-2} \text{ M}$$

$$V_f = 1 \text{ mL}$$

$$X = 10$$

$$N = 5$$

Step 1. Prepare a solution of 1×10^{-2} M "mitogen" (a few milliliters, or the smallest quantity that is practical).

$$\text{Place } 1 \text{ mL} + \frac{V_f}{X-1} = 1 + \frac{1 \text{ mL}}{10-1} = 1.111 \text{ mL} = 1,111 \mu\text{L in vessel 1.}$$

Step 2. Determine the concentrations of each dilution and label each vessel accordingly.

$$\begin{aligned} \text{Vessel 1} &= 1 \times 10^{-2} \text{ M} \\ \text{Vessel 2} &= 1 \times 10^{-3} \text{ M} \\ \text{Vessel 3} &= 1 \times 10^{-4} \text{ M} \\ \text{Vessel 4} &= 1 \times 10^{-5} \text{ M} \\ \text{Vessel 5} &= 1 \times 10^{-6} \text{ M} \end{aligned}$$

Step 3. Place 1 mL (V_f) of solvent (water) in vessels 2 to 5.

Step 4. Set a pipette to $\frac{V_f}{X-1} = \frac{1 \text{ mL}}{10-1} = 0.111 \text{ mL} = 111 \mu\text{L}$

Step 5. With a new pipette tip, pick up 111 μL of solution from vessel 1 and place it in vessel 2. Mix well. Discard the pipette tip.

Step 6. With a new pipette tip, pick up 111 μL of solution from vessel 2 and place it in vessel 3. Mix well. Discard the pipette tip.

Step 7. With a new pipette tip, pick up 111 μL of solution from vessel 3 and place it in vessel 4. Mix well. Discard the pipette tip.

Step 8. With a new pipette tip, pick up 111 μL of solution from vessel 4 and place it in vessel 5. Mix well. Discard the pipette tip. Each vessel contains 1 mL of solution, except for vessel 5 which has 1.111 mL.

19.3 Statistical tools

19.3.1 Determining the normal cut-off for FISH validation

An important function in FISH probe validation is determining the normal cut-off value. The results of FISH studies requiring a normal cut-off are binary, for example, positive or negative, normal or abnormal. The question to be answered is how many times in a known normal population is an “abnormal” signal pattern observed. Since the individual with the “abnormal signal pattern” is normal, this is considered to be a “false positive” result. The normal cut-off is, then, the number of “false positive” cells that are allowable within the normal population *determined by the validation studies performed in each individual laboratory*, and above which the result is considered to be truly positive. False positive cells may be observed for a variety of reasons, mostly due to technical artifact, such as incidental overlap, extreme split signals, fluorescent debris, and ill-advised cell selection.

Inverse beta distribution (betainv)

One method that can be used to determine the normal cut-off uses a formula that calculates the upper confidence limit for the 95th percentile of the binomial distribution. The mean and standard deviation *should not* be used since the FISH results (positive or negative) do not fit a Gaussian (bell-shaped) curve, but rather are binomial (a skewed bell-shaped curve). A statistical function that is related to the binomial distribution is called the beta distribution. In the beta distribution, the percentile is an unknown quantity. Since we know our target percent (95%), the inverse beta distribution is used. In this, the percentile is known, along with, for example, the number of times a result is observed in a given number of observations. The inverse beta function will provide the number of times a result can be observed to be within the chosen confidence limit (95%). This becomes the normal cut-off. If a patient value falls above the cutoff value, it is considered abnormal, while if it is at the cutoff value or below, it is considered within the normal range.

If this calculation sounds complicated, be assured that it is easily mastered with the use of an Excel spreadsheet. The formula to be inserted into an open Excel spreadsheet field is:

=BETAINV(CONFIDENCE LIMIT (0.95),LARGEST # OF FALSE POSITIVE CELLS OBSERVED + 1,# OF CELLS SCORED)

NOTE: If Excel 2003 is used, once = betainv is entered, the rest of the equation will be prompted. Note that [A] and [B] are optional entries and are not used in this application. The entry of “betainv” is not case sensitive.

EXAMPLE 19.27 DETERMINE THE NORMAL CUT-OFF FOR VALIDATION OF THE ENUMERATION PROBE, CEP 8.

In 20 normal patients, 200 cells were scored for each analysis. The largest number of cells that exhibited a signal pattern, indicating the presence of three copies of chromosome 8 (false positive), was 4.

In Excel, select a field and type “=betainv(“. If a version of Excel 2003 or later is used, a box will appear underneath this field prompting the entry of additional data. The first is probability, indicated in bold letters. If the 95th percentile is the desired confidence limit, enter 0.95 after the left parenthesis and enter a comma.

```
=betainv(  
    BETAINV(probability, alpha, beta, [A], [B])
```

After the comma is entered, the word “probability” will unbold and the word “alpha” will become bolded.

```
=betainv(0.95,  
    BETAINV(probability, alpha, beta, [A], [B])
```

Alpha is a parameter of the distribution. In this case it is the maximum number of times a false positive signal pattern has been observed in a FISH analysis of a normal individual **plus 1**. In this example, the most times that cells with three signals were observed were 4, so the number 5 is entered immediately after the comma, and is followed by another comma. The word “alpha” will unbold, and the word “beta” will become bolded.

```
=betainv(0.95,5,  
    BETAINV(probability, alpha, beta, [A], [B]))
```

Beta is another parameter of the distribution. In this case it is the total number of cells that were scored for each analysis. In this example the number was 200. This number is placed immediately following the last comma, followed by a right parenthesis. The A and B in brackets are optional and not needed for this application.

```
=betainv(0.95,5,200)
```

The parentheses will become bolded to indicate that an expression that meets the criteria of the function has been entered. When the “Enter” key is pushed, a number will appear in the field. Change this number to a percent by selecting the % icon on the toolbar. This is the normal cut-off percentage. If a percentage higher than this is observed, the result is considered to be abnormal.

0.044306

4.4%

Select the field below (or in the vicinity of) the field that contains the percent. Enter the following:

=200*[select the field with the percent] and press “Enter.” This is the maximum number of cells in 200 that are still considered to be normal. If more cells with a signal pattern consistent with three copies of chromosome 8 are observed in the test population, the result is considered to be abnormal.

4.4%

=200*A1

4.4%

8.86

Since fractions of cells cannot be scored, round any amount over to the next integer.

In this example, the normal cut-off percent for the detection of trisomy 8 is 4.4%. The normal cut-off is 9 cells with three CEP8 signals. If any more than 9 are observed, the result is abnormal.

NOTE: If a version of Excel before 2003 is used, the box with prompts will not appear. Enter the data as described above. The answer will appear.

EXAMPLE 19.28

One way to lower the normal cut-off (increase sensitivity) is to analyze more cells. What will happen in the above example if 500 cells per individual are scored during validation instead of 200 without seeing any more than 4 false positive cells?

```
=betainv(0.95,5
BETAINV(probability,alpha,beta,[A],[B])
```

```
=betainv(0.95,5,500)
```

0.018069

1.8%

1.8%

```
=500*A1
```

1.8%

3.61

If 500 cells are scored with no concomitant increase in false positive cells, the normal cut-off will be lowered to 1.8% or 4 cells of 500 analyzed.

EXAMPLE 19.29

When a probe strategy involves two colors with two fusion signals indicating the abnormal rearrangement, i.e., 2R 2G is normal and 1R 1G 2F is abnormal, the number of times an abnormal signal pattern is observed in a normal population is usually zero. What are the normal cut-offs when 200 cells are scored? 500 cells?

```
=betainv(0.95,1
BETAINV(probability,alpha,beta,[A],[B])
```

```
=betainv(0.95,1,200)
```

0.014867

1.5%

1.5%

```
=200*A1
```

1.5%

3

If 200 cells are scored, the normal cut-off is 3 cells.

For 500 cells,

```
=betainv(0.95,1
BETAINV(probability,alpha,beta,[A],[B])
```

```
=betainv(0.95,1,500)
```

0.005974

0.6%

0.6%

=500*A1

0.6%

2.99

When 500 cells are scored, >0.6% (>3 cells/500) of cells with a 1R 1G 2F signal pattern will be considered abnormal. What happens to the normal cut-off when the cells counted are increased to 1000? 6000? 100,000? 1,000,000? **Should even one cell with a 1R 1G 2F signal pattern be considered abnormal when the FISH study is being performed for the detection of minimal residual disease?**

Betainv and the multiplication rule

When FISH is being performed on newly diagnosed, untreated patients, the expectation is that the number of cells with an abnormal signal pattern is much greater than the normal cut-off. However, when a treated patient is being studied, the possibility exists that a very small number of abnormal cells may be present, and their detection becomes all the more crucial. In these cases, it is important to use a probe strategy that will allow the smallest number of cells with an abnormal signal pattern to be considered truly abnormal (with the least amount of technical artifact contributing to false positive cells). This situation has raised some concerns about using “betainv” that should be addressed. It seems that “betainv” allows up to 3 cells with 1R 1G 2F signal patterns to be considered normal when 200 cells are scored using a dual color, dual fusion probe strategy. How can this be justified when even one such cell may indicate residual disease? A discussion about general probe strategy is warranted and a refinement of our statistical method may lead to increased confidence in statistical methods.

As a preface to this discussion, it is important to remember that the recognition of a single cytogenetic rearrangement (a reciprocal translocation) is the goal of a dual color/dual fusion probe strategy. The requirement for identification of two derivative chromosomes is the basis for the proposed refinement. There are basically two types of probes, those that need only one “event” to occur in order to change the scoring of a cell from normal to abnormal, and those that need two “events” to occur in order to be scored as abnormal. The first type is exemplified by single target probes (with or without a second control probe), such as probes to determine the presence of additional chromatin (trisomy/duplication), or loss of chromatin (monosomy/deletion), where gain of one signal or loss of one signal is enough to score the cell as abnormal. Another example is a break-apart strategy where evidence of one separation is sufficient to score the cell as abnormal. Yet another example is a dual color, single fusion strategy where the presence of one fused signal is considered to be abnormal. What do all these probe strategies have in common? They are all subject to “impersonation” by technical artifacts, such as incidental overlap of two same color probes being scored as a single signal, or extremely split signals (same color probe or break-apart probe) being read as two separate signals, or incidental overlap of two different color probes being scored as a single fusion, or the presence of fluorescent debris in just the right plane that is scored as a legitimate probe signal. In these cases, it is important to recognize the limitations of the probe strategy and to set a cut-off that precludes the classification of normal individuals as having a disease that they, in fact, do not. Betainv performs this function. It is possible to identify a “gray area,” defined as the range from the number inserted into the betainv formula (the largest number of false positive cells observed in the laboratory’s normal population) to the number of cells defined by the betainv formula (the normal cut-off). For those instances when the number of abnormal signal patterns lies in the “gray zone,” additional information may be gathered (concerning treatment status of the patient or whether minimal residual disease is suspected), additional cells may be scored, the FISH assay may be repeated, etc.

The second type of probe is exemplified by the dual color, dual fusion probe strategy, where the presence of two fusions (“events”) is necessary to call the cell abnormal. In this case, the possibility of technical artifact “impersonation” is extremely unlikely. Can you think of examples of how this could happen? For this reason, the need for a “gray zone” is minimized and

the normal cut-off would be expected to be a very small number, less than what seems to be possible by using betainv alone. How can this be resolved statistically? It just requires the use of the multiplication rule:

The multiplication rule is used to determine the probability that two events, A and B, (or fusion 1 and fusion 2) both occur. Remember that even though one abnormality is present, two independent derivative chromosomes are identifiable.

For independent events, that is, events which have no influence on one another (i.e., a probe hybridizes to each derivative chromosome of a reciprocal translocation at the same time, in no particular order, and under no governing influence), the rule simplifies to:

$$P(A \cap B) = P(A) \cdot P(B)$$

That is, the probability of the joint events A and B (fusions 1 **and** 2) is **equal to the product of the individual probabilities for the two events**.

Betainv calculates the probabilities (cut-offs) for each individual “event,” which works fine for single event probes. By applying betainv and the multiplication rule, a more reasonable cut-off can be defined for dual event probes:

When 200 cells are scored for a dual color, dual fusion probe:

$$\begin{aligned} 0.015(\text{fusion1}) \times 0.015(\text{fusion 2}) &= 0.000225(\text{both fusions in the same cell}) \\ 0.000225 \times 100 &= 0.0225\% \\ 200 \times 0.0225\% &= 0.045 \text{ cells} \end{aligned}$$

Since a fraction of a cell does not exist, any cell (even only one) exhibiting two fusion signals would be considered to be positive (or perhaps falling into a “gray zone” that requires further examination). What would this look like in Excel?

0.015	for each probe individually
0.0002	for both probes at the same time
0.02%	
0.04	per 200 cells or >0 = normal cut-off

When considering a data set, such as the number of times an “abnormal” FISH signal pattern is observed in a “normal” population (false positives), it is important to consider the maximum and minimum values, and the range of values. These descriptive statistics are important tools when considering the impact of extreme outliers in the data set. In addition, having these values readily available can quickly alert the technologist to when a value close to the normal cut-off is encountered in a patient sample. A value that is above the MAX (largest value), but was not seen in enough cells to exceed the normal cut-off, might be considered to fall in a “gray zone,” which in turn might require additional counts and clinical correlation (new diagnosis or treated disease) in order to fully evaluate its significance, and/or may require the inclusion of a cautionary statement in the final report, or a recommendation for additional molecular-based confirmatory testing, when available. These MAX values can be determined by selecting the largest and smallest numbers within the data set. They can also be easily determined and documented by performing the following steps in the same Excel spreadsheet that was used for calculating the normal cut-off (see Table 19.2):

=MAX (select the cells containing the false positive data) or indicate the alphanumeric value for the first cell and the last cell separated by a colon (i.e., A5:T5)

=MIN (select the cells containing the false positive data) or indicate the alphanumeric value for the first cell and the last cell separated by a colon (i.e., A5:T5)

The range is equal to the maximum value minus the minimum value.

=MAX(A5:T5)-MIN(A5:T5)

For the more statistically minded technologist, when the prompt box appears after “betainv(“” is entered, and the cursor is placed over the BETAINV in the box, a link becomes available. When the link is selected, information about the beta inverse function is displayed at the right of the screen.

It is very efficient to keep a spreadsheet with validation data on each probe. One worksheet with tabs (located on the bottom of the spreadsheet) for each different probe can be easily maintained even beyond validation to ensure that each probe is performing correctly on an ongoing basis. An example is shown in Table 19.2. The number in the TOTAL fields can be easily

Table 19.2 Excel spreadsheet with probe validation data

Validation	1	2	3	4	5	6	7	8	9	10	Total
2R 2G (Normal)	195	190	189	192	190	188	196	193	200	196	1929
1R 1G 1F ("Normal")	5	10	11	8	10	12	4	7		4	71
1R 1G 2F (Abnormal)											0
Total	200	2000									
For 1R 1G 2F:	0.014867										
	1.5%										
	3	cells/200									
For 1R 1G 1F:	0.090098										
	9.0%										
	18	cells/200									
Maximum (MAX):	12										
Minimum (MIN):	0										
Range:	12										
Probe quality assurance											
Case FS-15	11	25	34	37	40	49	52	60	61	65	Total
2R 2G (Normal)	195	189	191	195	192	192	186	197	195	193	1925
'	5	11	9	5	8	8	14	3	5	7	75
1R 1G 2F (Abnormal)											0
Total	200	2000									
Probe 1	Probe 2	Probe 3									
Keeping a spreadsheet with validation data can be easily maintained to ensure that each probe is performing correctly on an ongoing basis. The number in the TOTAL field has been determined by using "=SUM()"; select range by highlighting all pertinent boxes by holding and dragging). Once the first set is established, the next (or previous) group can be filled in by selecting the first and dragging it in the appropriate direction.											

determined by using =SUM(select range by highlighting all pertinent boxes by holding and dragging). Once the first set is established, the next (or previous) group can be filled in by selecting the first and dragging it in the appropriate direction.

19.3.2 Other statistics of use in the cytogenetics laboratory

Statistics are used to describe or explain the meaning of a set of data points. Hundreds of statistical methods exist, so it is extremely important to apply the correct method for a given data set. In the previous section the upper limit of the 95% confidence interval of the binomial equation (as the related *betainv* function in Excel) was used to determine the normal cut-off for FISH probes. Other data sets found in the cytogenetics laboratory are the number of days that are required to release the results of a chromosomal analysis (turn-around-time), the number of chromosomal bands that are demonstrated per karyogram (band resolution) by a given technologist, and the number of metaphase plates observed per 100 cells on a slide used to judge the effectiveness of a culture medium (mitotic index). Statistical methods that are useful for these data sets are the arithmetic mean and the standard deviation, two expressions that are generally found together (mean \pm standard deviation).

Arithmetic mean and standard deviation

The arithmetic mean, \bar{X} , or simply “mean”, is the arithmetic average of a set of values. The formula is:

$$\bar{X} = \frac{1}{n} \sum_{i=1}^n X_i = \frac{X_1 + X_2 + \dots + X_n}{n}$$

where x is the numerical value of each data point and n is the number of data points.

For example, for the number set {4, 5, 6, 7, 8} the mean is:

$$= \frac{4+5+6+7+8}{5} = \frac{30}{5} = 6$$

It is obvious that when a set of numbers has a range of values there are numbers less than and greater than the mean. The extent of the variation from the mean is described by the *standard deviation*. Standard deviation is the most common measure of statistical dispersion, measuring how widely distributed are the values in a data set. If the data points are all close to the mean, then the standard deviation is close to zero. If many data points are far from the mean, then the standard deviation is far from zero. If all the data values are equal, then the mean is equal to the data values and the standard deviation is zero. The standard deviation, σ , is the square root of the mean of the squares of the data point values. The equation is:

$$\sigma = \sqrt{\frac{1}{n} \sum_{i=1}^n (X_i - \bar{X})^2}$$

For our previous data set {4, 5, 6, 7, 8}, the calculation of the standard deviation is as follows:

Replace n with 5, the quantity of numbers in the data set, and \bar{X} with 6, the mean.

$$\begin{aligned}\sigma &= \sqrt{\frac{1}{5} \sum_{i=1}^n (X_i - 6)^2} \\ \sigma &= \sqrt{\frac{1}{5} [(X_1 - 6)^2 + (X_2 - 6)^2 + (X_3 - 6)^2 + (X_4 - 6)^2 + (X_5 - 6)^2]} \\ \sigma &= \sqrt{\frac{1}{5} [(4-6)^2 + (5-6)^2 + (6-6)^2 + (7-6)^2 + (8-6)^2]} \\ \sigma &= \sqrt{\frac{1}{5} [(-2)^2 + (-1)^2 + (0)^2 + (1)^2 + (2)^2]} \\ \sigma &= \sqrt{\frac{1}{5} [4+1+0+1+4]} \\ \sigma &= \sqrt{\frac{10}{5}} \\ \sigma &= \sqrt{2} \\ \sigma &= 1.41\end{aligned}$$

The mean and standard deviation of the data set {4, 5, 6, 7, 8} is 6 ± 1.41

In a normally distributed set of data points, there are as many values above the mean as are below the mean, and the set of data points are described by a bell-shaped (Gaussian) curve. In some cases the curve described is skewed, with more values either above or below the mean. In the cytogenetics laboratory, it is more desirable to have the turn-around-time curve (TAT) with a small standard deviation and with the values skewed toward the fewer number of days! It is also more desirable to simplify the calculation of mean and standard deviation! These calculations can be easily performed with the aid of an Excel spreadsheet.

•	A	B	C
1	4	•	•
2	5	•	•
3	6	•	•
4		•	•
5	8	•	•
6	=average	•	•
	•	•	•

To calculate the mean, enter the data points into a row or column. Select a free cell and enter the following:
=average(select the range of data points by dragging) and <Enter>.

NOTE: Although Excel uses the term “average” for the calculation of the arithmetic mean, in formal statistics the two terms are not strictly interchangeable. Since “average” can refer to several other statistical methods, such as mode and median, the term “mean” will be used in this discussion.

The following will appear near the cell as soon as the left parenthesis is entered:

AVERAGE(number 1,[number 2],...)

When the enter key is pressed the mean will appear in the cell. When the cell in which the mean is displayed is selected the following will appear in the menu field at the top of the worksheet:

=AVERAGE(A1:A5)

Another way to select the data is to indicate the alphanumeric identity of the starting cell and the ending cell with a colon placed between the two, as seen in the box above.

4	
5	
6	
7	
8	
6	MEAN

As with any statistic in Excel, once the box appears after the “=statistic()” is entered, the statistic name becomes a link to information about the properties of the statistic.

Standard deviation also can be easily calculated in Excel. The data points are the same as were used to calculate the mean. In a free cell the following is entered:

=stdevp(select the data range) and <Enter>

4	
5	
6	
7	
8	
6	MEAN
1.41	STDEV

In this case, if all data points are included in the calculation, the standard deviation of the population is used (stdevp).

Graphically, data that are amenable to mean and standard deviation produce a bell-shaped (Gaussian) curve. Figure 19.5 is the graph associated with the following simple series of data points. The lines representing one and two standard deviations above the mean are shown at 7.6 and 9.2. The lines representing one and two standard deviations below the mean are shown at 4.4 and 2.8. If, as represented here, the data is present in a normal distribution, that is, if most of the data points are located close to the mean, then about 68% of the values fall within one standard deviation of the mean, about 95% of the values are within two standard deviations and about 99.7% lie within 3 standard deviations.

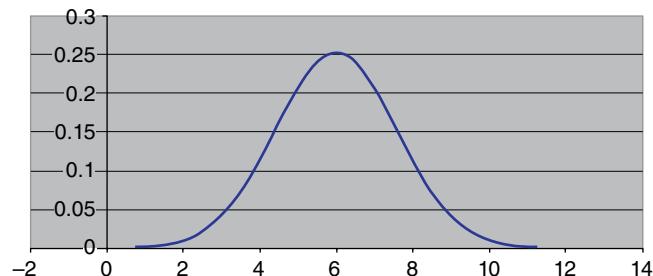


Figure 19.5 Bell-shaped (Gaussian) curve. Standard deviation is the most common measure of statistical dispersion, which determines the distribution of values in a data set. Data amenable to mean and standard deviation produce a bell-shaped (Gaussian) curve. The lines representing one and two standard deviations above the mean in this graph are shown at 7.6 and 9.2. The lines representing one and two standard deviations below the mean are shown at 4.4 and 2.8. The plotted values were:

As another example, consider the following set of data points. They may represent the TAT (turn-around-time) of a number of cytogenetic analyses:

7	
8	
7	
7	
8	
7	
7	
9	
10	
6	
8	
8	
7	
7	
6	
9	
10	
7	
8	
10	
7.8	MEAN
1.2	SD
7	MODE

The graph associated with this series is shown in Figure 19.6.

The shapes of the two graphs are similar. The steepness of the curve will vary depending on how close the data points are to the mean. The smaller the standard deviation, the steeper the curve will be.

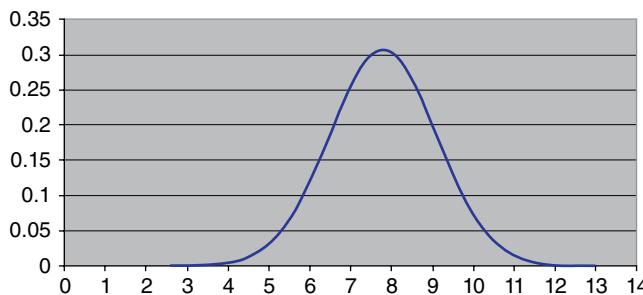


Figure 19.6 Another bell-shaped curve. This bell-shaped curve simulates a standard deviation value for a specific set of turn-around time values. A smaller deviation will show as a steeper curve. Plotted values were:

Modal number

Another useful statistic is the mode, or modal number. This concept is familiar to cytogenetic technologists since the modal chromosome number, 46, is commonly documented. The mode is, simply, the data point that is observed the most number of times. In Excel, this is calculated by entering “=mode(select data range)” into a free cell. Upon pressing <Enter>, the mode will be displayed.

A common misuse of mean and standard deviation is in the determination of the normal cut-off for FISH analysis. In some ways it makes sense that these statistics may be suitable. If the mean plus two standard deviations of the number of false positive signal patterns that are observed in a known normal population is used, then it may seem logical to conclude that this will account for 95% of occurrences. Since the “95% confidence interval” is established as acceptable for this purpose, why is this actually unsuitable?

As seen previously, the data for which a mean is calculated includes values that are greater than and less than the mean. The standard deviation is used to describe the extent of variance around the mean, implying in both directions. When using a mean and standard deviation, it is important to recognize the contribution of all data points in the determination of these values. Since FISH analysis is a qualitative assay, the result is reported as either positive or negative (a binary function). To determine when a result will be considered to be negative, a population that is known to be unaffected by the phenotype/disease for which a FISH test is specific is tested. This will determine the normal cut-off, the value above which an analysis is considered to be abnormal. During the course of this testing, signal patterns other than the expected “normal” may be encountered. By accounting for all “abnormal” signal patterns in the normal population, an expectation for what will be considered truly abnormal is attained. The goal is to gain confidence that the correct interpretation is being made without generating an inordinate number of false positive or false negative results. False positive results may occur if the normal cut-off is set too low and false negative results may occur if the normal cut-off is set too high. The graph in Figure 19.7 depicts the results of a FISH assay on a population of normal and abnormal individuals. Note that the normal cut-off is free of interfering false positive and false negative results.

19.3.3 Choosing the right method to fit the situation

In reality, technical issues may contribute to the appearance of false positive results in a true negative population (attributes of the probe set (single signal, single fusion, dual fusion, etc.), technical artifacts (overlapping signals, fluorescence from nonchromosomal sources, lost chromosomes)). An ideal normal cut-off establishes that all abnormal cases are found on the positive side of the graph without an inordinate number of false positive cases entering from the negative side. In actuality, in the initial diagnosis of untreated patients, the normal cut-off is rarely an issue, with most FISH results exhibiting a large number of abnormal signal patterns. However, in treated patients and in hematological disorders with a small population of abnormal cells, the normal cut-off is an important issue and the data may not be as clear as is depicted in the graph above. The question becomes “How confident am I that the FISH result I have is truly positive or truly negative?”

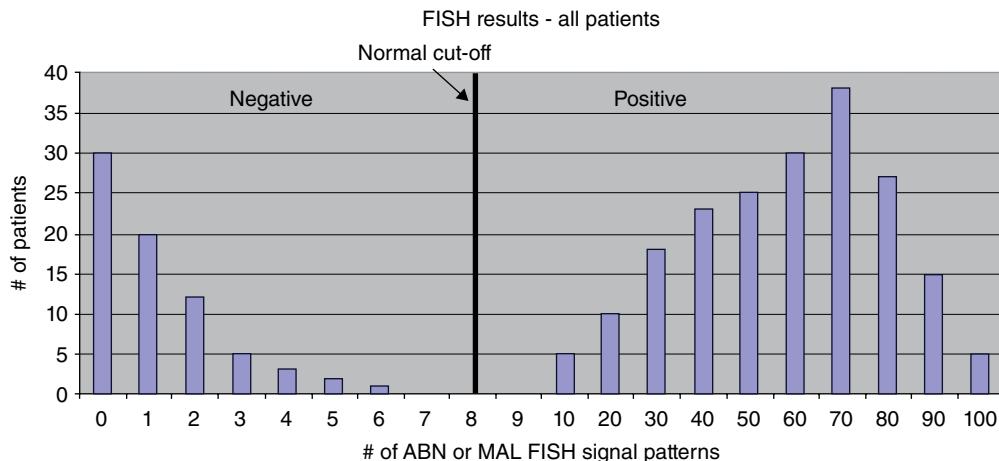


Figure 19.7 Depiction of a FISH assay on a population of normal and abnormal individuals. This graph depicts the results of a FISH assay on a population of normal and abnormal individuals. The normal cut-off is free of interfering false-positive and false-negative results.

In scientific matters, the approved standard is that “I am 95% confident that my FISH result is above the normal cut-off.” How can the 95% be determined? One method uses the upper bound of a one-sided 95% confidence interval of the binomial distribution as the appropriate statistic for determination of the normal cut-off for FISH. By using this method, one can be assured with 95% confidence that all abnormal results lie above the established cut-off level. It is important to note that, since every “normal” individual in a population is not studied during a probe validation and since technical artifacts can occur, that a 100% confidence cannot be established. In cases where FISH results are close to the normal cut-off, an extra measure of caution should be employed, as demonstrated in the following examples.

EXAMPLE 19.30

2R 2G	199	196	195	194	197	198	195	194	200	194	194	197	200	200	198	196	196	196	198	198	MEAN	ST DEV	+1 SD	+2SD
1R 1G 1F	1	4	5	6	3	2	5	6	6	6	6	3	2	4	4	4	2	2	2					
	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	3.8	1.7	5.5	7.2

In the first example, FISH was performed on 20 normal individuals. The expected normal signal pattern is 2 red signals and 2 green signals (2R 2G). One alternative signal pattern is 1 red signal, 1 green signal, and 1 fusion signal (1R 1G 1F). This could represent a genuine abnormal signal pattern or the chance overlap of two signals in an otherwise normal cell. Since the study population is defined as normal, these occurrences are instances of false positives. The question is, how many can be tolerated as normal? In this example, the mean and standard deviation of the “false positive” occurrences is calculated to be 3.8 and 1.7, respectively. If the mean plus two standard deviations is used as the normal cut-off, then signal patterns observed above 8 (cells are present in whole numbers and acknowledging that a bit more than 7 is 8) would be considered to be abnormal. However, there are instances where 6 “abnormal” cells are present in some normal individuals and this is dangerously close to 8 as truly abnormal!

Consider the next examples:

Example 19.31

2R 2G	185	190	187	182	188	183	184	190	188	182	190	190	188	188	190	190	190	191	190	190	MEAN	ST DEV	1 SD	2SD
1R 1G 1F	15	10	13	18	12	17	16	10	12	18	10	10	12	12	10	10	10	9	10	10				
	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200	12.2	3.0	15.2	18.1

Here the mean plus two standard deviations is actually equal to the number of false positive signal patterns (18) observed in one individual.

Example 19.32

2R 2G	200 200	MEAN	ST DEV	1 SD	2 SD
1R 1G 2F					
	200 200	#DIV/0!	#DIV/0!	#####	#####

In this case, all normal individuals exhibited the expected normal signal pattern. What is the normal cut-off? One cell?

While these examples may not conform exactly to real life, they are not impossible and do reveal some insight into why selecting the appropriate statistic is important. For the initial diagnosis of untreated hematological disorders, the number of cells with an abnormal signal pattern will likely be very much higher than the normal cut-off. It is when studying patients for the presence of minimal residual disease that values nearer to the normal cut-off are observed and for which interpretation may be more complicated. It may be more prudent to view a range near the normal cut-off as a gray zone or an equivocal area, when it is decided that additional cells should be scored or when an alternate method (i.e., PCR-based testing) is recommended. This becomes most crucial when dealing with probes that use a dual color, dual fusion methodology. In these cases, even one cell with a positive signal pattern may be consistent with minimal residual disease, as will be seen below.

When using the upper bound of a one-sided 95% confidence interval of the binomial distribution (as the beta inverse function (betainv) in Excel), the pitfalls exemplified above are not seen. Returning to the first example above, the largest number of cells with a 1R 1G 1F signal pattern is 6. When “=betainv(” is entered in Excel, the following is observed:

BETAINV(probability,alpha,beta,[A],[B])

For “probability”, 0.95 is entered.

For “alpha”, 7 is entered. This is the maximum number of false positive signal patterns observed in the normal population plus 1.

For “beta”, 200 is entered. This is the number of interphase cells that were scored for each patient.

For this application, [A] and [B] are not required.

Press <Enter> and convert the cell to percent. Multiply the percent by 200 to determine how many cells are “allowed” to have an “abnormal” signal pattern before it is certain with 95% confidence that all occurrences with this signal pattern are truly abnormal.

MEAN	ST DEV	+1 SD	+2SD
3.8	1.7	5.5	7.2
5.7%	•	•	•
11	200	•	•

0.056675		
5.7%		
11	/200 cells	

For the second example,

MEAN	ST DEV	1 SD	2 SD
12.2	3.0	15.2	18.1

0.119983		
12%		
24	/200 cells	

For the third example,

MEAN	ST DEV	1 SD	2SD
#DIV/0!	#DIV/0!	#####	#####
0.014867			
1.5%			
3			

This case is not unusual to observe with dual color, dual fusion probe strategies. Even one instance of a 1R 1G 2F signal pattern in a treated patient being studied for the presence of minimal residual disease should trigger a higher cell count, reporting the finding with an “interpret with caution” or borderline explanation and/or recommendation for PCR-based testing. In cases where the clinical history is unknown or unclear, this information should be ascertained before a result is released. It appears that the requirement that two positive signal patterns be present in the same cell in order to call the cell abnormal is accompanied by an increased confidence that the result is truly positive when very small numbers of abnormal cells are observed. Keeping in mind that the presence of a single cytogenetic rearrangement (a reciprocal translocation) is being studied, the use of another statistical method in addition to beta inverse may lend. In any case, results that are close to the normal cut-off should be accompanied by caution and contemplation.

19.4 Using a hemacytometer

19.4.1 Determining cell count by using a hemacytometer

Many laboratories performing chromosomal analysis determine cell counts prior to culture initiation to ensure an optimal seeding concentration, usually 1×10^6 cells per mL of culture medium. This is especially true for culture initiation of peripheral blood or bone marrow specimens and for nonculturing solid tumor cells. These tasks can be accomplished by using a hemacytometer. These instruments are constructed so that a fixed volume of suspended cells are allowed into the device. In this way, a representative sample is taken from the original specimen, cells are counted in a prescribed manner, and the total cell count is extrapolated. In the case of solid tumors, a vital dye, like trypan blue, may be added to determine the number of living cells. Several types of hemacytometer are available. A commonly used version is the improved Neubauer hemacytometer (see Figure 19.8). **It is important that the protocol for hemacytometer use for each laboratory be consulted prior to use since specimen preparation, dilutions, and counting conventions may differ.**

The special cover glass is held in position by two raised bars such that the depth between the counting grid and the cover glass is 0.1 mm (see Figure 19.9). The cover glass that comes with the hemacytometer cannot be replaced by an ordinary coverslip since the weight of an ordinary coverslip is not heavy enough to displace the fluid to the required depth.

While protocols for preparing cells for counting may vary, one version is included here as an example. (Refer to the appropriate protocol in use for this application in your laboratory).

1. The peripheral blood or bone marrow specimen may be centrifuged at 800-1000 RPM for 8-10 minutes and the buffy coat (along with serum or medium) removed to a sterile tube. This centrifugation step may be eliminated for bone marrow specimens.
2. For blood or bone marrow specimens, it is necessary to remove the red blood cells prior to counting the mononuclear white blood cells. This can be done by using 3% acetic acid (3 mL of glacial acetic acid is added to 97 mL of de-ionized water) (add 3 mL of acetic acid to 85 mL of de-ionized water in a graduated cylinder and Q.S. to 100 mL).
3. To simultaneously lyse the red blood cells and perform a 1:20 dilution, add 0.025 mL (25 μ L) of blood or bone marrow to 0.475 mL (475 μ L) of 3% acetic acid in a small tube. Mix thoroughly to facilitate red cell lysis and to ensure an even suspension of mononuclear white blood cells. A Unopette capillary tube can be used for this step. Refer to the manufacturer's information or the laboratory protocol for detailed instructions.
4. It is extremely important that the cells be evenly suspended in liquid to ensure an accurate representation of the original specimen. The cells should be suspended in enough liquid so that they are not touching or clumped. This is achieved by diluting the cells to an appropriate concentration [35–50 cells per 0.1 μ L square (one of nine 1 mm \times 1 mm squares) or 350–500 total cells]. If the initial 1:20 dilution is not sufficient, a second dilution should be made. The magnitude of the additional dilution can be approximated and performed as such:

For 1:2 – add 25 μ L of the diluted cells to 25 μ L of 3% acetic acid

For 1:5 – add 25 μ L of the diluted cells to 100 μ L of 3% acetic acid

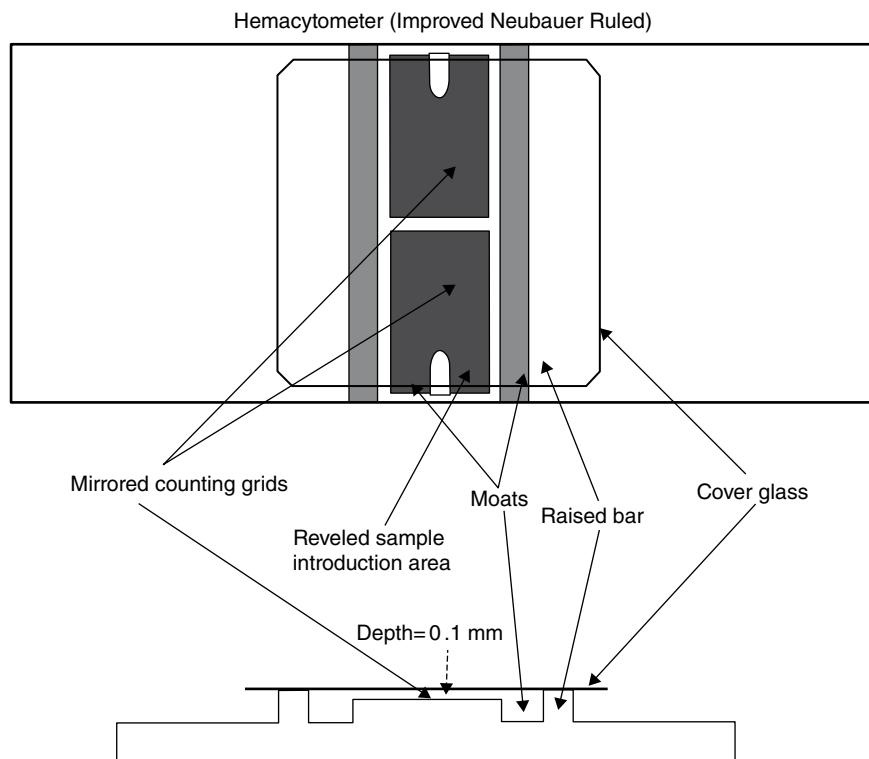


Figure 19.8 Hemacytometer (side view). The hemacytometer determines cell counts prior to culture initiation in order to ensure an optimal seeding concentration. These instruments count the cells within a fixed volume of cell suspension. In the case of solid tumors, a vital dye, like trypan blue, may be added to determine the number of living cells.

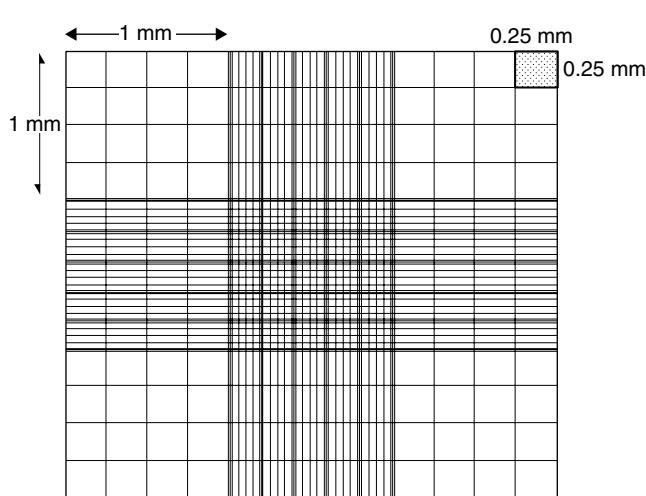


Figure 19.9 Hemacytometer, counting grid. Each mirrored surface contains one counting grid for a total of two per hemacytometer. The grid is made up of nine larger squares. Each large square is $1\text{ mm} \times 1\text{ mm}$ (1 mm^2). With the cover glass in place, the volume of each large square is $0.1\text{ }\mu\text{L}$. The four outer squares are subdivided into 16 smaller squares, each $0.25\text{ mm} \times 0.25\text{ mm}$ (0.0625 mm^2). Two sides of each outer large square are bounded by a triple line. The actual boundary is the middle of the three lines. When the hemacytometer is loaded with the cover glass in place, each of the 9 larger squares contains $0.1\text{ }\mu\text{L}$ of fluid ($1\text{ mm} \times 1\text{ mm} \times 0.1\text{ mm} = 0.1\text{ mm}^3 = 0.0001\text{ mL} = 0.1\text{ }\mu\text{L}$) ($1\text{ mm}^3 = 1\text{ }\mu\text{L}$).

For 1:10 – add 25 μL of the diluted cells to 225 μL of 3% acetic acid

For 1:20 – add 25 μL of the diluted cells to 475 μL of 3% acetic acid

The dilution factor/s will be needed in the final cell count determination, so record it/them for later use.

5. The sample is introduced into the hemacytometer by capillary action. With the cover glass in place on a clean, dry hemacytometer, a small quantity of well-suspended cells (work quickly once the cells have been resuspended) in a pipette are placed on the beveled sample introduction area. Do not force the liquid into the slot, but allow it to be pulled in by capillary action. Remember that a very small volume of liquid (~20 μL total) is used to load the hemacytometer. Repeat the process on the second side. If liquid is seen in the moats surrounding the mirrored surfaces, remove the cover glass, clean and dry the cover glass and the mirrored surface, and begin again. Place the hemacytometer on the microscope and view using a 4 \times objective (40 \times) to confirm the correct cell density. Count the cells at 100 \times using the prescribed laboratory protocol.

One possible counting strategy is to count the cells in the 4 outer large squares as well as the center square for a total of five per side and 10 per hemacytometer (see Figure 19.10). To avoid double counting cells that are located on a line, define two of the four sides of each square as “counted” and the remaining two as “not counted”. For example, the top and right side are counted and the bottom and left side are not counted. Perform counts in the same manner (for example, start at the top right corner of each large square and “snake” down and around until the final square at the lower right corner is reached) each time. This will promote consistency and reliability of counts. If the counts in each of the five squares differ by more than 10, or if the counts on one side of the hemacytometer differ by more than 20, then the cells were not evenly distributed and the counts should be repeated.

To summarize the steps to take when counting cells with a hemacytometer:

1. Place the special cover glass on a clean, dry hemacytometer.
2. Resuspend the cells in the diluted sample just prior to loading the hemacytometer.
3. Using a pipette, pick up a small amount of the cell suspension.
4. Place a drop of the sample onto the triangular depression of the sample introduction area on one side of the hemacytometer. Allow the drop to be taken up into the device by capillary action. Do not force. Repeat on the other side of the hemacytometer. If liquid is seen in the moats, start over.
5. Place the hemacytometer on the microscope stage. View the counting grid using the 4 \times objective. Confirm the correct cell concentration of approximately 35–50 cells per large square. If too many cells are present or if clumps are observed, perform an additional dilution and repeat the process (make a note of all dilution steps and dilution factors).
6. Count the cells in 10 of 18 squares (5 on each side of the hemacytometer) using the prescribed counting strategy and methodology.
7. Add the 10 counts to determine the number of cells contained in one microliter (1 μL).
8. Multiple the number of cells in 1 microliter by 1000 (10^3) to estimate the number of cells in one milliliter (1 mL).
9. Multiple the number of cells in 1 milliliter by the dilution factor(s) to determine an estimate of the number of cells per milliliter of the original specimen.

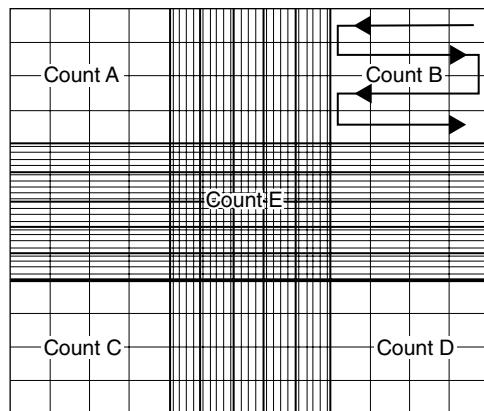


Figure 19.10 Counting strategy. The sum of the 10 squares is the number of cells contained in 1 μL ($10 \times 0.1 \mu\text{L}$). This number is multiplied by 10^3 to determine the number of cells in 1 mL. If the original specimen was diluted, the number of cells in 1 mL is multiplied by the dilution factor/s to yield the number of cells per milliliter in the original specimen (e.g., if the sample of the original specimen was diluted 1 : 20, the dilution factor is 20).

To determine cells per milliliter:

$$\left(\sum_{i=1}^{10} \text{Cells in square} \right) \times 10^3 \times D = \text{cells / mL}$$

where D = dilution factor; a dilution of 1: D = 1 part concentrate + $D - 1$ parts diluent.

EXAMPLE 19.33

Determine the quantity of a bone marrow specimen to add to a culture to ensure that approximately 1×10^6 cells per mL of culture medium are present. A 1 : 20 dilution was performed to prepare the sample for cell count (see Figure 19.11).

Patient: _____ Date: _____

Case #: _____

Dilution(s):

1st: 25 µL buffy or b.m. + 475 µL 3% acetic acid = 1:20

2nd: ____ µL of 1:20 + ____ µL 3% acetic acid = 1:____

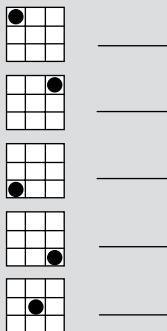
For 1:2 – add 25 µL of the 1:20 to 25 µL of 3% acetic acid

For 1:5 – add 25 µL of the 1:20 to 100 µL of 3% acetic acid

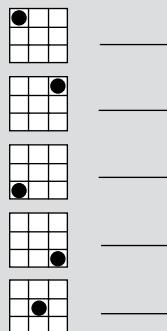
For 1:10 – add 25 µL of the 1:20 to 225 µL of 3% acetic acid

For 1:20 – add 25 µL of the 1:20 to 475 µL of 3% acetic acid

Side 1



Side 2



Total

Side 1 _____

Total

Side 2 _____

Intraside 1Δ <10 _____

Intraside 2Δ <10 _____

Interside Δ <20 _____

TOTAL CELL COUNT _____ (cells per µL)

× 1000 = _____ (cells per mL)

× ____ 20 ____ 1st Dilution

× _____ 2nd Dilution

_____ Cells per mL in original specimen

For 5 mL culture, add $\frac{5 \times 10^6}{\text{cells per mL (original)}}$ = _____ mL = _____ µL

Figure 19.11 Hemacytometer count sheet. Sample form used to determine the needed inoculation volume of a specimen in order to obtain an anticipated cell volume of 1×10^6 cells per mL of culture medium.

- Following the protocol for hemacytometer use, the following cell counts are recorded:

	Side 1	Side 2
A	45	41
B	42	46
C	38	42
D	42	38
E	44	47
	211	214

- $211 + 214 = 425$ cell per microliter of diluted specimen.
- $425 \times 10^3 = 4.25 \times 10^5$ cells per milliliter of diluted specimen.
- $4.25 \times 10^5 \times 20$ (dilution factor) = 8.5×10^6 cells per milliliter of original specimen.
- To add approximately 1×10^6 cells to each milliliter of culture medium:

$$\frac{1 \times 10^6}{8.5 \times 10^6} = 0.118 \text{ mL} = 118 \mu\text{L} \times \# \text{mL of culture medium of original specimen is required}$$

If 5 mL cultures are initiated, then 5×118 , or 590 μL of the original specimen are added to the culture medium. If 10 mL cultures are initiated, then 10×118 , or 1180 μL (1.18 mL) of the original specimen are added to the culture medium. Remember to resuspend the cells in the original specimen before culture initiation.

19.4.2 Determining cell viability using a hemacytometer

When subculturing solid tissue cultures or when initiating cultures from cryopreserved cells, it is important to determine the percent of viable cells that will be contributing to subsequent cultures. Living cells can be distinguished from dead cells by the introduction of a vital dye, like trypan blue, to the liquid used to dilute the cell suspension prior to hemacytometer counting. Trypan blue is excluded from live cells but is taken up by dead cells. The dead cells will appear blue while the live cells will appear clear in a background of blue liquid.

The following scenario is presented as an example. Consult the appropriate protocol for instructions pertinent to your laboratory. Trypan blue is a mutagen (see SDS). Use protective clothing, gloves and eyewear.

- Prepare a cell suspension following the appropriate trypsinization/neutralization protocol. Place the cells in a sterile 15 mL conical tube. Pipette the cell suspension up and down 5–7 times in a 5 mL or 10 mL pipette to ensure proper suspension. Cells that have been cryopreserved in a smaller volume are resuspended by pipetting 7–10 times using a 1 mL pipette.
- Prepare a 1:2 dilution of the cell suspension in trypan blue. Place 15 μL of trypan blue into a conical microfuge tube. Resuspend the cells just prior to dilution. Using a sterile pipette tip, transfer 15 μL of the cell suspension into the microfuge tube containing trypan blue. Mix well by pipetting up and down with the same pipette tip used to transfer the cells. The cell suspension should be kept sterile at all times, however, the cells placed in trypan blue do not require a sterile vessel.
- Load the hemacytometer as described above. For an accurate cell count, the total number of cells contained within one 1 mm^2 should range between 35 and 50. If necessary, perform secondary dilutions. If fewer than 35 cells are present, start over with a less dilute cell suspension.
- Count the cells as described above, keeping track of live (clear) and dead (blue) cells. Add these numbers to obtain the total cell count (see Figure 19.12).
- Divide the number of live cells by the total cell count and multiply by 100. This is the percent of the cells that are viable.
- To calculate the viable cell concentration:
 - Total live cells in 10 squares = live cells per mm^3 (μL)
 - Live cells per $\mu\text{L} \times 10^3 \times$ dilution (2) = live cells per mL
 - Live cells per mL \times total volume of cell suspension = total viable cells recovered

Patient: _____	Date: _____						
Case #: _____							
Dilution(s): 1 st : 15 µL cell suspension + 15 µL trypan blue = 1:2 2 nd : ___ µL of 1:2 + ___ µL trypan blue = 1:___							
Side 1	Clear Live	Blue Dead	Total	Side 2	Clear Live	Blue Dead	Total
	____	____	____		____	____	____
	____	____	____		____	____	____
	____	____	____		____	____	____
	____	____	____		____	____	____
	____	____	____		____	____	____
Total Side 1	____	____	____	Total Side 2	____	____	____
	Live	Dead	Total		Live	Dead	Total
TOTAL LIVE CELLS		TOTAL CELL COUNT					
Side 1 _____	Side 1 _____						
Side 2 _____	Side 2 _____						
Total _____	÷ _____	× 100 = _____ % viable					
TOTAL LIVE CELL COUNT (10 squares) _____ (cells per µL)							
× 1000 = _____ (cells per mL)							
× ____ 2 ____ 1 st Dilution							
× ____ 2 ____ 2 nd Dilution							
= _____ Cells per mL in original suspension							
× mL of original suspension = _____ Total live cells recovered							

Figure 19.12 Hemacytometer viability/count sheet. Sample forms used to determine the viability of a specimen.

19.5 Quantification and purity determination of DNA using spectroscopy

With the introduction of chromosome microarray analysis testing performed in the cytogenetics laboratory, mastery of new techniques beyond the recognition of chromosomes and targeted regions on chromosomes is required. One step in the performance of chromosome microarray analysis is determining the purity and quantity of DNA that has been isolated from a patient blood or tissue sample. UV/visible light spectroscopy is one of the most commonly used techniques to quantify solutions of nucleic acids by characteristic absorption spectra. Many different spectrophotometers are available that use varying quantities of specimen and with varying levels of automation. It is important to follow the protocol that is appropriate to the equipment used in the laboratory. The following discussion is generally applicable to all methods of nucleic acid quantification and purity determination.

The aromatic ring structure of the purine and pyrimidine moieties that make up the nucleoside bases of DNA are responsible for absorbance of UV light at 260 nm. Although each specific base has a maximal absorbance at a slightly different

wavelength, nucleic acids taken collectively will absorb maximally very near 260 nm. The approximate purity of DNA can be determined by examining the ratios of spectrophotometric absorbance of the sample at A260/A280. Pure preparations of DNA have an A260/A280 ratio of 1.8. This method for purity determination cannot be used if phenol has been used in the processing of the sample since this chemical also absorbs at 260 nm.

The quantity of DNA in a sample is derived from the fact that an A260 of 1.0 is equal to 50 µg/mL of DNA. So, the following equation is used to determine the concentration of DNA in the original sample:

$$\text{OD}_{260} \times 50 \text{ }\mu\text{g / mL} \times \text{dilution ratio (e.g., 100 or 400)} = \text{concentration of DNA}$$

Reference

1. Spencer-Adams D. *Lab Math*. New York: Cold Spring Harbor Laboratory Press, 2003.

Additional readings

The above and following books provide insight and are useful guides for mathematically-inclined cytogenetic technologists.

1. Cann AJ. *Maths from Scratch for Biologists*. New York: Wiley, 2003.
2. Stephenson FH. *Calculations for Molecular Biology and Biotechnology*. New York: Academic Press, 2003.

CHAPTER 20

Selected topics on safety, equipment maintenance, and compliance for the cytogenetics laboratory

Helen Jenks and Janet Krueger

(retired), UC Davis Health System, Sacramento, CA, USA

20.1 Introduction

Safety plays an integral role in the operation of the cytogenetics laboratory. Safety practices have come a long way since the early 1970s, when mouth pipetting and flame sterilization were common practices. Each employer is responsible for formulating a laboratory safety program that will minimize the risk of injury or illness to laboratory personnel by ensuring proper training, administrative support, and safe equipment usage. Creating a safe environment requires teamwork between management and staff in order to comply with federal and state laws and regulations. Each employee must know the location of the safety manuals, emergency telephone numbers/contacts, and evacuation routes. Additionally, the staff must know the function and location of the safety equipment, which includes, but is not limited to, pull fire alarms, fire extinguishers, eye washes, safety showers and spill kits. Periodic checks of the safety equipment must be performed and documented.

This chapter will review the major components of laboratory safety, equipment maintenance, and compliance in the cytogenetics laboratory. For a more comprehensive description of quality systems, which includes quality control, proficiency testing, and process control, the reader is referred to Chapter 21, A system approach to quality.

20.2 Biological hazard safety

20.2.1 Safety program

Good laboratory practice requires that all laboratory personnel be knowledgeable in the identification, containment, and prevention of exposure to biological hazards. Bloodborne pathogens, which include but are not limited to hepatitis B (HBV), hepatitis C (HCV), and human immunodeficiency virus (HIV), pose health and safety hazards to those exposed. These pathogens are potentially present in human blood, blood components, body fluids, and solid tissues. All specimen types, therefore, must be handled with caution.

Occupational Safety and Health Administration (OSHA) is the federal agency under the US Department of Labor which publishes and enforces health and safety regulations for most industries, including healthcare. OSHA Standard 29 CFR 1910.1030 addresses the regulation of bloodborne pathogens [1]. The employer is required to enforce a safety plan, including a mandatory training program at initial hire, with annual review sessions, a system for reporting and monitoring accidents, and a written exposure control plan that addresses safety and operating procedures. The plan must be reviewed and updated annually, and must be accessible to all laboratory personnel. The employer must also offer the hepatitis B vaccine and vaccination series to all laboratory personnel at no cost. If an employee declines the hepatitis B vaccine, an appropriate refusal form should be signed by the employee, witnessed, and kept on file to document that the vaccination series was offered.

The laboratory supervisor bears the primary responsibility for monitoring issues that could affect the health and safety of the laboratory staff. The following contact information, hazard warning signs and labels must be posted: (a) emergency telephone numbers; (b) warning labels on biohazard and medical waste containers; (c) location signs for safety showers, eyewash stations, and first aid equipment, and d) warning labels on equipment, such as refrigerators and freezers, containing potential biohazard materials (see Figure 20.1). The international biohazard symbol shown in Figure 20.2 is usually displayed in bright orange or on a bright orange background [2].



Figure 20.1 Signs around the cytogenetics “wet-lab.” Special signs ensure safe operational usage of equipment. Some signs will define, while others may caution the operator of the dangers to be considered. (a) Warnings for flammables: Flammable reagents, such as methanol or fixed pellets, require special warnings when stored within a laboratory or storeroom. Two signs are posted on the cryo-freezer. (b) Warning for storing food: Following standard precautions, biohazard materials must be handled, stored and discarded as if they carry infectious pathogens. Food, therefore, should never be stored in a laboratory refrigerator.



Figure 20.2 International biohazard symbol. Originally proposed by Dow Chemical in the mid 1960s, this symbol has become internationally recognized as the symbol for biological material that may be carrying a potential health risk.

The five major methods of compliance which minimize exposure to bloodborne pathogens include the following:

- Implement work practice controls.
- Utilize personal protective equipment (PPE).
- Practice standard precaution.
- Establish engineering controls.
- Implement housekeeping procedures.

20.2.2 Work practice controls

The Centers for Disease Control and Prevention (CDC) states that hand washing is the single most important measure to prevent acquired infections [3]. All laboratory personnel must wash their hands before and after removal of gloves or other personal protective equipment and following contact with blood or potentially infectious materials. Using soap and water, hand hygiene involves vigorously rubbing hands for about twenty seconds. Rinse thoroughly under running water and completely dry hands using a clean paper towel.

Other restrictions set by the CDC include:

- Do not eat, drink, chew gum, or apply cosmetics, lip balm or contact lenses in the laboratory.
- Do not store food and drink in the same refrigerators, freezers, or cabinets as specimens, chemicals, or reagents.
- Do not mouth pipet. Use a pipette bulb, pipette aid or an automatic pipette.
- Do not bend, recap, or remove contaminated needles or sharps. Sharps are defined as wastes that cause punctures or cuts to personnel (i.e., needles, blades, glass pipettes, and sharp metal). Avoid using needles, scalpel blades and other sharps whenever possible. Dispose of contaminated sharps in appropriate puncture-resistant containers after use.
- Shield suction flasks properly. Trap and contain disinfectant used for biological wastes [4].
- Transport blood, body fluid, and solid tissue specimens in leak-proof containers labeled with proper handling and storage information (see Figure 20.2).

20.2.3 Personal protective equipment

Personal protective equipment (PPE) is safety equipment designed to protect employees from injury and illness. PPE acts as a primary barrier between the biohazard and the individual. Follow the safety protocol from your institution for safely removing PPE without contaminating your clothing, skin, or mucous membranes with potentially infectious materials. Laboratory personnel must wear fluid-resistant laboratory coats to protect themselves from direct exposure to chemicals and infectious materials. Laboratory coats cannot be worn outside of the laboratory and should never be taken home for laundering. The employer must provide a service to launder soiled lab coats or have a facility on-site for laundering.

Gloves provide protection against infectious materials, but should be discarded after each individual task. Remove gloves before opening refrigerators, incubators, doors, or the telephone, unless the item is designated “Gloves Only” (see Figure 20.3), and put on fresh gloves before resuming laboratory tasks. Latex gloves should be powder-free to avoid disseminating the latex protein into air. Nonlatex gloves should be available to workers who are allergic to latex.

Other protective personal equipment includes masks, goggles/glasses, or face shields to protect mucous membranes when performing procedures where splashing of the face may occur. Proper footwear, such as closed heel and toe leather shoes, will also minimize slips and falls and protect the feet from specimen or caustic reagent spills.

20.2.4 Universal (or standard) precautions

Practicing universal (or standard) precautions reduces transmission risks by combining the handling considerations for both recognized and unrecognized bloodborne pathogens (universal precaution) and pathogens from moist body substances (body substance isolation). Regardless of a patient’s diagnosis or presumed infection status, healthcare personnel must handle all blood and body substances as if they could be infectious for HBV, HCV, HIV, and OPIM (other potentially infectious materials). Moist body substances include all body fluids, secretions, and excretions except sweat; nonintact skin; and mucous membranes.

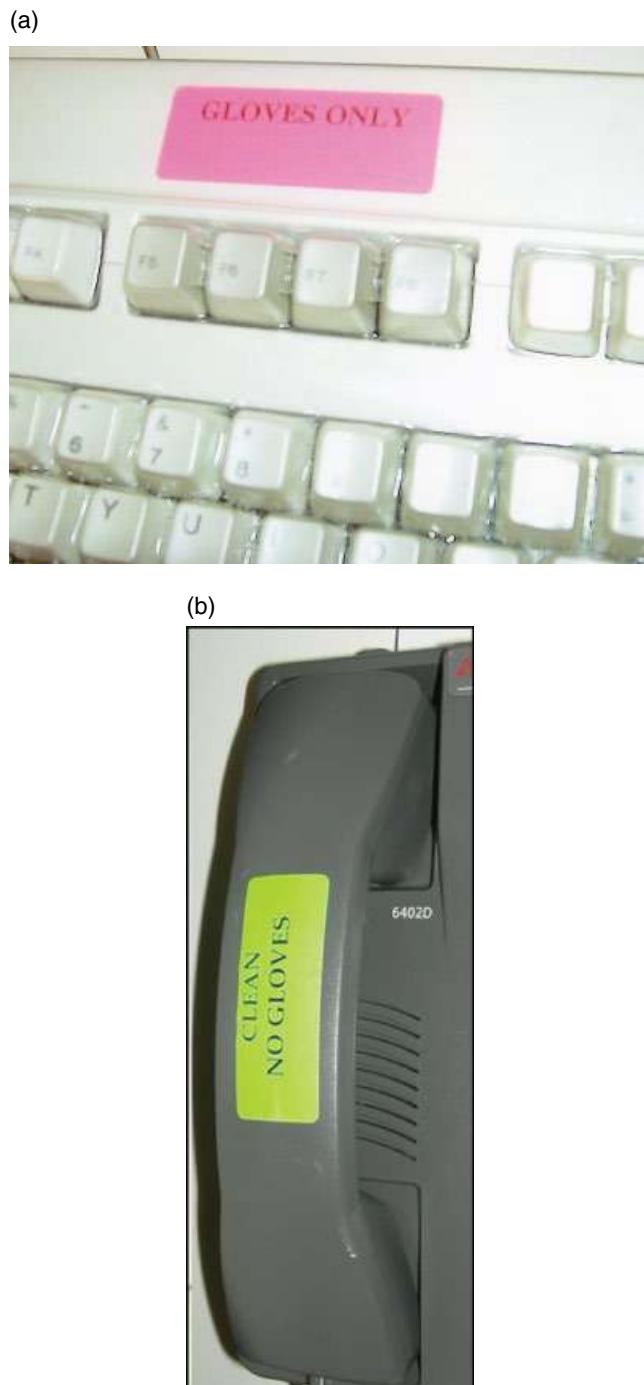


Figure 20.3 Labeling potential biohazard items. Sometimes the most commonplace apparatus can pose the most dangerous health threat. Telephones and computer keyboards should be clearly labeled as “Gloves Only” (a) or “CLEAN No Gloves” (b) in order to ensure that everyone in the laboratory follows the same protocol, thus ensuring that each individual is protected.

20.2.5 Engineering controls

Engineering controls isolate or eliminate employee exposure to bloodborne pathogens by either removing the hazard at the source or by protecting laboratory staff from the hazard. Examples include:

- Hand washing facilities or antiseptic towels must be accessible to those handling specimens.
- Eye wash stations must be accessible within 10 seconds of a work area in the event of splashes of blood or other potentially infectious or caustic materials.
- Contaminated needles, sharps, and broken glass must be discarded in puncture-resistant and leak-proof receptacles, appropriately labeled and color-coded.
- Certified biological cabinets (Class I, II, or III) should be used in order to reduce the risk of exposure to cytogenetics laboratory personnel by directing airflow through a high efficiency filter. Class II cabinets, which are the most common type used in cytogenetics laboratories, serve as the initial primary barrier to infectious agents.

20.2.6 Housekeeping procedures

Choosing a disinfectant will depend upon the type of organism being targeted. Most disinfectants require that the laboratory work surfaces and instruments be pre-cleaned. Concentration and exposure requirements, however, for any commercial product, such as Oxivir Tb, should follow the manufacturer's guidelines. Laboratories should also have a spill control plan that will explain the use and rationale behind each disinfecting agent used by the laboratory, including contact time and precautions [5].

The two most commonly used generic disinfectants are 70% alcohol with a shelf life of six months, and 10% household bleach (sodium hypochlorite), with a shelf life of one day. Exposure time to 70% alcohol is up to 10 minutes, but because of its rapid evaporation rate, it is less effective on residual organic material. A 10% household bleach solution is more effective with ten minutes of exposure, but it is also more corrosive, especially on stainless steel, and is a potential irritant to the laboratory worker. Because of its corrosive nature, household bleach should be wiped off a metal surface with water or alcohol after decontamination. Once opened, the stock shelf life will also decrease to six months after the open date [6].

OSHA Standard 29 CFR 1910.1030 outlines specific housekeeping practices that are required in order to maintain a clean and sanitary environment, for example:

- Regularly decontaminate the work environment for the protection of healthcare professionals.
- Decontaminate all work surfaces between usage, after contact with potentially infectious blood or body fluid, and at the beginning and end of the work shift, using the appropriate disinfectant.
- Clean and decontaminate all equipment surfaces after contact with potentially infectious blood or body fluids and prior to servicing or shipping [7,8]. Caution, however, should be taken in using corrosive disinfectants, like chlorine, to clean stainless steel surfaces, as it can burn a hole through the metal [6].
- Perform routine maintenance of incubators, including shelving, water pans and accessories, as well as hoods, centrifuges, refrigerators/freezers, water baths, and other processing equipment, as outlined by the laboratory's procedure manual, and/or follow manufacturers' recommendations.
- Decontaminate eyewash apparatus on a weekly basis, as outlined by the American National Standards Institute (ANSI), which defines testing, installation, recommended maintenance, and training guidelines for safety equipment. ANSI Z358.1-2004 specifies that, in addition to an annual inspection, weekly maintenance should include flushing the drench hose and spray heads long enough to clear the line of any sediment and debris.
- Discard contaminated waste in containers with puncture-resistant lids, leak-proof containment and appropriate biohazard labeling (see Figure 20.4). Do not overfill. Use a secondary container if the first shows any evidence of leakage. Replace biohazard waste as soon as feasible or as specified by laboratory's procedure manual.

20.2.7 Biological hazard spills

In the event of surface contamination, isolate the contaminated area and put on the appropriate PPE. Wear puncture-resistant gloves to clean up broken glass. Disinfect blood or body fluid spills with 10% bleach solution. Discard all contaminated material into the appropriate puncture-proof biohazard containers.

If laboratory personnel are exposed to biohazard contamination, immediately wash the exposed surface with soap and water and use the eyewash for eye exposure. Refer to the incident reporting section for further information.



Figure 20.4 Waste receptacles. Disposal of medical waste depends on whether the object had been in contact with a live biological specimen or if it has the capability to puncture a bag. (a) All “sharps,” including needles, scalpels and glass, must be discarded in a puncture-proof bucket, irrespective of whether they have been in contact with a biological specimen or not. (b) Plastic and paper materials that have been in contact with a live specimen must be discarded in a regulated medical waste container. If this container is cardboard, it is recommended that it also rest on a platform off the floor surface in order to minimize the risk of being punctured or compromised in the event of a spill. As long as there is no patient identification, plastics and paper that have had NO contact with a live biological specimen can be discarded in a normal waste container. This includes pipettes that have been in contact with fixed cell pellets.

20.3 Chemical safety

The Occupational Safety and Health Administration (OSHA) regulates employee safety in the workplace. It classifies chemicals as health hazards when they fall into the following categories: carcinogens, corrosives, hepatotoxins, mutagens, nephrotoxins, neurotoxins, and teratogens [9]. OSHA’s Chemical Sampling Information provides a general description of each chemical, which includes its Permissible Exposure Limit (PEL), Recommended Exposure Limit (REL), and In Danger to Life or Health Limit (IDLH). The PEL is defined as the average concentration of a chemical in the air to which individuals can be exposed during an entire 8-hour day without adverse effects. Any substances that have a PEL less than 50 mg/m³ should be handled in a laboratory chemical hood.

20.3.1 Know your chemicals

Owing to the vast amount of information available for a reagent, chemical safety is far easier to maintain than biohazard safety. All chemicals should be stored in their original packaging, which comes from the manufacturer clearly labeled with the following information (see Figure 20.5):

- name of the chemical
- expiration date

- appropriate hazard warnings
- proper handling
- storage
- emergency response

When making solutions, containers used for storage must also be clearly labeled with the pertinent information. The major sources of information about chemicals are described below.

Hazard Communication Standard

The Hazard Communication Standard (HCS) (29 CFR 1910.1200 (g)), revised in 2012, is associated with the Globally Harmonized System of and Classification and Labeling of Chemicals (GHS). It requires that the chemical manufacturer provide Safety Data Sheets (SDS), formerly known as Material Safety Data Sheets (MSDS), in a format that will now be internationally used for all hazardous chemicals. This new SDS format is presented in 16 sections. Sections 1 through 8 include information about the chemical, identification, hazards, composition, safe handling practices, and emergency control measures. Sections 9 through 11 and 16 describe the physical and chemical properties, stability, exposure control, and dates of preparation and last revision. OSHA does not enforce sections 12 through 15 as they are enforced by other agencies.

The following list offers a brief description of the 16 sections:

- Section 1: **Identification** includes chemical identification, recommended uses, and manufacturer contact information.
- Section 2: **Hazard(s) Identification** includes chemical hazards and warning information.
- Section 3: **Composition/Information on Ingredients** includes information on chemical ingredients.
- Section 4: **First-Aid Measures** includes symptoms and treatment for chemical exposure.
- Section 5: **Fire-Fighting Measures** lists recommendations for extinguishing a chemical fire.
- Section 6: **Accidental Release Measures** lists emergency procedures for handling chemical spills, leaks, or releases, including containment and cleanup.
- Section 7: **Handling and Storage** lists safe handling and storage of chemicals.
- Section 8: **Exposure Controls/Personal Protection** lists OSHA's Permissible Exposure Limits (PELs) engineering controls (i.e., exhaust ventilation), and personal protective equipment (PPE) to minimize chemical exposure.
- Section 9: **Physical and Chemical Properties** lists the characteristics of the chemical, that is, appearance and odor.
- Section 10: **Stability and Reactivity** lists chemical stability and reactivity hazards information.
- Section 11: **Toxicological Information** includes routes of exposure and symptoms.
- Section 12: **Ecological Information** (nonmandatory) describes the environmental impact of the chemical.
- Section 13: **Disposal Considerations** (nonmandatory) describes the proper disposal practices.
- Section 14: **Transport Information** (nonmandatory) describes transport of hazardous chemicals.
- Section 15: **Regulatory Information** (nonmandatory) identifies health, safety, and environmental regulations.
- Section 16: **Other Information** states chemical preparation and last revision dates [9].

Chemical Hygiene Plan

A Chemical Hygiene Plan (CHP), which is required by OSHA, defines the safety procedures for all hazardous chemicals used in the laboratory. The plan must list carcinogenic potential, reproductive toxicity and acute toxicity of the chemicals in use. The laboratory director must ensure that the Safety Data Sheets (SDS) are accessible to the laboratory staff. A detailed description of the SDS content is accessible using the following link: www.OSHA.gov/1910.1200, Appendix D.

Laboratory Chemical Safety Summaries

Laboratory Chemical Safety Summaries (LCSS) are an excellent source of safety data for chemicals commonly used in laboratories and applies specifically to the laboratory environment. These summaries are included as Appendix B in the National Research Council's report, *Prudent Practices in the Laboratory: Handling and Disposal of Chemicals* [10]. The LCSS addresses toxicity, flammability, reactivity, and explosive capability date; directions for handling, storage, and disposal; and first aid and emergency response instructions [11].

National Fire Protection Agency

The National Fire Protection Agency (NFPA) provides a four-color hazard identification label, which may be found on the label or SDS of some chemicals. The symbol (see Figure 20.6) is diamond shaped, with each of the four colors representing a class of hazard risk [10] and its associated hazard rating from 0 (no hazard) to 4 (greatest hazard).

(a)



Hazard Communication Standard Labels

OSHA has updated the requirements for labeling of hazardous chemicals under its Hazard Communication Standard (HCS). As of June 1, 2015, all labels will be required to have pictograms, a signal word, hazard and precautionary statements, the product identifier, and supplier identification. A sample revised HCS label, identifying the required label elements, is shown on the right. Supplemental information can also be provided on the label as needed.

For more information:



1-800-321-OSHA (6742)
www.osha.gov

SAMPLE LABEL	
CODE _____ Product Name _____	Product Identifier
Company Name _____ Street Address _____ City _____ State _____ Postal Code _____ Country _____ Emergency Phone Number _____	Supplier Identification
Hazard Pictograms	
Signal Word Danger	
Keep container tightly closed. Store in a cool, well-ventilated place that is locked. Keep away from heat/sparks/open flame. No smoking. Use explosion-proof electrical equipment.	
Take precautionary measures against static discharge. Ground and bond container and receiving equipment. Do not breathe vapors. Wear protective gloves.	
Do not eat or drink or smoke when using this product. Wash hands thoroughly after handling. Dispose in accordance with local, regional, national, international regulations as specified.	
In Case of Fire: use dry chemical (BC) or Carbon Dioxide (CO ₂) fire extinguisher to extinguish.	
First Aid If exposed call Poison Center. If an eye is hurt: Take off immediately any contaminated clothing. Rinse skin with water.	
Precautionary Statements	
Highly Flammable liquid and vapor. May cause liver and kidney damage.	
Hazard Statements	
Supplemental Information Directions for Use _____	
Fill weight: _____ Lot Number: _____ Gross weight: _____ Fill Date: _____ Expiration Date: _____	

OSHA 3492-02 2012

(b)



Hazard Communication Standard Pictogram

As of June 1, 2015, the Hazard Communication Standard (HCS) will require pictograms on labels to alert users of the chemical hazards to which they may be exposed. Each pictogram consists of a symbol on a white background framed within a red border and represents a distinct hazard(s). The pictogram on the label is determined by the chemical hazard classification.

HCS Pictograms and Hazards

Health Hazard	Flame	Exclamation Mark
 <ul style="list-style-type: none"> Carcinogen Mutagenicity Reproductive Toxicity Respiratory Sensitizer Target Organ Toxicity Aspiration Toxicity 	 <ul style="list-style-type: none"> Flammables Pyrophorics Self-Heating Emits Flammable Gas Self-Reactives Organic Peroxides 	 <ul style="list-style-type: none"> Irritant (skin and eye) Skin Sensitizer Acute Toxicity (harmful) Narcotic Effects Respiratory Tract Irritant Hazardous to Ozone Layer (Non-Mandatory)
Gas Cylinder	Corrosion	Exploding Bomb
 <ul style="list-style-type: none"> Gases Under Pressure 	 <ul style="list-style-type: none"> Skin Corrosion/ Burns Eye Damage Corrosive to Metals 	 <ul style="list-style-type: none"> Explosives Self-Reactives Organic Peroxides
Flame Over Circle	Environment (Non-Mandatory)	Skull and Crossbones
 <ul style="list-style-type: none"> Oxidizers 	 <ul style="list-style-type: none"> Aquatic Toxicity 	 <ul style="list-style-type: none"> Acute Toxicity (fatal or toxic)

For more information:

OSHA® Occupational Safety and Health Administration
U.S. Department of Labor
www.osha.gov (800) 321-OSHA (6742)



Pictograma para la norma sobre la comunicación de peligros

A partir del 1.º de junio de 2015, la norma de comunicación de peligros (HCS, por sus siglas en inglés) exigirá pictogramas en las etiquetas para advertir a los usuarios de los peligros químicos a los que puedan estar expuestos. Cada pictograma representa un peligro definido y consiste en un símbolo sobre un fondo blanco enmarcado con un borde rojo. La clasificación del peligro químico determina el pictograma que muestra la etiqueta.

Pictogramas y peligros según la HCS

Peligro para la salud	Llama	Signo de exclamación
 <ul style="list-style-type: none"> Carcinógeno Mutagenicidad Toxicidad para la reproducción Sensibilización respiratoria Toxicidad específica de órganos diana Peligro por aspiración 	 <ul style="list-style-type: none"> Inflamables Pirofóricos Calentamiento espontáneo Desprenden gases inflamables Reaccionan espontáneamente (autorreactivas) Peróxidos orgánicos 	 <ul style="list-style-type: none"> Irritante (piel y ojos) Sensibilizador cutáneo Toxicidad aguda (dañino) Efecto narcótico Irritante de vías respiratorias Peligros para la capa de ozono (no obligatorio)
Botella de gas	Corrosión	Bomba explotando
 <ul style="list-style-type: none"> Gases a presión 	 <ul style="list-style-type: none"> Corrosión o quemaduras cutáneas Lesión ocular Corrosivo para los metales 	 <ul style="list-style-type: none"> Explosivos Reaccionan espontáneamente (autorreactivas) Peróxidos orgánicos
Llama sobre círculo	Medio ambiente (No obligatorio)	Calavera y tibias cruzadas
 <ul style="list-style-type: none"> Comburentes 	 <ul style="list-style-type: none"> Toxicidad acuática 	 <ul style="list-style-type: none"> Toxicidad aguda (mortal o tóxica)

Para más información:

OSHA® Administración de Seguridad y Salud Ocupacional
Departamento de Trabajo de los EE. UU.
www.osha.gov (800) 321-OSHA (6742)

Figure 20.5 Chemical labels. OSHA has changed chemical labeling to provide more safety information. These Quick Cards (a) and pictogram explanations (b) are available on their website at: https://www.osha.gov/Publications/HazComm_QuickCard_Labels.html and pictograms at https://www.osha.gov/Publications/HazComm_QuickCard_Pictogram.html. Reproduced with the kind permission of OSHA publications.

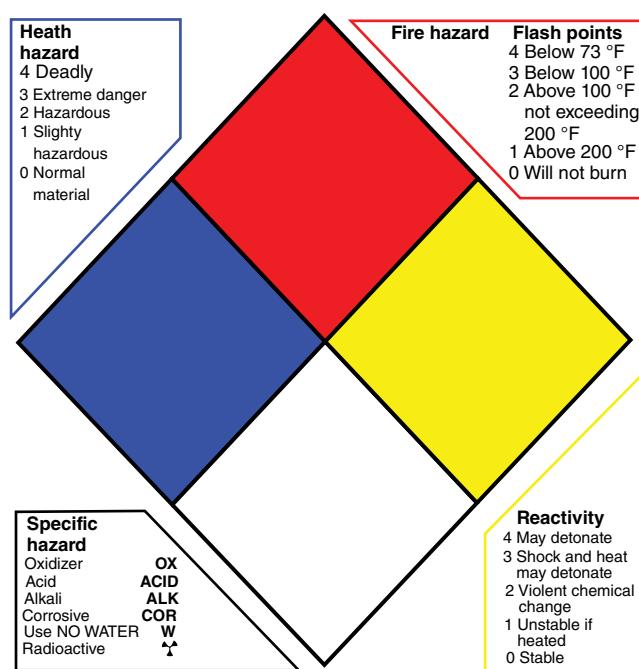


Figure 20.6 Four-color hazard identification symbol. The degree of hazard is rated from 0 to 4 in each field, with 4 representing the greatest hazard and 0 indicating no hazard. Reprinted with permission from NFPA. This reprinted material is not the complete and official position of the NFPA on the referenced subject, which is represented solely by the standard in its entirety. The classification of any particular material within this system is the sole responsibility of the user and not the NFPA. NFPA bears no responsibility for any determinations of any values for any particular material classified or represented using this system. See insert for color representation of this figure.

- B – Blue indicates health hazard
- R – Red indicates flammability
- Y – Yellow indicates reactivity
- W – White indicates Special Information

- Blue indicates a health hazard, with 2 reflecting hazardous health risks and 4 warning of potential deadly features.
- Red indicates flammability, where 3 warns that the chemical can ignite at temperatures below 100°F (13.78°C), and a 4 rating is ignitable at room temperature (73°F, 22.78°C) or below.
- Yellow represents reactivity characteristics, which ranges from 0 (stable) to 4 (may detonate).
- White will reflect specific hazard characteristics for that individual chemical, such as OX (oxidizer), ACID, ALK (alkali), COR (corrosive), W (use NO water), or radioactive.

20.3.2 Proper chemical storage

Safe chemical storage provides for the effective management of chemicals, lessens the risk of fire, prevents the accidental mixing of incompatible chemicals, and minimizes exposure to hazardous chemicals. Knowledge of each chemical and its potential hazard (see Figure 20.7) is required to determine the most appropriate storage requirements.

- Store chemicals on shelves with raised edges, NOT in the fume hood. Store only the amount of each chemical that can easily be stored and used before the manufacturer's expiration date.
- Store chemicals in original containers that are clearly labeled with the date of receipt, date put into use and expiration date.
- Discard all chemicals with improper labels or of uncertain date of receipt.
- Separate the chemicals into compatible groups, using the manufacturer's color-coded labels, and store alphabetically within the groups.
- Dry chemicals may be stored together. Separate organic and inorganic chemicals.



CHEMICAL NAME	USE	NFPA HAZARD				CAS#
		H	F	R	C	
5-Bromo-2-deoxyuridine	Cell Culture	0	0	0		59-14-3
5-Fluoro-2-deoxyuridine	Cell Culture	2	0	0		50-91-9
Acetic Acid	Cell Harvest	3	2	2	4	64-19-7
Barium Hydroxide Octahydrate	Staining	3	1	1		12230-71-6
Buffer Solution pH 7.0	pH Indicator	1	0	0		7778-77-0
Cement (Rubber)	FISH	2	4	0		142-82-5
Citric Acid	Buffer	2	0	0		77-92-9
KaryoMax Colcemid®	Cell Harvesting	0	0	0		
Collagenase	Cell Culture	3	0	0		12-1-9001
Cytoseal	Mounting Coverslips	2	3	0		80-56-8
Ethyl Alcohol	Cleaning/Staining/FISH	2	3	0		64-17-5
Formamide	FISH	2	1	0		75-12-7
Formic Acid	Staining	3	2	1		64-18-6
Fyrite (Potassium hydroxide)	CO ₂ Maintenance	3	0	1		1310-58-3
Gentamicin	Cell Culture	3	0	0		1405-41-0
Hydrochloric Acid (33%–38%)	C-banding, FISH	3	0	1	acid	7647-01-0
Lectin	Cell Culture	2	0	0		63231-57-2
Magnesium Chloride	FISH	1	0	0	1	7786-30-3
Methanol	Cell Harvesting	1	3	0		67-56-1
Methotrexate hydrate (Amethopterin)	Peripheral Blood	2	0	0		133073-73-1
Potassium Chloride	Cell Harvesting	2	0	0		7447-40-7
Silver Nitrate	Staining	3	0	1	ox	7761-88-8
Sodium Citrate	Harvesting	0	0	0		68-04-2
Sodium Hydroxide	Staining	3	0	1	cor	1310-73-2
Trypsin-EDTA (.25%)	Staining; Cell Culture	1	0	0		
Wright Stain	Staining	2	0	0	0	61-73-4 17372-87-1



Figure 20.7 Know your chemicals. Most laboratories maintain an inventory log of all chemicals used in the laboratory, which would include their NFPA rating (see Figure 20.6) and their unique CAS chemical registry number, which is assigned by the Chemical Abstracts Service (CAS) of the American Chemical Society. Laboratory personnel must be aware of the characteristics for all chemicals being handled in the laboratory and must be able to access any SDS for a reagent used in the laboratory. The examples in this list may not apply to the reagent used within your laboratory.

- Store acids in trays or tubs that will catch spills and provide for proper segregation. Do not store above shoulder height.
- Store flammables in an approved fire cabinet away from heat and flames. Flammable chemicals that require cold storage must be stored in refrigerators approved for flammable storage.
- Store highly toxic chemicals in a separate designated storage area with appropriate warning signs.
- Store hazardous chemicals near a chemical fume hood to facilitate use of the hood when using the chemicals.

20.3.3 Fume hoods

Chemical fume hoods are as important to a laboratory technologist as personal protective equipment. Fume hoods are designed to protect the operator from exposure to chemical fumes, aerosols, and gases generated from chemicals used within the hood by pulling room air into the hood and venting to the outdoors, thus diluting its toxic concentration. Hoods should be used when handling any flammable, hazardous, or toxic chemical.



Figure 20.8 Safety bottle carrier. Storing chemical reagents in a stockroom that is located apart from the general laboratory creates the need to transport bottles as needed. Special rubber carriers are used to transport commonly used acids and bases, such as methanol and acetic acid. Note: Never carry a container by its cap, especially those containing biological specimens or hazardous chemicals.

For proper operation, plan ahead and gather all material needed in the hood. Raise the sash to the manufacturer's specified level to ensure best control and to serve as a physical barrier to protect you from splashes or explosions within the hood. Avoid obstructing the airflow with unnecessary equipment or chemicals in the hood.

20.3.4 Working with chemicals

General principles [12]

- Wear appropriate PPE to minimize all chemical exposures, and avoid skin contact with chemicals.
- Avoid underestimation of risk, even for substances of no known significant hazard.
- Use special precautions when working with substances presenting special hazards.
- Purchase chemicals in shatter-resistant bottles, if available.
- Transport large bottles of chemicals in a safety bottle carrier (see Figure 20.8).
- Assume that any mixture will be more toxic than its most toxic component, and that all substances of unknown toxicity are toxic.
- Provide adequate ventilation.
- Prevent the escape of chemicals into the working atmosphere by use of hoods and other ventilation devices.
- Institute a chemical hygiene program. A mandatory chemical hygiene program designed to minimize exposures should be a regular, continuing effort, not merely a standby or short-term activity.
- The Permissible Exposure Limits (PEL) of OSHA and the Threshold Limit Values (TLV) of the American Conference of Governmental Industrial Hygienists should not be exceeded.

20.3.5 Chemical emergencies

In the event of a chemical exposure or spill, refer to the SDS and implement the following guidelines:

- **Eye contact:** Flood eyes with water immediately and continue flooding for at least 15 minutes, including under the eyelids. Wearing contact lenses should be avoided, but if they are worn, remove lenses if possible, or move to the corner of the eye.

- Any eye injury, no matter how small, may be serious. Seek immediate medical attention. **Know the location of the nearest eyewash station.**
- **Skin contact:** Flood the affected area with water immediately and continue flooding for at least 15 minutes. Do not rub or abrade the skin. If a substantial portion of the body is involved, use a safety shower. Remove contaminated clothing. If the chemical is toxic, or if its toxic properties are unknown, seek immediate medical attention. **Know the location of the nearest emergency shower.**
 - **Inhalation:** If inhalation has occurred, move to fresh air immediately. An unconscious individual may have to be removed from the area by coworkers. Call for immediate attention as described by your institution's emergency protocol or call 911. Loosen clothing and check breathing. If the individual is not breathing, apply artificial respiration until breathing is restored. Seek immediate medical attention.
 - **Ingestion:** For ingestion of chemicals, call poison control or the doctor immediately. Rinse the mouth repeatedly with water, but do not swallow. If the chemical has been swallowed, drink water to dilute the chemical. Do not give liquid to an unconscious person. Seek immediate medical attention.
 - **Chemical exposure:** Refer to the SDS for appropriate first-aid treatment.
 - **Chemical spill:** Check the container or SDS for instructions. If no instructions are immediately available, encircle the spill with Safestep Spill Absorbent or equivalent (see Figure 20.9). If you suspect the spill is flammable, extinguish all ignition sources. If the spill is large, or has definite or unknown corrosive, explosive, or toxic properties, evacuate the area and call 911 [3].

20.4 Fire safety

20.4.1 Fire prevention unit

Flammable chemicals/solvents and malfunctioning electrical equipment are common causes of laboratory fires. Each employer's fire prevention unit offers services to the staff, including fire safety training and inspections, testing fire protection systems (e.g., fire alarms), and investigating potential problems [3].

20.4.2 Fire drills

Code names and their corresponding indications may vary per institution, but every workplace should have a standard coding system for emergency situations. For example, some may use Code Red to designate a fire situation. Laboratory staff is required to respond to a Code Red. Federal and state agencies require mandatory fire drills for all laboratory staff. Fire drills should be treated in the same way as an actual Code Red (see Figure 20.10).

The acronym **RACE** as defined by **OSHA** is used to aid staff in remembering how to respond to a fire situation.

R – RESCUE all persons from the area.

A – Activate ALARM and call 911.

C – CONFINE or close doors to the fire area.

E – EXTINGUISH only if the fire area is small.

20.4.3 Fire extinguishers

Fires are classified as A, B, and C.

Class A fires involve ordinary combustibles such as paper, wood or cloth.

Class B fires involve flammable liquids.

Class C fires involve electrical equipment.

Use the appropriate fire extinguisher corresponding to Class A, B, or C fires. The multipurpose fire extinguisher can be used on any class of fire. Fire extinguishers must be inspected annually.

PASS is the acronym used for the operation of a fire extinguisher.

P – Pull the pin from the fire extinguisher.

A – Aim at the base of the fire.

S – Squeeze the handle.

S – Sweep the base of the fire.

(a)

Infectious (or Biological) Spills

1. Notify supervisor or safety officer and staff in the immediate area.
2. Put on appropriate PPE.
3. Cover an area twice the size of the spill with germicide/disinfectant-soaked paper towels. Set timer for a 20-minute contact period.
4. After 20 minutes, use forceps or broom to remove broken glass and other solid items. Discard all clean-up material in sharps container or red biohazard bag.
5. Clean area with fresh disinfectant.

(b)

ACID Splashes and Spills

- Eyes: • Flush thoroughly with water for 15 minutes.
Remove contact lenses immediately.
• Go to emergency room.
- Skin: • Remove contaminated clothing.
• Flush skin with large amounts of water for 15 minutes.
• Go to emergency room.
- Spills: • Wear protective clothing.
• Use spill control pillows to absorb spills.
• Notify laboratory safety officer and/or supervisor.

(c)

CHEMICAL SPILL Clean-up StepsMAJOR SPILLS*

- Dial 55
- Assist injured persons
- Contain spill, if possible
- Move to clear area until Safety/Security arrives
- Locate SDS^[1]
- Clean-up spill as instructed using PPE^[2]
- Place clean-up materials in Hazardous Material Disposal Bags and give to Facilities
- Notify supervisor

MINOR SPILLS

- Wear appropriate PPE**
- Consult SDS^[1]**
- Clean spill
- Discard clean-up material in sealed bag & consult Lab Chemical Hygiene Officer for disposal
- Notify supervisor

*NOTE: A MAJOR SPILL is defined either as greater than one liter of a general solvent or chemical reagent OR any unmanageable volume of an acutely toxic/hazardous substance, extremely flammable, volatile, carcinogenic or radioactive material.

^[1]SDS = safety data sheet FRONT SHELF^[2]PPE = personal protective equipment

Figure 20.9 (a–c) Laboratory safety signs. Being prepared for an accident before it occurs is the safest way to operate a laboratory. Signs are an effective means for immediate reminders.

20.5 Electrical safety

The clinical engineering department is responsible for electrical safety testing on all electrically powered laboratory equipment. Safety testing is required when the equipment is placed into service and when equipment is periodically inspected. Document all safety testing, repairs, and inspections. Plug all critical equipment, such as computers, incubators, and refrigerators/freezers into emergency electrical outlets, and consider an alarm system to alert personnel of power outages or temperature fluctuations. Emergency lighting must be available in the event of any interruption of normal lighting. The laboratory must comply with the following guidelines:

- Ground electrical equipment with a three-pronged plug.
- Replace frayed wires and plugs.
- Avoid using extension cords.
- Inspect each piece of electrical equipment routinely, and remove faulty equipment from service immediately.
- Never place equipment on wet surfaces [13].



Figure 20.10 (a-d) Safety plan. In order to be prepared to handle emergency situations, technologists must know what safety tools are available to them. Safety training and periodic drills provide the technologist with this critical information, e.g., location and operation of fire extinguishers, fire blankets, eyewash station, showers, and emergency exits.

20.6 Disaster plan

A disaster plan is designed to respond to external or internal events which cause a major disruption (i.e., natural disasters like earthquakes, external acts of terrorism or internal acts of civil disobedience). Each institution is responsible for the development and maintenance of response plans, policies, and procedures, in conjunction with local, state and federal government public health policy and procedure.

20.7 Equipment operation, maintenance, and safety

NOTE: This section does not include image analysis system, metaphase finder system, metaphase chromosome harvester, chromosome microarray, and genome sequencing equipment use or maintenance.

Equipment-related injuries can be minimized or eliminated with proper training and adherence to the manufacturer's operation instructions. Improper handling and use of gas cylinders, autoclaves, and centrifuges may lead to injury.

A medical equipment management plan is required to ensure safe operation and minimize both physical and clinical risks of each piece of equipment. The clinical engineering department is responsible for the repairs, inspection, labeling, and inventory of medical equipment [13]. Each technologist must demonstrate and document competency for each piece of equipment.

The following guidelines should be followed:

- Schedule routine inspections and preventive maintenance (see Table 20.1) for instruments and equipment in order to maintain performance quality and prolong functional utility.
- Check clinical laboratory equipment for adequate electrical grounding and current leakage [14].
- Document routine cleaning, replacement of parts, maintenance service, calibrations, and any repairs in the equipment maintenance manual. If a piece of equipment is malfunctioning, remove it from service immediately and report it to the clinical engineering department.

Table 20.1 Equipment maintenance schedule

Daily

- Clean work area/biological cabinet with disinfectant before and after use
- Record temperatures, CO₂/O₂ levels and humidity for incubators
- Record temperatures for refrigerators, freezers, ovens and water baths
- Monitor gas levels and pressure for CO₂/O₂ gas cylinders
- Monitor temperature, humidity, and water level for environmental controlled chamber
- Robotic harvester
 - Drain and clean liquid lines
 - Clean end of pipetting tip
 - Clean Petri dishes, racks and work table
- Clean microscope objectives and cover microscope when not in use

Weekly

- Record incubator CO₂/O₂ using a fyrite gas analyzer
- Check water level in incubator humidity pan

Monthly

- Clean and disinfect centrifuges and water baths
- Replace water and add Roccal in humidity pans in incubators
- Robotic harvester
 - Clean Z-toothed rack and Y-slides
 - Examine valves and tips

Biannually

- Have biosafety cabinets inspected and recertified professionally
- Service and calibrate pipettes, pH meters, scales and analytical balances
- Clean and disinfect incubator walls and trays and replace filters if discolored
- Have centrifuge timers and RPM verified by clinical engineering or the equivalent
- Perform preventative maintenance on environmental control chamber

Annually

- Have microscopes professionally cleaned and serviced
- Inspect all equipment power cords
- Have fume hoods inspected and recertified professionally
- Inspect centrifuge brushes and electrical components
- Robotic harvesters
 - Wipe down arm assembly
 - Clean and lubricate syringe pump and X and Y bearings
 - Inspect/replace drive belt, tubing, valves, syringes, block and probe tip

Follow manufacturer's guidelines for the operation and maintenance of all laboratory equipment, and refer to the owner's manual as the primary reference. The following section discusses the operation, maintenance, and safety of specific equipment commonly found in or used by the cytogenetics laboratory.

20.7.1 Autoclaves

Operation and Safety

- Properly load the autoclave. Before starting the cycle, make sure the door is fully closed and the correct cycle has been selected.
- Autoclaves create both physical (e.g., heat, steam, and pressure) and biological hazards. Wear heat-resistant gloves when removing autoclaved items.
- Vent the autoclave to release steam before fully opening the door after use.
- Use an indicator tape to ensure proper decontamination of items placed in the autoclave.

Maintenance

- Check seals around the door on a routine basis.
- Maintenance should include periodic efficiency tests, such as *Bacillus stearothermophilus* spore testing, to ensure proper operation [15].

20.7.2 Compressed gas cylinders

Operation and safety

- Gas cylinders supply incubators with CO₂, O₂, and N₂.
- Transport and secure cylinders on a cart.
- Avoid rolling, dragging, or sliding the cylinders.
- Valve safety covers should remain on the cylinder until the regulator is ready to be installed.

Maintenance

- Store the cylinders in a cool, well-ventilated location and secure with chains to a wall or solid structure [13].
- Do not use lubricants on valve regulators or fittings.

20.7.3 Centrifuges

Operation and safety

- Always operate the centrifuge with the top down and locked.
- Always cap and balance test tubes before centrifugation.
- Stop the centrifuge immediately and check the tube balance if an unusual noise or vibration begins.
- Do not exceed a safe rotor speed.
- Allow the centrifuge to come to a complete stop before opening, and wear gloves to remove specimens.
- Check the inside of the centrifuge, including the buckets, regularly for possible spills and leaks. Clean with noncorrosive disinfectant if necessary (biological hazard).
- If a centrifuge malfunctions while in operation, turn it off and unplug (physical hazard).

Maintenance

- Keep the centrifuge clean and dry.
- Wipe the outside and inner rim with a disinfectant (i.e., 70% alcohol) daily. Clean all spills immediately. Do not use harsh cleansers, for example, bleach, unless approved by manufacturer.
- Routinely clean buckets with detergent and grease pivots monthly.
- The clinical engineering department/outside contractor checks the timer/RPM biannually using a tachometer, inspects the brushes and electrical components annually, and maintains the service records [16].



Figure 20.11 Min–max thermometer. Maintaining an appropriate temperature range is critical to both human cell cultures and temperature-dependent reagents. In order to ensure that the temperature has remained stable overnight in a critical apparatus like culture incubators, refrigerators and freezers, minimum and maximum temperatures are monitored and documented on the daily QC log (see Chapter 21: A system approach to quality).

20.7.4 Thermometers

Operation and safety

- Laboratory thermometers are used to measure temperatures with a high degree of precision and usually measure temperatures in degrees Celsius (see Figure 20.11).
- Liquid-in-glass thermometers are made of sealed glass and contain a fluid, usually mercury or red alcohol. Note: Replace mercury thermometers with alcohol thermometers to eliminate health and environmental hazards.
- Thermometers are classified as total, partial, or complete immersion [17]. Use the appropriate type of thermometer following equipment or procedure guidelines.
- TempTale RF temperature monitors record temperatures in real time. The time, temperature and location for refrigerators, freezers and incubators are recorded and transmitted wirelessly to a local computer where the information can be downloaded to a graphic display. These monitors serve as a valuable tool for initial validation and semi-annual calibration of the equipment.

Maintenance

- The National Institute of Standards and Technology (NIST) and the American Society of Testing Material (ASTM) calibrate laboratory thermometers and provide traceability.
- Record date of calibration or purchase calibrated thermometers.

20.7.5 Refrigerators/freezers

Operation and safety

- Record temperatures daily on a chart, and retain charts with other maintenance records.
- Notify clinical engineering/outside vendor if equipment malfunction occurs, and document action on the temperature chart (see Figure 21.5 in Chapter 21, A system approach to quality).

- Normal temperature ranges:

Refrigerators: 4–8 °C

Freezers: –15 °C, –20 °C, –80 °C

Maintenance

- Decontaminate with disinfectant or defrost as needed.

20.7.6 Ovens

Operation and safety

- Record temperature on the Quality Control chart when in use and retain charts with other maintenance records.

Maintenance

- Refer to the owner's manual.

20.7.7 Water baths

Operation and safety

- Record the temperature on a chart when in use and retain charts with other maintenance records.
- Check the water level daily to ensure safe operation and to avoid overheating.

Maintenance

- Clean monthly. Turn the power switch off and unplug the unit.
- Empty the water bath, wipe clean with disinfectant and replace with deionized water and a disinfectant or fungicide to retard microbial growth.

20.7.8 Microscopes

Operation and safety

- Refer to the owner's manual for operation and safety.

Maintenance

- Clean objective lenses, eyepieces, and condenser daily after use with a commercial lens paper and a small amount of cleaner (e.g., mild glass cleaner) to remove immersion oil and dust.
- Cover the microscope with a dust cover to prevent exposure to dust.
- Microscopes should be professionally cleaned and serviced annually.
- Document the service records.

20.7.9 Environmental control chamber

The environmental control chamber is designed to provide a controlled temperature, humidity, and air velocity environment for drying slides and in situ coverslips during the harvest procedure. Use of the chamber allows optimum chromosome spreading in a consistent manner.

Operation and safety

- Record temperature and humidity on a quality control chart when in operation and retain charts with other maintenance records.
- Monitor the water level in the tank which provides the humidity, and refill with water as needed.

- Monitor the water level in the waste reservoir and empty as needed.
- Wipe inside of the chamber daily.
- If a spill occurs and the chamber has a removable floor, shut down the chamber, open the door, and pull out the removable floor. Wipe chemicals off the drain floor and removable floor, and resume operation.

Maintenance

- Disinfect interior of chamber as needed (i.e., for contamination).
- Follow preventative maintenance safety instructions to flush the vapor generator tank, clean the air-cooled condenser, and perform checks on blower, heaters, and other components. Perform these checks on a quarterly cycle.

20.7.10 Biological safety cabinets

Biological safety cabinets serve as a primary barrier when working with infectious agents. Class II (Types A, B1, B2, and B3) cabinets are recommended for use in cytogenetics laboratories and provide personnel, product and environmental protection. Class II cabinets utilize high efficiency particulate air (HEPA) filters. The vertical downward flow of HEPA-filtered air provides personnel and product protection, and the exhaust HEPA filter provides environmental protection.

Operation and safety

- Spray the interior surface of the cabinet with disinfectant (i.e., 70% alcohol) before and after use. Spills should be wiped immediately.
- Turn on blowers for a minimum of 5 minutes before using the cabinet.
- Do not place items over the front intake grills.
- The use of toxic, explosive, or flammable substances should be evaluated by appropriate safety personnel before placing them in the cabinet.
- Separate clean and dirty materials in the work area to minimize contamination.
- Minimize the number of times the technologist enters and leaves the cabinet to prevent disruption of the air curtain.
- Utilize aseptic technique to minimize contamination and exposure to biohazardous material.
- Minimize room activity in front of the cabinet when in use.
- The ultraviolet (UV) lamp is optional for germicidal control in the cabinet. If used, turn on the UV lamp at the end of the day, and avoid direct viewing at all times, as ultraviolet light will injure your eyes. A warning sign should be in clear view explaining the danger of UV lamps. Clean lamps weekly to remove dust, which could block the UV light.

Maintenance

- Check and remove any paper from paper catch weekly. It is located in the work surface area behind the rear wall.
- A qualified technician must inspect and recertify the biosafety cabinets biannually and repair as needed. If a UV light is used for germicidal control, the radiation output of the UV light should be tested with a UV meter to ensure that the proper intensity of light is reaching the work surface. UV lamps should be replaced if the bulb falls below the minimum requirement for protection.
- Document maintenance and repair in the equipment manual [17–19].

The Laboratory Response Network at New York State Department of Health Wadsworth Center [<http://www.wadsworth.org/testing/lrn/>] provides an excellent video, “Essentials in Biosafety,” that offers both an overview of biosafety principles and a step-by-step demonstration for the proper use of a biological safety cabinet. The video can be accessed by going to <http://www.wadsworth.org/testing/lrn/resources.html> and selecting Biosafety Video at the bottom of the page.

20.7.11 Pipettes

Maintenance

Pipettes must produce both reproducible and accurate sample results.

- Calibration testing for precision and accuracy should be performed biannually by either manual calibration or computerized testing.

- For manual testing, transfer at least 10 consecutive samples of deionized or distilled water to a balance. Record the sample weight, and tare the balance for the next sample. When all weights have been recorded, convert the sample weights to volumes, adjust for temperature, and calculate the mean, standard deviation, and correlation of coefficient. If the pipette passes the test, document results on the calibration log sheet, and place into use. If the pipettes fail the test, document the failure and repeat calibration. If the repeat test also fails, document the results and send the pipette to a company for maintenance and recalibration. If the pipette is not working properly at any time, either recalibrate or send it for maintenance or repair.
- Computerized testing automates the tracking and validation of pipette performance. The computer software automatically corrects for environmental variables. After each run, the software calculates the mean, standard deviation, and the correlation of coefficient. The results are recorded in the database and the pipettes are assigned either a pass or fail status. If a pipette fails validation, it should be sent out for maintenance and repair [20,21].

20.7.12 pH meter

Operation and safety

- Calibrate pH meter with each use following manufacturer's guidelines.

Maintenance

- Service biannually and retain maintenance records.

20.7.13 Scales

Maintenance

- Service and calibrate biannually and retain maintenance records.

20.7.14 Timers

Maintenance

Timer accuracy is also important, especially for those procedures where cells can be significantly compromised if left beyond the validated parameters that have been outlined in the laboratory's procedure.

- Check timer accuracy biannually, or as otherwise instructed, retain maintenance records (see Protocol 20.5).

20.7.15 Incubators

Operation and safety

Both CO₂ and three gas system (CO₂, O₂, and N₂) incubators are used for culturing cells. If amniotic fluid or chorionic villi specimens are being processed, the specimen should be divided between at least two independent incubators, which are ideally connected to an emergency electrical circuit. If an emergency line is not available, the incubators should be connected to separate electrical circuits. It is critical to monitor the temperature, gas levels, and relative humidity to protect the viability of the cultured cells. Acceptable ranges include 37–37.5 °C, 4–6% CO₂, 3–5% O₂ (by infusion of nitrogen gas) and relative humidity close to saturation. Incubators should be equipped with an independent electrical circuit, backup gas sources, and an emergency alarm system.

Maintenance

- Record all maintenance on the quality control log and document any deviations and corrective action taken.
- Daily maintenance includes recording the temperature, gas content, and tank levels and pressure. Check the digital display temperature with an internal thermometer as an additional precaution.
- Weekly maintenance includes verifying and recording the CO₂/O₂ content with a fyrite gas analyzer and checking the water level in the humidity pan.
- Monthly maintenance includes replacing the water in the humidity pan with distilled water and a fungicide or germicide (e.g., ½ to 1 ounce of SigmaClean® water bath treatment #S5525 per gallon water) to prevent contamination.

- Biannual maintenance includes cleaning and disinfecting the incubator by autoclaving all removable parts or wiping down the interior with disinfectant. Check all filters and replace if discolored.
- Annual maintenance includes inspecting the power cord for wear, draining the water-jacketed incubator and cleaning its interior. Refill with distilled water and a biocide [17,22].

20.7.16 Fume hoods

Fume hoods are utilized for handling volatile solvents and other irritating toxic materials. Fume hoods are designed to protect the technologist from chemical exposure. The two main types are ducted and recirculating. Air is drawn in from the front of the cabinet and is expelled to the outside of the building or made safe through filtration and circulated back into the room.

Operation and safety

- Always work with the sash as low as possible as it is considered a physical barrier and provides additional protection to the technologist.

Maintenance

- Fume hoods are inspected annually to check the filters and air flow.
- Record the maintenance and repair in the equipment manual [17].

20.7.17 Automated hybridization system

The automated hybridization system performs fluorescence in situ hybridization (FISH) on slides prepared for chromosome and interphase analysis. The technique involves co-denaturation of the probe solution and specimen via the use of heat. Each type of probe (e.g., LSI, CEP, and WCP) utilizes a specific temperature and time for co-denaturation and hybridization.

Operation and safety

- Record the high and low temperature verification using the reversible temperature indicator.
- Fill all unused slide positions with blank slides for proper operation.
- Line edges of the system (i.e., Hybrite or ThermoBrite) with water-dampened KimWipes or paper towels.
- Avoid spilling fluid on or into the instrument. Clean spills immediately with a water-dampened cloth.
- Use caution when touching the heating surface to prevent burns.

Maintenance

- The heating surface of the unit should be kept clean using a water-dampened cloth.
- If the instrument does not start, check both cord ends and the fuse.
- Unplug the electrical cord if the fuse needs replacing [23].

20.7.18 Robotic harvester

The robotic harvester instruments are reliable platforms that require very little maintenance. Daily and monthly schedules can be undertaken by the laboratory users. Annual preventive maintenance should be carried out by a qualified technician.

Note: Do not allow alcohol to come in contact with the Z-bearing while cleaning the instrument.

Daily maintenance

- Run the flush program to drain and clean liquid lines.
- Clean the end of pipette tip with a lint free cloth dampened with isopropyl alcohol.
- Remove and clean the Petri dish racks. Clean the worktable.
- Empty waste container.

Monthly maintenance

- Clean the Z-toothed rack with a dry, lint free cloth.
- Clean Y-slides with a dry lint free cloth.
- Run random XYZ movement test.
- Examine valves for wear and leaks; replace if necessary.
- Examine suction and dispensing tips for problems; replace if necessary.

Annual maintenance

- Wipe down arm assembly and flex cable channel with a lint free cloth dampened with isopropyl alcohol (Note: Alcohol should not enter the Z bearing).
- Clean and lightly lubricate (white lithium grease) syringe pump lead screws.
- Clean and lightly lubricate (white lithium grease) X and Y bearings and clean only Z axis rack.
- Inspect and replace when needed, syringe pump drive belt, tubing, valves, syringes, insulation block, and probe tip. Replacement of the parts may either be performed by the in house staff with a company kit or the company technical representative [24,25].

20.8 Ergonomics

Ergonomics is the applied science of equipment design intended to maximize efficiency and ensure safety and comfort to personnel in the work environment [26]. Technologists are at risk for repetitive motion injuries (RMIs) at the laboratory bench, biological safety cabinets, microscope and computer. Both awkward positions and repetitive motions result in ergonomic problems.

The cytogenetics technologist performs tasks that include cell culturing, microscopy, image analysis, and computer data entry. These tasks use repeated motions creating risks for RMIs such as carpal tunnel syndrome and lower back pain. Common symptoms include radiating pain, numbness, tingling, soreness, and loss of strength. Employers are required to establish a safety program which includes worksite evaluation of equipment design, control of RMIs, training of personnel, and reporting symptoms and injuries to the employer. The proper training of personnel to properly use laboratory equipment will minimize work-related injuries and improve productivity. Regulatory agencies which set guidelines for ergonomics include OSHA and the National Institute for Occupational Safety and Health (see Table 20.2).

Routine stretches and exercises along with good posture will minimize the risk of injuries. Keeping the body in a neutral position and avoiding awkward positions are essential for maintaining proper posture. In addition, frequent breaks are important to prevent

Table 20.2 Take ergonomics personally

Identify risks
✓ Posture
✓ Repetition
✓ Environment (i.e., temperature, vibration, lighting)
✓ Duration of activity
✓ Recovery time – time required to rest between repetitive tasks and awkward postures
Reduce risks
✓ Keep body in a neutral position to maintain proper posture
✓ Work within 14 to 18 inches of your body, depending on your size
Monitor risks
✓ Reduce risk factors by designing and organizing a workstation which accommodates the specific technologist
Preventative maintenance exercises
✓ Total body stretches are helpful when your task creates awkward postures
✓ Upper body stretches are helpful for neck, shoulder, and back pain while working at the microscope and computer workstations
✓ Arm and wrist stretches are also helpful while working at the microscope and computer workstations
✓ Prevent eye fatigue from microscopy and computer work by taking breaks and focusing on distant items to change eye muscle position

From Banks RJ. *Ergonomics and You*. Krames Communication. (Pamphlet), 1995. Permission to reprint has been granted by author.



Figure 20.12 Anti-fatigue mat. Anti-fatigue floor mats may help relieve back stress when tasks require long periods of standing. Shifting your weight periodically from one leg to the other may also help.

muscle fatigue. Workstations should be customized to each individual technologist to avoid poor posture and movement, such as twisting the wrist and bending at the waist. Lift objects safely, keeping your feet shoulder width apart. Bend at your knees and hips, using your legs, face the load you are lifting, hold it close to your body at waist height, and avoid twisting your body.

The following are recommendations for proper use of equipment and work areas to minimize RMIs:

Laboratory benches, safety cabinets, and hoods

- The work bench height must be appropriate for the tasks performed.
- Use anti-fatigue floor mats (see Figure 20.12) and proper shoes with support when tasks require standing for long periods of time.
- Adjust chair (see later).
- Position specimens and reagents as close as possible.
- Take breaks to minimize forearm and wrist injuries.

Laboratory chairs

- Laboratory chairs must provide adequate back support with adjustable height, tilt, and seatbacks.
- The technologist's feet must remain on the floor or footrest for leg support.

Pipetting

- Refer to the suggestions in the laboratory bench and chair sections for proper sitting and standing.
- Do not twist or rotate your wrist while pipetting and use a relaxed grip when holding the pipette. Use ergonomically designed pipettes which are lighter and shaped properly to allow neutral posture and minimize carpal tunnel pressure [27].
- Take frequent breaks.

Microscopes

- Use a proper height of desk and chair, and if necessary, use a footrest.
- Maintain an upright head position, and avoid rounding neck and shoulders.
- Use adjustable eyepieces for easier viewing.
- Pad forearms and edges of desk to avoid leaning on hard edges or use a cut-out work table to provide forearm support.
- Keep elbows close to sides.
- Maintain wrists in a straight, neutral position.
- Relax grip when using microscope knobs.
- Take frequent eye and stretch breaks to prevent eye fatigue and injuries to the back and neck.

Computer workstations

- Maintain good posture with an upright head position (see Figure 20.13). Avoid sustained bending of your neck.
- Work with keyboard and/or mouse at elbow height and keep wrists straight with hands curved and relaxed [28].
- Relax grip on mouse. Type quietly and be soft on the keys [29,30].

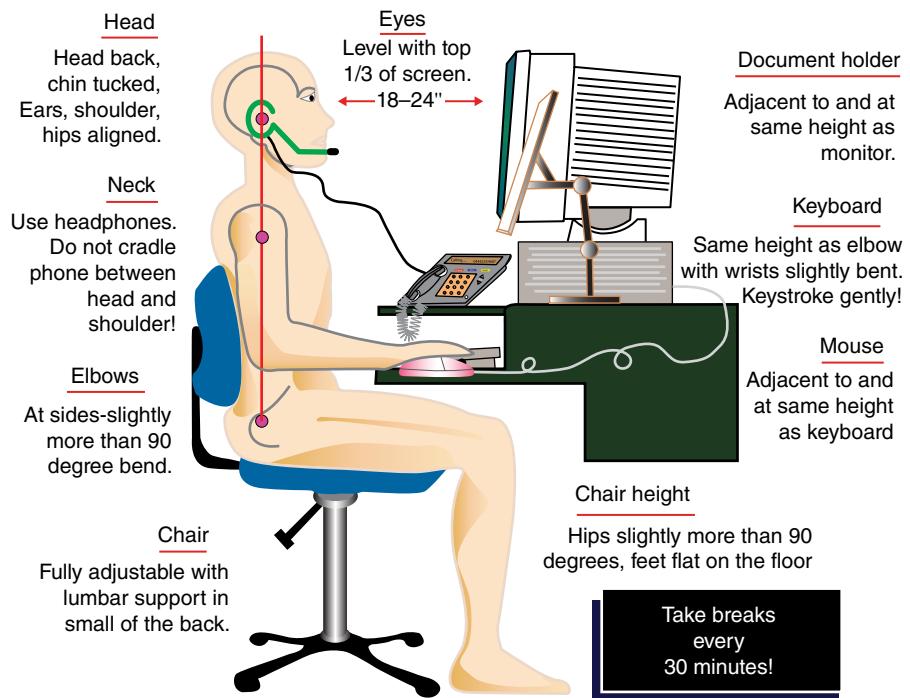


Figure 20.13 Posture neutral position. This illustration gives several good pointers to minimize injuries. Reprinted with permission from UCAR University.

20.9 Regulatory considerations

20.9.1 Incident reporting

A work-related injury must be immediately reported to the supervisor. The supervisor is then responsible for investigating the incident and initiating an incident report describing the injury. The report form includes sections to be completed by the employee and the institution's occupational health personnel or safety officer and documentation of the cause and corrective action [13]. If the incident involves exposure to potential bloodborne pathogens, it is the responsibility of the employee to promptly report the exposure incident and initiate the work-up. Both the employee and the source patient's blood must be tested for infection, and the employee is entitled to medical care. Health records are kept confidential [31].

A chemical's Safety Data Sheets (SDS) is the primary reference for first-aid treatment of injuries sustained by chemical exposure. The health risk of exposure depends on the toxicity and the type of exposure. The type of exposure depends on the following routes of entry: (a) skin and eye contact, (b) inhalation, (c) ingestion, and (d) injection.

20.9.2 Privacy act

The Standards for Privacy of Individually Identifiable Health Information ("Privacy Rule") establishes, for the first time, a set of national standards for the protection of certain health information [32]. The US Department of Health and Human Services (HHS) issued the "Privacy Rule" to implement the requirement of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). This Rule addresses the use and disclosure of an individual's health information—called "protected health information (PHI)". Within HHS, the Office for Civil Rights (OCR) has responsibility for implementing and enforcing the Privacy Rule with respect to voluntary compliance activities and civil money penalties.

A major goal of the Privacy Rule is to assure that an individual's health information is properly protected, while still allowing the flow of any information needed for high quality health care for that individual. Information includes not just a

person's physical or mental condition, but also the individual's provision of health care, as well as past, present or future payment for this provision of health care. This Rule thus permits important uses of information, while protecting the privacy of people who seek care and healing.

The effects of this Rule reach beyond simply reporting a patient's results to an unauthorized person, even if that person is a physician. The Rule affects how we speak in an elevator and what we discuss among our peers, that is, it relates to any individually identifiable information that is held or transmitted in any form or media, whether electronic, paper, or oral. The HHS website provides a comprehensive summary of this Rule (<http://www.hhs.gov/ocr/privacy/hipaa/understanding/summary/index.html>), and the reader is encouraged to refer to their website for a more thorough understanding.

20.9.3 Safety inspections/drills/training

The prevention of laboratory-related accidents and injuries begins with periodic inspections and training, including safety drills. The laboratory safety committee is responsible for developing policies and procedures to monitor laboratory-related tasks for safety compliance and job specific safety training. Safety inspections and training are required for the accreditation process by agencies such as The Joint Commission (TJC, formerly known as the Joint Commission on Accreditation of Healthcare Organizations or JCAHO) and its international branch, Joint Commission International (JCI), as well as the College of American Pathologists (CAP), and Commission of Office Laboratory Accreditation (COLA) (see Chapter 21, section 21.4.2, External audits/accreditation). Training in all aspects of safety and compliance policies must be documented in both the institution and employee records.

Safety inspections include both documented review of manuals and records and physical on-site inspections to observe personnel practices and environmental safety compliance in the following areas: (a) biological safety (e.g., OSHA); (b) chemical safety (e.g., SDS manual); (c) fire prevention, and (d) physical safety. Safety policies and procedures must be accessible to all personnel, and all personnel should be knowledgeable in the laboratory safety policies and procedures. All laboratory-related accidents and occupational injuries or illnesses that require medical treatment must be reported and documented (e.g., hazardous substance spills and RMIs). Evaluate incident and accident reports as part of the laboratory's quality improvement plan to avoid recurrence of the accidents. The laboratory safety officer is responsible for correcting the noted deficiencies identified during the inspection.

Fire prevention and disaster plans include both written policies and exit drills. All personnel must participate in exit drills at least once a year to respond safely in the event of fire or emergency. The exit drill educates the staff about the location and use of fire extinguishers, fire alarms, and evacuation plans. Staff participation is essential to ensure an effective response during an emergency situation, and it is crucial to know one's role prior to the actual event. Both training and drills must be documented.

All new staff members are required to attend a safety orientation. Job and departmental-specific safety training is required annually with documentation. It is provided in a variety of ways, including drills, in-service training, bulletin boards, and educational posters. [32].

20.9.4 Compliance

As part of the inspection process, the Joint Commission (TJC) may follow a specimen's trail from registration to reporting, in order to ensure that each step has been properly executed. Management is responsible for ensuring that the laboratory is compliant with state and federal regulations, and for ensuring that staff members are aware of those laws that are relevant to their operation. Mistakes happen, but when they do, we all can play a role in catching and correcting them.

The Office of Inspector General (OIG) of the Department of Health and Human Services (HHS) and other federal agencies provide guidelines for clinical laboratories to voluntarily implement a compliance plan. This program should include documentation requirements emphasizing internal monitoring and prompt corrective action to deal with errors and/or violations. The plan must address marketing plans, Current Procedural Terminology (CPT) and Healthcare Common Procedure Coding System (HCPCS) coding issues, ICD-10 diagnosis coding, and improper claims submission [33].

The False Claims Act (31 U.S.C. §3729) was put into legislation in 1986 in order to safeguard against submitting or causing the submission of false or fraudulent claims for payment or approval. It specifies that anyone who "knowingly" submits false claims to the government is liable for damages up to three times the amount of the erroneous payment, plus mandatory penalties between \$5,000 and \$10,000 for each false claim submitted. The act defines "knowingly" to mean that a person (1) has actual knowledge of the false claim, (2) acts in deliberate ignorance of the truth or falsity of the information, or (3) acts in reckless

disregard of the truth or falsity of the information. Violations under this Act include (but are not limited to): billing for medical services not rendered (see Duplicate claims, later); misrepresenting the level of services rendered (see Upcoding, later); automatically running a lab test whenever the results of some other test fall within a certain range, even though the second test was not specifically requested (see Excessive testing, later); falsely certifying compliance with federal laws; or submitting a claim for payment that is contrary to Medicare or Medicaid payment requirements (see Unbundling, later).

The following list describes just a small selection of laws which could affect cytogenetics laboratories, in particular, those that accept Medicare/Medicaid reimbursements. These regulations specifically address United States law, but the basic premise may be covered by other countries, as well. The theoretical examples used below are NOT actual incidences, but they represent *mistakes or situations that could happen in the cytogenetics setting and could affect improper billing.*

- **Unbundling:** Submitting separate bills for a service that also has a group code *at a reduced cost*, in order to receive a higher reimbursement for the same test or procedure. FISH panels, for example, could fall within this category if the reimbursement rate is higher when billing separating for each probe in a specific panel than what would be received if they were billed together as one panel.
- **Upcoding:** Billing for a more costly CPT (Current Procedural Terminology) procedure code than what was actually performed. For example, your LIS system could have been designed to automatically bill CPT code CG88264 (analyzing 20–25 metaphases) upon accessioning hematological specimens; however, after completing the analytical step on a bone marrow aspirate, you were only able to analyze 10 cells, either due to poor quality or extensive complexity. If the CPT code CG88264 is not credited and replaced with the correct code, the resultant bill would be upcoding to a higher reimbursement than what was actually performed (CG88261, for analyzing five cells and one karyogram with banding). Another example of upcoding is billing at doctor rates for work that was actually performed by a technologist or resident intern.
- **Duplicate claims:** Billing twice for the same procedure. This can inadvertently occur if bills are systematically generated when accessioning a specimen and a mistake is made that requires re-accessioning the same sample. If a credit is not submitted for the erroneously generated bill, duplicate claims could be billed. Sometimes, however, the appearance of duplication may not be real. For example, logging in amniotic fluid specimens from a twin pregnancy could signal a false flag of duplication. Institutional guidelines should be followed for accessioning and reporting these prenatal specimens – for example, accessioning separately, using different time-stamps, maintaining separate records, and clearly differentiating specimens with a unique twin identifier (e.g., Twin A, Twin B).
- **Excessive services:** Billing for a test that was performed but which exceeds the acceptable level of care determined for that particular condition. For example, billing for high-resolution studies is excessive when a Down syndrome patient is referred with no other clinical or karyotypic suspicion to warrant higher level testing. Another potential situation that could fall under “Excessive Services” is automatically performing FISH when the referring physician had only ordered chromosome analysis, even if FISH studies are warranted. For example, after finding normal chromosomal results on a bone marrow specimen with a high suspicion of CML (see Chapter 11, section 11.4.1, Chronic myelogenous leukemia), you cannot perform FISH for *BCR/ABL1* fusion to rule out low-level mosaicism of the t(9;22) or a cryptic form of the *BCR/ABL1* fusion (see Chapter 16, section 16.10.2, Scoring cells), because the doctor did not order a reflex (perform subsequent testing dependent upon certain conditions) to this specific FISH test. You must receive written orders from the physician before proceeding.
- **Unnecessary Services:** Performing a test that has no relation to the patient’s condition; for example, billing for stimulated B-cell karyotyping when the patient has a T-cell neoplastic disorder.

Other federal laws that have severe fine and felony consequences include:

- **Federal Anti-Kickback Statute (AKS)** (42 U.S.C. §1320a-7(b)): Soliciting, receiving, offering or paying remuneration (monetary or otherwise) in exchange for referring patients, predominately for those covered by the government (e.g., Medicare/Medicaid).
- Similarly, the **Stark Law** (42 U.S.C. §1395nn) refers to a Medicare or Medicaid beneficiary for health services which the physician, or the physician’s immediate family member, has a financial relationship (unless that relationship is protected under a statutory exception). Under the Stark Law, a “financial relationship” can include ownership interest, investment interest, and/or a compensation arrangement.

20.9.5 US regulatory and accreditation agencies

Listed below are some of the federal regulatory and accrediting agencies in the United States:

1. Occupational Safety and Health Administration (OSHA) – federal agency that regulates employee safety in the workplace.
2. College of American Pathologists (CAP) – provides laboratory accreditation through education, standard setting and proficiency testing. Annual inspections assess management, laboratory operations, and regulatory compliance of the laboratory.

3. The Joint Commission (TJC) – is a not-for-profit organization that accredits and certifies health care organizations in the United States. The goal is to provide safe and effective care for the public and ensure patient safety of the highest quality and value.
4. US Food and Drug Administration (FDA) – approves new laboratory tests, technology, and instruments.
5. Centers for Medicare and Medicaid Services (CMS) – federal agency responsible for administration of Clinical Laboratory Improvement Act of 1988 (CLIA '88) and Medicare/Medicaid services.
6. US Department of Transportation (DOT) – administers the Hazardous Materials Standard which requires procedures for shipping of biohazardous materials and employee training.
7. US Postal Service (USPS) – administers instructions for packaging biological products and clinical specimens.
8. Centers for Disease Control and Prevention (CDC) – national advisory agency that develops disease prevention standards and guidelines.
9. US Department of Health and Human Services (HHS) – oversees the FDA, CMS, and CDC.
10. Office of the Inspector General (OIG) of the US Department of Health and Human Services (HHS) – provides a compliance program to assure that the laboratory adheres to regulations established by CMS for fraud and abuse prevention.
11. Equal Employment Opportunity Commission (EEOC) – an independent federal law enforcement agency that enforces job discrimination.
12. The Board of Certification (BOC) of the American Society of Clinical Pathology (ASCP) – provides a certification examination for technicians, technologists, specialists, and diplomats in laboratory management, in many laboratory specialties, including cytogenetics and molecular cytogenetics [34]. Please see Chapter 25, Credentialing and guidelines, for a more thorough list of international organizations and websites. Several states have implemented their own employee requirements and licensure; check with your state for any state-dependent regulations.

Acknowledgments

We would like to acknowledge Marilyn Arsham, Dr. Jeanna Welborn and Dr. Jane Bruner for their expert review and guidance in writing the chapter. We would also like to thank Turid Knutsen, author of the chapter in the previous edition, for providing a most valuable reference.

References

1. Hopkins S. Safety and the stick. *Advance* 2005 10: 37–39.
2. OSHA Occupational Exposure to Bloodborne Pathogens Standard Code of Regulations, Title 29, Part 1910.1030 Bloodborne Pathogens, 2003.
3. University of California Davis Health System, Center for Nursing Education, Employee Annual Training 2007 Document.
4. www.cdc.gov/od/ohs/manual/labsfty.htm. General Laboratory Health and Safety 2000; 1–25.
5. Chosewood LC, Wilson DE, eds. Appendix B: Decontamination and disinfection. *Biosafety in Microbiological and Biomedical Laboratories* (BMBL), 5th ed. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institutes of Health. HHS Publication No. (CDC) 21-1112; December 2009: 326–335. <http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf>
6. Ono M. Everything you always wanted to know about bleach but were afraid to ask. *News & Views* 2006 13:6(5). http://www.scripps.edu/newsandviews/e_20060213/bleach.html
7. Phelan K. Laboratory safety. In: Dunn B, Mouchrani P, Keagle M, eds. *The Cytogenetic Symposia*, 2nd ed. 2005; 23-1-23-8.
8. University of California Davis Health System Bloodborne Pathogen Exposure Control Plan, 2005; 1–11.
9. OSHA, 29 CFR 1910.1200(g) and Appendix D. United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS), third revised edition, United Nations, 2009. www.OSHA.gov. Accessed 1/3/14.
10. National Research Council. *Prudent Practices in the Laboratory: Handling and Disposal of Chemicals*. Commission on Physical Sciences, Mathematics, and Applications (CPSMA) 1995.
11. www.med.yale.edu/caim/hhmi/public/, HMMI Laboratory Safety Training. Knowing How to Practice Safe Science. 1.0, 1999.

12. www.compliance signs.com/nfpadiamonds.shtml. Accessed 10/28/07.
13. Gile TJ. A Safety Plan for Laboratories, Course #056–906, California Association for Medical Laboratory Technology Distance Learning Program, 2006, 1–7.
14. <http://www.mc.uky.edu/Clinical Engineering/MEMP.htm> Clinical Engineering, University of Kentucky Medical Equipment Management Plan. Accessed 8/13/06.
15. www.med.cornell.edu/ehs/updates/autoclave_safety.html, 1–2, April, 2003. Accessed 8/20/06.
16. www.ibc.umn.edu/centrifuge.html, 1–3. Accessed 8/20/06.
17. Knutsen T. Laboratory safety, quality control, and regulations. In: Barch MJ, Knutsen T, Spurbeck JL, eds. *The AGT Cytogenetics Laboratory Manual*, 3rd ed. Philadelphia: Lippincott-Raven, 1997; 597–646.
18. NuAire Operation and Maintenance Manual for Biological Safety Cabinets. NuAire Inc, 2004.
19. www.niehs.nih.gov/odhsb/biosafe/bsc/section 3.html, 1–5. Accessed 8/20/06.
20. NCCLS Proposed Guideline, Determining Performance of Volumetric Equipment Vol. 4, No. 6, 1984.
21. www.labtronics.com/resources/art122_pt.asp -thermometers, 1996. Accessed 9/10/06.
22. www.mw-labs.com/id14.html, CO₂ incubator operation. Accessed 10/28/07.
23. Vysis Hybrite TM User Guide, Abbott Molecular ThermoBrite TM Operator's Manual, 2005. www.abbott.com (Karla Bellett).
24. Spurbeck J. Mayo Clinic Procedure Manual, Preventive Maintenance Schedule for Scinomix Instruments: Cyto Prep 56/84, Tecan Mini Prep 75 and Tecan 5051, 2007.
25. www.scinomix.com Maintenance schedule for cyto instruments (Nigel Malterer), 2006.
26. Dul J, Weerdmeester B. *Ergonomics for Beginners*. Taylor and Francis Ltd. 1994.
27. Gile TJ. Ergonomics in the laboratory. *Laboratory Medicine* 2001; 32: 263–267.
28. Graham K. Injury Prevention. *Advance* July 30, 2007; 19: 18–19.
29. University of California Davis Health System Ergonomics Program, 2006.
30. www.ergonomics.ucla.edu/exercises.html. Accessed 10/9/06.
31. Rasmussen J. Laboratory self-inspection: a guide to effective safety and health auditing. *Laboratory Medicine* 2002; 33: 123–126.
32. Lawrence L. Laboratory safety and regulations. In: Jarreau PC, ed. *Clinical Laboratory Science Review: A Bottom Line Approach*, 3rd ed. New Orleans: Louisiana State University Health Sciences Center Foundation, 2005; 239–243.
33. <http://oig.hhs.gov/fraud/docs/complianceguidance/cpcl.html> or <http://oig.hhs.gov> and search for HHS = MODEL COMPLIANCE PLAN FOR CLINICAL LABORATORIES. Accessed 1/13/12.
34. <http://www.ascp.org/PDF/BOC-PDFs/procedures/Examination-Procedures.aspx> for examination eligibility or the BOC of ASCP website at <http://www.ascp.org/certification>. Accessed 1/15/12.

Contributed protocols section

IMPORTANT: No protocol included in this manual should be used for clinical testing unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements for all chemicals used in the laboratory.

Protocol 20.1 Autoclave sterilization, liquid nitrogen, pro-par

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Autoclave sterilization

1. Place instruments to be sterilized in sterilizing bags and seal.
2. Place Pasteur pipettes, cotton stuffed, in metal canisters.
3. Run autoclave at 20 minutes (15 to 18 lbs. pressure, 121 °C) with a 30-minute dry cycle.
4. Run a test (either steam stripe or biological test culture weekly).
5. Record the sterility biological indicator results in the notebook.
6. Dry instruments in drying over or leave in autoclave (door open) for several hours until dry.
7. Distribute sterilized equipment to their appropriate tissue culture room

II. Liquid nitrogen

1. Fill the liquid nitrogen tanks weekly from the large LN Tank.

Wear protective gloves and face protection.

III. Slide de-oiling

1. Place slides in glass boats to drain excess oil.
2. *Run each boat of slides through 3 changes of Propar clearant using about 5 to 10 minutes per change.
3. After several runs (approx. 5) discard the first wash, rotate to second wash to the #1 position and put fresh Pro-Par into the third wash position.
4. Discard the original first wash Pro-Par into a collection container for future pick up and disposal by Environmental Safety.

*Conduct this operation in the fume hood located in the cytogenetics lab.

Pro-Par should be handled in a fume hood with chemical resistant gloves (Nitrile) and skin and face protection to avoid skin contact.

IV. Additional readings

1. John Paul, ed. *Cell and Tissue Culture*, 5th ed. London: Livingstone, 1975.
2. Willmered EN, ed. *Cells and Tissues in Culture*. NY: Academic Press, 1965.
3. Kruse Jr PF, Petterson Jr MK, eds. *Tissue Culture, Methods and Applications*. NY: Academic Press, 1965.
4. Barch M, Knutsen T, Spurbeck J, eds. *AGT Cytogenetics Laboratory Manual*, 3rd ed., 1997.

Protocol 20.2 Dishwashing procedure

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Principle

Glassware, plastic and metal items in the cytogenetics laboratory are sanitized once they have been used and/or dirtied. Items are sanitized by washing with soap and water. Any presence of soap residue is identified by performing a random check of glassware using the pH indicator Bromothymol Blue.

II. Materials

1. Alconox Detergent: Alcojet Detergent Powder or Liqui-Nox. Contains sodium metasilicate, avoid contact with skin, eyes, or mucous membranes. Wear gloves and a lab coat or apron when washing dishes. Store under sink in dishwashing area. Can be ordered through Lab Stores.
2. Bromothymol Blue: Dry reagent ordered through Sigma. To make for dishwashing soap residue check, mix 0.1 g of Bromothymol Blue per 1 L of de-ionized water. Store at room temperature. Avoid contact with clothes, skin, eyes, or mucous membranes. Wear gloves and lab coat when using or preparing.

III. Method

1. All dirty dishes and miscellaneous items to be washed are placed in buckets/plastic containers in the Cytogenetics Lab and Tissue Culture area. Collect all dishes and place on the cart. Use the cart to take the dishes to the dishwashing station, located on the second floor.
2. Place dishes to be washed in the sink and fill the sink with hot water. Add approximately 1 tablespoon of detergent. Let dishes soak for 30 minutes.
3. While dishes are soaking, the drying baskets can be set up. To do so, place paper towels or 2–3 Chux absorbent pads (purchased through Lab Stores) on the counter next to the sinks. Place metal drying baskets on top.
4. After soaking, use a brush or scouring sponge to scrub beakers, flasks, graduated cylinders, cups, scoops, tubes, etc.
5. Place scrubbed dishes in the rinse sink.
6. One by one, rinse each item with regular tap water seven times.
7. After rinsing an item, place it in the de-ionized water sink. One by one, rinse each item with de-ionized water three times.
8. Before finishing the entire dishwashing process, pick a piece of glassware that has been rinsed in both regular and de-ionized water and test it to make sure no soap residue remains (see Note 1, Quality control). Partially fill the item with de-ionized water and squirt enough Bromothymol Blue into it to see a color change. The water should turn a yellow-orange-ish color. Be sure to use de-ionized water, whereas tap water will result in a blue color with or without soap residue. If the de-ionized water turns blue, soap residue is present and all items already rinsed should be re-rinsed and the check repeated.
9. After the de-ionized water rinse and the Bromothymol Blue check, items can be placed in the drying basket and allowed to air dry.
10. When all washing and rinsing is done, the full drying baskets can be placed on the cart and taken back up to the lab to finish drying.
11. When dry, dishes are placed back in or on the shelf, cabinet, drawer, or area where they belong.
12. After completing the dishwashing procedures, make sure to initial the Glassware Check record sheet next to the day and month the dishes were washed and checked for soap residue.

IV. Notes

1. **Quality control:** Each time dishes are washed, a piece of glassware is picked at random and checked with Bromothymol Blue after the final de-ionized water wash. A yellow-orange color indicates no soap residue has been left (a nonbasic pH) and the dishwashing technique is deemed adequate for all pieces being washed at that time. A blue color indicates a basic pH and therefore, the presence of soap residue. If a blue color appears, all dishes must be re-rinsed and the check repeated. Record day and month dishes were washed and checked with Bromothymol Blue on the Glassware Check sheet.

V. Additional readings

1. Alconox detergent package instructions.
2. Bromothymol Blue SDS.

Protocol 20.3 Eppendorf pipette calibration

Anonymous contribution

I. Principle

In order to ensure that all pipettes are accurate when measuring reagents, they must be assessed for accuracy. *Accuracy* relates to some absolute known value. Degree of accuracy differs with the type of pipette. A tolerance of $\pm 0.5\%$ can be expected of Class A glass volumetric pipettes, SMI positive displacement pipettes, and pipettes with electronic settings. Manual micro-pipettes can be

expected to have a tolerance of +/- 1.0 percent. The degree of tolerance must be considered when selecting the type of pipette to be used for a certain function – e.g., whether the function requires great accuracy and precision or if precision with reasonable accuracy is sufficient. This QC procedure is performed annually.

II. Materials

1. Micro-pipettors (Eppendorf) and tips
2. Water
3. Analytical balance

III. Method

A. Gravimetric technique to check accuracy

1. Be familiar with the operation of the pipette. If it has variable settings, check the calibration of the settings frequently used on that particular pipette.
2. Be sure that the sampler is lubricated according to the manufacturer's directions.
3. Use an analytical balance mounted on a vibration-free bench in a draft-free, temperature controlled area. Keep the balance and the area around it immaculate; check the balance with the NS 100 mg weight, if it has not been done within the preceding month.
4. Place a flask containing ambient temperature deionized water next to the balance. Place a thermometer into the water. If the pipette to be calibrated is stationary in another room, place a second thermometer into the second flask of ambient-temperature, deionized water that is to be used with that pipette. The temperature at the two locations should be the same. If not, use the temperature at the pipette to calculate the weight of water; work rapidly when at the analytical balance.
5. Check your weighing bottle to make sure that is immaculate, dry and free from fingerprints. Handle the weighing bottle and cover very carefully. Handle only with tissues to avoid fingerprints. (When finished, very carefully clean the bottle with Micro cleaning solution and return it to the drawer).
6. Record type of pipette, volume used and water temperature.
7. Weigh the weighing bottle with glass stopper. Record weight or tare.
8. From the flask of deionized water containing a thermometer, measure a sample of water employing your best technique. Transfer the sample carefully into the weighing bottle or cup; use a tissue to remove the stopper; replace the stopper as quickly as possible. Weigh the bottle plus the water.
9. Measure at least five (5) samples from each pipette or setting. You can weigh one sample after another in the same bottle and subtract each weight from the preceding one (or tare) in order to determine the weight of each measured sample. Alternate procedure could be weighing five (5) empty bottles and measuring one sample in each, then subtract the weight of the bottle from the weight of the bottle plus the water to obtain the weight of the water delivered by the pipette. Continuously monitor the temperature of the water and record any change.

B. Calculations

10. Consult Table 20.3 to locate the weight of exactly 1 mL of water at the temperature of the water being used; interpolate, if necessary. Multiply this weight by the volume of the pipette being checked to obtain the theoretical weight. If the allowable error is $\pm 0.5\%$, multiply the theoretical weight by 0.5 percent (0.005). This is the absolute plus or minus error allowed. Add and subtract this amount from the theoretical weight to determine the allowable range. If the allowable error is $\pm 1.0\%$, multiply the theoretical weight by 0.01 to determine the allowable range.

Summarize results:

- a. pipette is accurate $\pm 0.5\%$
- b. pipette is accurate $\pm 1.0\%$
- c. pipette is not accurate, but is precise
- d. pipette is not accurate and is not precise and should not be used.

C. Reporting results

Accuracy of pipettes can be evaluated by gravimetric technique. All results (good and bad) must be recorded and reviewed by the supervisor. Forms for recording data can be found in the QC Logbook.

Table 20.3 Weights of water in air

Temperature °C	Weight (g) of 1.0mL of water
20	0.9972
20.5	0.9971
21	0.9970
21.5	0.9969
22	0.9968
22.5	0.9967
23	0.9966
23.5	0.9965
24	0.9964
24.5	0.9962
25	0.9961
26	0.9959
27	0.9956
28	0.9954
29	0.9951
30	0.9948

Values include effects of buoyance and dissolved oxygen.

IV. Reference

College of American Pathologists Commission on Laboratory Accreditation Inspection Checklist, Laboratory General Section: 1, 2000.

Protocol 20.4 NIST thermometer calibration

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

Thermometer label	Instrument	Clinical engineering or serial no.	Error (\pm) of working thermometer to the NIST thermometer
A	TC LRG FREEZER	562467	-0.6
B	MAIN OVEN	508666	-0.9
C	TC SM FREEZER	RM517730	-0.5
D	AF H ₂ O BATH	522204	+0.1
E	TUMOR H ₂ O BATH	524236	-0.3
F	AF INCUB. #2	511523	+0.1
G	PARAFFIN OVEN	509N0326	-0.4
H	BLD/BM INCUB.	522217	\pm 0.0
I	AF/BLD STORAGE REF.	561222	-0.9
J	BLD/BM H ₂ O BATH	090408	-0.9
K	BM STORAGE REF.	WA82200423	-1.0

L	AF SLIDE WARMER	508674	-0.3
M	BLD/BM FREEZER	508663	-0.8
N			
O	AF INCUB. #1	511523	-0.1
P	(CALIBRATED)		-0.6
Q	TC REFRIG.	RM517730	-0.5
R	FIBRO SLIDE WARMER	508673	+0.9
S	(NOT CALIBRATED)		
T	(NOT CALIBRATED)		
U	(NOT CALIBRATED)		
V	(NOT CALIBRATED)		
W	FISH INCUB.	527929	+0.7
X	(NOT CALIBRATED)		
Y	(NOT CALIBRATED)		
Z	(NOT CALIBRATED)		
AA	(NOT CALIBRATED)		
BB	AF INCUB. #3	510256	+0.2
CC	RESEARCH FREEZER	562489	-0.2
DD	BIOCHEM INCUB. TOP	525064	-0.6
EE	BIOCHEM INC. BOTTOM	525064	-0.3
FF	FISH REFRIG.	561212	-0.3
GG	FISH REF. FREEZER	561212	-0.4
HH	FISH H ₂ O BATH (40)	1598061005218	-0.2
II	(NOT CALIBRATED)		
JJ			
KK	FISH FREEZER	561213	-0.6
LL	FISH HEAT BLOCK	561207	-1.1
MM	BLD/BM REFRIG	508663	-0.8
NN			
OO	(CALIBRATED)		-0.6
PP	FISH H ₂ O BATH (37)	561211	+0.6
QQ	(CALIBRATED)		+0.5
RR			
SS	TUMOR INCUB.	511520	-0.2
TT	TUMOR REFRIG.	562469	+0.2
UU			
VV	FISH H ₂ O BATH (VARIABLE)	1598061005221	-0.4

Note: All thermometer calculations are noted in degrees Celsius

Protocol 20.5 Thermometer calibration

Anonymous contribution

I. Principle

All new thermometers should be calibrated against a National Institute of Standards and Technology (NIST)-certified thermometer and re-calibrated annually or when needed [1]. NIST is a nonregulatory federal agency under the U.S. Department of Commerce.

NIST-certified thermometers are expensive and difficult to replace; therefore, handle reference thermometers with care.

II. Materials

1. NIST-certified thermometer
2. Water bath, adjustable temperature, up to 95 °C
3. Ice slush bath
4. Refrigerator

III. Method

1. Assemble, label and log each thermometer calibration. Ensure no air bubbles can be seen in shaft.
2. Fill a large beaker with shaved ice and add enough water to tightly pack the shavings into a slush suspension that will have contact with the thermometer, but not float the ice. Carefully place reference and nonreference thermometers in the slush bath, and record all thermometer readings after the reference device registers 0 °C. If stem-temperature corrections are required, see NCCLS standards [2].
3. Gently remove thermometers from ice bath. Isolate any thermometer that failed the zero temperature test as it will register incorrectly for all subsequent readings. Allow thermometers to reach room temperature and record readings.
4. Place in a second beaker of water in a water bath at ambient temperature. *Slowly* increase the temperature of the water bath up to 90 °C; record reference vs. other thermometer temperatures at various points of interest (dependent upon applications). Example of recorded readings may include: 0 °C (required); 3 °C, 37 °C, 60 °C.
5. Record calibration results on a QC Equipment Maintenance log.

IV. References

1. College of American Pathologists Commission on Laboratory Accreditation Inspection Checklist, Laboratory General Section: 1 2000.
2. NCCLS Tentative Standard: TS1-2 National Committee for Clinical Laboratory Standards.

Protocol 20.6 Timer calibration

Anonymous contribution

I. Principle

Timers or stopwatches that are used independently or as part of an instrument must be certified when initially placed into production, and then subsequently verified every six months or when the battery has been changed. To validate a test timer, timing values are compared with a National Institute of Standards and Technology (NIST) traceable stopwatch in order to ensure that timing is within an acceptable tolerance range for the test timer. The validation process compares time interval, *not* actual time; this value should be present on the label of each timer apparatus, along with the date of verification and the person performing the validation. Acceptable tolerance range discrepancies vary, depending on the type of timer being tested. Tolerance limits for external reference timers are more stringent (± 1 second) than those that are internal to an apparatus, such as a centrifuge (± 5 seconds).

II. Materials

1. NIST-certified stopwatch/timer or one validated against a NIST-traceable (e.g., NIST website) timer
2. Test timer(s)

3. Timer Maintenance Log (see Note 1: Designing a timer/stopwatch QC maintenance Log).
4. 70% ethyl alcohol
5. Gauze
6. Label
7. Tape

III. Procedure

Warning: Timers used at the bench are considered contaminated; therefore, the technologist handling the timer must wear gloves and other proper protective equipment. Timers must also be disinfected before performing this procedure.

1. Remove battery and thoroughly disinfect all surfaces by using a gauze sufficiently saturated with 70% ethyl alcohol to allow the timer's surface to be in contact with the disinfectant for 5 minutes; repeat if the alcohol evaporates too early. Re-install the battery. If another timer has already been NIST-verified into laboratory production, proceed to step 5.
2. For a new timer, assign a unique identifier from the Timer QC Maintenance Log (see Note 1: Designing a timer/stopwatch QC maintenance log). Label the timer with its unique identifier, and cover the label with protective tape.
3. If a NIST-certified timer is available, you may proceed to step 8.

Using the NIST-traceable website [1]

4. Go to the NIST traceable website at <http://www.nist.gov/index.html>. Scroll down to the NIST clock (right-hand side at the bottom). If desired (optional), follow the instructions to post the "Official US time" widget to your computer.
5. Change time zone to your area by using left or right arrows.
6. As soon as the NIST website timer begins the hour, immediately start the timer to be verified. Record the start time for both the NIST website and the timer being verified. *Suggestion: Set any second timer for 55 minutes to ensure that you are back at the computer before the full hour has completed.*
7. As soon as the NIST website timer reaches :00, indicating the end of the hour, stop the test stopwatch/timer. Record the end time from both the NIST-website and the timer being tested.

Using a NIST-certified timer

8. Set the NIST-certified timer and the test timer at either 60 minutes, or at the setting that the timer is being used in a procedure. Start both as simultaneously as possible, and stop as equally as possible when the set time is complete (the alarm will resound).

Calibrate validation results

9. Compare the two values (see Note 2: Tolerance ranges for time deviations). If the deviation between the NIST-certified timer and the test timer is within ± 1 (one second), the test timer is NIST-certified for 6 months and can now be used to check other timers in the laboratory. Proceed to step 8 if other timers must be calibrated. If the deviation exceeds a deviation from ± 1 (one second), check the battery and repeat the procedure. Note the action on the log. Because this process can be influenced human reaction time [2], a second person can be asked to repeat the test. If the setting on the timer can be adjusted to reliably achieve the correct timing, mark the corrected setting with the verified time. If results continue to be unacceptable, remove the timer from use; indicate within the log that the timer failed validation and has been removed.
10. Label the validated timer with the date of verification, the deviation value, and the initials (or other acceptable identifier) of the laboratory staff member who performed the validation. Expiration is 6 months.

IV. Notes

1. **Designing a timer/stopwatch QC maintenance log:** This log (or spreadsheet) should record the timer/stopwatch's unique serial number, acceptable tolerance range for verifying timing deviation, as well as the actual verification tolerance values and its resulting deviation. Additional information that will later require recording either within the same or separate maintenance logs are subsequent maintenance, including validations that follow battery-changes and semi-annual maintenance. Repetitive fields for ongoing maintenance include dates, NIST- and test-timer readings, test deviation value, acceptable status, and identifiers of performing person(s) for each validation.

2. **Tolerance ranges for deviations in nonreference timers:** Acceptable range values between the test timer and the NIST-certified reference timer are $\pm 2\%$ for timers used for assays, $\pm 10\%$ of the total test timer on centrifuges, or $\pm 5\%$ for serofuges.

V. References

1. Timer Calibration Verification SOP at Johns Hopkins website <http://resources.psmile.org/resources/equipment/general-equipment/timers>
2. Gust JC, Graham RM, Lombardi MA. Stopwatch and Timer Calibrations. Special Publication 960-12. NIST May 2004:38. <http://tf.nist.gov/general/pdf/1930.pdf>

CHAPTER 21

A system approach to quality

Peggy J. Stupca¹ and Sheryl A. Tran²

¹(retired), Mayo Clinic, Rochester, MN, USA

²Mayo Clinic, Rochester, MN, USA

21.1 Quality system

Virtually every chapter in this manual has a quality component. The recognition that each step in the process of producing a laboratory result has multiple quality components became more evident with the transformation of many laboratories from a service at a single institution into a commercial, business-oriented one. This integration of all quality components into all phases of testing is called a quality system in the United States and International Organization for Standardization (ISO) standards in European countries. Every laboratory director and their staff want to produce the best quality preparations, have a zero percent error/event rate, use equipment that is properly calibrated and maintained, and have adequate and well-trained personnel. The quality system was designed to make these goals achievable.

In 1988 Congress passed the Clinical Laboratory Improvement Amendments (CLIA '88) in order to establish quality standards for all laboratory testing, with an emphasis on patient tests being produced reliably, accurately and in a timely manner, no matter where they were being performed. CLIA '88 42 CFR 493.1200 [1] specifies that each laboratory that performs non-waived testing must establish and maintain written policies and procedures that implement and monitor a quality system for all phases of the workflow (that is, pre-analytic, analytic, and post-analytic), as well as general laboratory systems. The laboratory's quality systems must include ongoing monitoring and assessment to identify, evaluate and resolve problems and ensure continuous improvement of the laboratory's performance and services. The various components of the quality system are used to meet the requirements in this part and must be appropriate for the testing specialties and subspecialties performed, services offered, and clients it serves. Accrediting organizations such as the College of American Pathologists (CAP) and The Joint Commission (TJC) [2,3] have incorporated standards specifically designed to meet the CLIA requirements.

21.1.1 What is the quality system?

The quality system in healthcare identifies several components, called Quality System Essentials (QSE) [4,5], for each step in the path of workflow. The QSE include organization, facilities and safety, personnel, purchasing and inventory, equipment, process management, documents and records, nonconforming event management (error detection/reporting), assessments, customer focus, information management, and continual improvement. For laboratory testing, the operation systems are patient assessment, test request, specimen collection, specimen transport, specimen receiving and processing, testing and review, laboratory interpretation, reporting, post-specimen management, information system, and clinical application/consultation.

As it would take an entire book to thoroughly outline each QSE, we will focus on those areas that many cytogenetic laboratory personnel are most concerned with.

21.1.2 Organization

This QSE outlines who is responsible for overseeing the laboratory's quality program, what responsibilities are included, the laboratory's mission and structure (organization chart).

21.1.3 Facilities and safety

This QSE provides information necessary to ensure an adequate and safe environment for employees, patients and visitors. Planning for facility needs, safety regulations/policies, assessing compliance (auditing), and education and training are addressed in this QSE.

21.1.4 Personnel

The QSE for personnel has several components: hiring, orientation, training, competency assessment, continuing education, performance appraisal, and end of employment. Please note that in this chapter we have opted to include competency assessments in the assessment portion, since we did not include a personnel section in this chapter.

21.1.5 Purchasing and inventory

This QSE ensures that reagents, supplies and services needed by the laboratory to perform the assigned tasks are adequate in quality and availability. Each analytical section of this book outlines which reagents and supplies are used in testing; therefore, we have opted not to include a detailed section on this QSE in this chapter.

21.1.6 Equipment

The equipment QSE addresses the selection, acquisition, calibration, maintenance, troubleshooting, and repair of equipment that is in service at the laboratory, as well as the removal of equipment from service. Equipment maintenance is addressed in Chapter 20: Selected topics on safety, equipment maintenance and compliance for the cytogenetics laboratory.

21.1.7 Process management

This QSE provides a mechanism to ensure that work processes are performed consistently and meet defined objectives. Documented processes should exist to define, validate, and implement processes and control changes to existing methodology.

21.1.8 Documents and records

This QSE requires that there is an established authority to write, revise, review, and approve documents and records. No changes to the documents can be made without following the laboratory's written procedure for such changes. It ensures that the most current versions of documents are available to the users. Obsolete documents are archived and retained according to applicable retention guidelines. The documents are reviewed annually by authorized individuals, and the documentation of the review should be traceable.

21.1.9 Nonconforming event management

Regardless of how hard we try to prevent them, mistakes (events) happen. In the quality system, events are documented, investigated, categorized and analyzed, and appropriate improvement measures are completed.

21.1.10 Assessments

Laboratory personnel employ several methods to systematically measure and evaluate the work and quality system elements throughout the path of work flow. This QSE provides guidance for assessing the laboratory performance.

21.1.11 Customer focus

The customer's needs, expectations and satisfaction should be assessed. In addition, customer complaints should be documented. Processes should be improved to meet the needs and expectations of customers when they change. Laboratories can obtain information about their customers by surveys, customer comments, feedback and consultation.

21.1.12 Information management

The Information Management QSE deals with appropriate use, management and protection of the laboratory's data and information. Key components of this QSE include, but are not limited to, password protection, levels of security (who can access which data), verification of software and policies for maintaining confidentiality. Many of the issues in this QSE are regulated by outside organizations, but the laboratory should have policies and procedures that describe how the laboratory will comply with the regulations. These policies and procedures should also include how the laboratory will respond when the protection of data and information has been compromised.

21.1.13 Continual improvement

Recognizing when to make improvements is an important aspect of the quality system. All laboratory personnel are responsible for identifying opportunities for improvement and for participation in improvement activities.

21.2 Process management

21.2.1 Validating new tests, changed tests, and new or moved equipment

For any clinical laboratory test performed in the United States, validation for the accuracy of each test result is required under CLIA '88 [1]. Validation establishes documented evidence with a high degree of assurance that a specific process *consistently* produces a result or product that meets its predetermined specifications and quality attributes (Table 21.1).

The laboratory management must determine and document the test system's calibration and control procedures based upon the performance specifications verified or established under this section. Once data has been collected that meets minimal criteria for these performance characteristics, continued monitoring of test results will increase the confidence levels for the test.

Furthermore, reagents and equipment must be validated for ability to perform as expected prior to use in patient testing. For FDA cleared, approved or exempt reagents, the manufacturer will supply the performance characteristics achieved during their evaluation process, but each laboratory must test the reagent before it is used in clinical testing. The equipment manufacturer often provides the installation validation needed for new equipment, but laboratory personnel should perform such validation if the equipment is moved, repaired or requires re-calibration.

21.2.2 Validation plan

Validation should be carefully planned (Box 21.1), regardless of whether it is being performed for new or modified tests, equipment or reagents. The validation plan should state why the validation is being conducted, for example, to verify the manufacturer's performance characteristics or to establish method performance characteristics for an in-house developed test (now referred to by FDA as laboratory developed test or LDT). The plan should describe the methodology and testing instrumentation that will be used.

Table 21.1 Test method validation requirements

Each laboratory that introduces an unmodified FDA-cleared or approved test system must do the following, before reporting patient results:	Each laboratory that:
<ol style="list-style-type: none"> Demonstrate that it can obtain performance specifications comparable to those established by the manufacturer for <ul style="list-style-type: none"> Accuracy Precision Reportable range of test results Verify that the manufacturer's reference intervals (normal values) are appropriate for the laboratory's patient population. 	<ol style="list-style-type: none"> Modifies an FDA-cleared or approved system, Introduces a test system not subject to FDA clearance or approval (including methods developed in-house and standardized methods such as text book procedures), or Uses a test system in which the performance specifications are not provided by the manufacturer, must establish performance specifications for each test system before reporting patient test results. Performance characteristics include, as applicable: <ul style="list-style-type: none"> Accuracy Precision Analytical sensitivity Analytical specificity to include interfering substances (conditions) Reportable range of test results for the test system Reference intervals (normal values) Any other performance characteristic required for test performance

Prior to performing patient testing, the laboratory must verify or establish the performance specifications for the test system being employed. The list of required performance specification is defined in the CLIA '88 regulations found in 42 CFR 1253 [1].

BOX 21.1 PROCESS VALIDATION OUTLINE

Process validation outline

Title: State what will be validated.

Purpose: Explain why the validation is being conducted and what is the desired outcome.

System description: Provide an overview of the new or changed process and what the process is expected to do. (What will measure success of the process being tested?) Flowcharts may be a useful way to display this information.

Study summary: Summarize the main points of what will be validated and how the validation will be conducted. This should include the number of specimens to be studied, the sampling method that will be employed and how the analysis will be done.

Responsibilities: State who will be responsible for developing the validation outline, approving it, conducting the validation study, calibrating and maintaining the equipment, reviewing the study data and approving the study results.

Validation plan: Provide step-by-step instructions on how to perform the validation (standard laboratory operating procedure, manufacturer's instructions/manual, or a new stepwise set of instructions). This section should include the equipment needed and the calibration/maintenance required. It should also include the reagents and supplies that will be needed to perform the validation, what samples/items will be used and how they will be obtained, and any support that will be needed from outside the laboratory (e.g., information systems, phlebotomy, etc.).

Acceptance criteria: State how the results will be evaluated and what will constitute an acceptable validation. This may include quantitative and/or qualitative indicators, conditions that will require that all or parts of the study need to be repeated, and allowable run-to-run and technologist-to-technologist variation.

References: Document all references used to develop the plan and establish acceptance criteria.

Study conclusions: Document records of the validation process, describe any deviations that occurred from the outlined process and summarize the results of the study. Include justification for failure to follow the planned validation process or for modifications to acceptance criteria. Describe any limitations or restrictions for use of the process. Determine if the process is acceptable, needs modification or should be rejected.

Final review and approval: Obtain the signatures necessary to complete the validation and move the process into clinical practice.

Prior to initiating validation for a new test, plan the process using a template such as this one [14]. The plan should include the number of specimens to be tested; reagents, supplies and equipment required to perform; expected results; procedure documents; and who will be responsible for various components of the validation.

It should indicate reagents, supplies, equipment, and documents to be used for the validation, and should include steps for recording reagent lot numbers, equipment calibration, maintenance, and quality control.

The plan should also list what is not in the scope of the validation, such as specimen stability tests, specimen transport systems, etc. Whenever possible, all phases of testing (pre-analytic, analytic and post-analytic) should be included in the plan.

It is also important to account for various responsibilities during the validation. Who will manage the project? Who will approve the validation outline and acceptance criteria? Who will conduct the studies? Who will review and summarize the study outcomes?

21.2.3 Specimen number for validation

The number and source of specimens required for process validation (normal and abnormal or affected and unaffected) may be determined by previously published information or may be a reasonable number suggested by the investigators. The number should be sufficient so that a stated confidence level can be established for the test's performance characteristics, such as reference intervals, analytical and clinical sensitivity, and analytical and clinical specificity (see Chapter 16, section 16.11, FISH probe testing and validation). The American College of Medical Genetics (ACMG) guidelines indicate the number of signals/cells to assess for validation of both metaphase and interphase cells for FISH methodology [6]. The Clinical and Laboratory Standards Institute (CLSI) [7] offers some suggestions for validation when large samples are not possible.

BOX 21.2 PROCESS VALIDATION: SUMMARY OF RESULTS

Results

Attachment(s):

- Attach all documents upon which records of the validation process have been recorded or displayed.

Deviations:

- Describe any deviations that occurred from the process as outlined in the study. Include justification for failure to follow the planned validation process or for modifications to acceptance criteria.

Summary of findings:

Summarize results of the validation studies.

- Point out all important aspects of the validation results (high level summary of findings)
- Evaluate data and prepare data visuals (e.g., charts and graphs)
- Conclude whether or not the process can be considered validated

Limitations:

Based on the results, describe any limitations or restrictions for use of the process as validated.

Conclusions:

- Acceptable, OK to implement
- Modify, Define modifications in comments section
- Reject

Reproduced with permission from Mayo Clinic Rochester.

21.2.4 Data analytical evaluation

Data should be analyzed using reasonable statistical tools and these methods should be listed in the validation plan. The confidence level (e.g., 95%) should be stated, and the acceptance criteria for the test should be described for each performance characteristic (accuracy, precision, analytical sensitivity, etc.). Wiktor et al. [8] suggest a multi-step approach for validation of new FISH tests, but their plan could easily apply to validation for other testing methodology as well.

When the validation is completed, prepare a summary of the results (Box 21.2). The report should include records of the validation, a description of any deviations that occurred from the process as outlined in the study, a justification for the deviation, if applicable, and a summary of the findings. The document should include whether the outcome was acceptable, and, if not, whether the validation will be reattempted with modification or should be rejected.

21.2.5 Reference range maintenance

Once the method, equipment or product is validated and put into clinical practice, it is important to periodically review the reference range. This can be done by evaluating a standard control specimen with each batch of patient specimens or by periodically testing samples that have no disease/syndrome, that have one hundred percent abnormal cells, and that have an abnormal cell ratio that is greater than zero but less than one hundred percent. If reference ranges have changed, there should be an investigation to determine if the cause can be identified. (This is described in the process improvement section of this chapter.) If the cause for the discrepancy cannot be corrected, new reference ranges should be established.

21.3 Documents and records

Documents and records are the most frequently cited problems during inspections and audits. In addition, laboratory staff rarely refer to written procedures, but more often rely on memory, co-workers, index cards or post-it notes for instructions. Often documents aren't used by the staff because they don't exist, they are too long and difficult to get information from, or they are not up-to-date. In a quality system, documents are managed to ensure that only the latest versions of approved documents are being used. This system includes a process to control the development, distribution and accepted changes to documents.

Laboratory documents are any written item that provides guidance for the performance of a process. Documents include policies, processes, procedures/work instructions and forms. Document management includes identification, version number, change control, a master index, distribution lists, and archives of obsolete or discontinued documents [9].

21.3.1 Document creation and control

When developing a QSE system, it is useful to establish a master index and a guideline indicating the minimal elements required for each type of document that your laboratory will create. A master index lists all laboratory documents and their status (i.e., draft, active, obsolete/archived, and date for review). It provides staff, auditors and assessors with a quick evaluation of the documents in your work area. Table 21.2 identifies possible elements for several types of documents and suggests

Table 21.2 Elements for policies, processes, procedures and forms. The elements that should be incorporated into laboratory documents are listed in the table

Types of documents					
Elements	Policies	Process	Non-analytical procedure	Analytical procedure	Forms
Title	R	R	R	R	R
Location of Laboratory	R	R	R	R	R
Author	R	R*	R	R	R*
Approving Authority	R	R*	R	R	R*
Approval Signature	R*	R*	R*	R*	R*
Effective Date	R	R	R	R	R
Tracking/Control Number	R	R	R	R	R
Version Number	R	R	R	R	R
Purpose	AA	AA	R	AA	AA
Principle (e.g., method)			AA	R	
Scope	AA		AA		
Policy Statements	R	AA	AA		
Responsibilities	R	AA	AA	AA	
Specimens			AA	R	
Reagents/Supplies			AA	R	
Equipment			AA	AA	
Calibration			AA	AA	
Quality Control			AA	AA	
Procedural Instructions			R	R	AA
Calculations			AA	AA	AA
Reporting/Interpreting Results			AA	R	
Procedural Notes			AA	AA	
Limitations			AA	AA	
Related Documents	AA	AA	R	R	
References	AA	AA	AA	R	
Annual Review	R*	R*	R*	R*	R*
Revision/Document History	R*	R*	R*	R*	R*

AA=as applicable

R=required

R*=required and traceable to the document but need not be on the source document

Reproduced with permission from Mayo Clinic Rochester.

whether these items might be considered as “required” or “possible” for that type of document. The author of the document should be familiar with these elements and should ensure that they are considered when developing a document.

Four types of laboratory documents exist in most laboratories. (1) Policy documents are based on regulations, accreditation requirements and/or organization requirements. They are a written statement of overall intentions and directions. (2) Process documents represent the flow of work (Figure 21.1). They are most effectively represented by a flowchart or table. Process documents describe who does what and when. (3) Procedure documents provide the step-by-step instructions to do the work. (4) Forms, either paper or electronic, capture results and become records when they are completed.

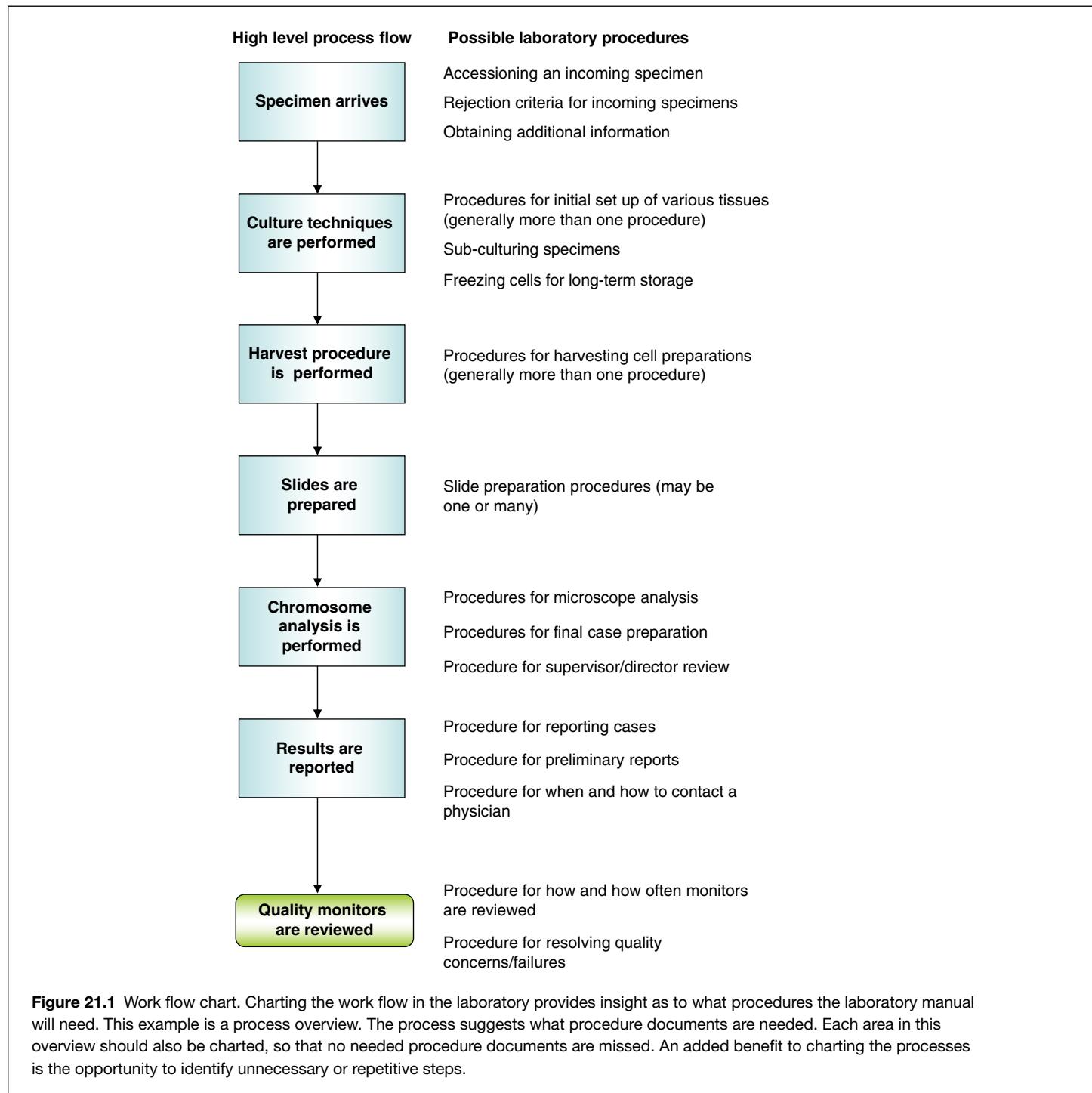


Figure 21.1 Work flow chart. Charting the work flow in the laboratory provides insight as to what procedures the laboratory manual will need. This example is a process overview. The process suggests what procedure documents are needed. Each area in this overview should also be charted, so that no needed procedure documents are missed. An added benefit to charting the processes is the opportunity to identify unnecessary or repetitive steps.

Refrigerator ID number: 42 Location: Room 552 Month/Year: November, 2012

Temperature expected range: 1°C to 6°C

Date	Initials	Top/front	Is alarm switch turned on	Is recording chart on right day and time	✓ if criteria are met	Supervisor notified of unmet criteria
1	PJS	2	✓ ^a	Yes	✓	
2	PJS	b	✓ ^a	No	b	No-adjusted
3	SAT	3 ^c	Yes	Yes	✓	
4	SAT	7 ^d	Yes	Yes	✓	

Incorrect completions/corrections:

- a. ✓ not acceptable for alarm and chart recording columns
- b. Columns do not have data
- c. Incorrect entry is not corrected appropriately
- d. Temperature above expected range and no correction indicated

Figure 21.2 Incorrectly entered temperature record – refrigerator. This record demonstrates several types of incorrect entries for a refrigerator temperature record. Most of these incorrect entries make it difficult to determine how this equipment was performing. These types of errors are commonly observed during an on-site laboratory inspection or audit.

When creating a document, identify the user. In the past, it was common to have documents that were written for inspectors or auditors. Thus, they had more information than necessary for the staff. (That's why the staff used index cards, post-it notes, etc.) Procedure documents should be written in short sentences and with recipe-style directions.

Use words with precision. The reader will understand what is written, not what was intended. For example, “analyze cells” has a different meaning to different people. Define or describe what is meant by this phrase with concrete tasks or parameters.

When writing policies, avoid promissory language. The words “will” and “shall” mean commitment to an action. If you use these words, there is no room for variation.

21.3.2 Records

A good record provides a trail for what happened. Characteristics of good records are (a) overall appearance, (b) permanence (use blue or black pen, not pencil), (c) designation of when to use initials or entire signature, (d) timely completion, (e) complete and correct information, and (f) appropriate method of correcting erroneous entries. Figure 21.2 illustrates a poorly completed record with explanations as to what is inappropriate.

Records should be neat and easy to follow. When decimals are used, a zero should precede the decimal point for values less than 1 and zeroes should follow the decimal point if the accuracy of the entry dictates it. No area of a record should be blank when it is completed. If a requested field is not applicable in a situation, record “NA” in the space. For instrument or equipment maintenance logs, enter “not in use” for those days and times when it is appropriate. If there are long periods when the instrument or equipment is not used, consider a form without defined dates or define on the form regular scenarios when it is not in use, for example, “equipment is not used on weekends.” The laboratory should determine how dates will be entered on forms, and this method should be used consistently by all personnel. Any acceptable abbreviations should be developed by laboratory management and should be written in the instructions for completing the form or in a standard operating procedure.

Errors should not be erased, obliterated, written over, taped over, or removed with white out. Instead, errors should have a single line through the incorrect information; the correct information should be placed proximate to the line through, with the corrector’s initials and date of the correction (Box 21.3). The record should also have the reason for the correction.

Records are documents of what was done and should be retained according to regulatory and accreditation requirements. In some cases, state laws may supersede national regulatory or accrediting agencies.

21.4 Assessments

Assessments are scheduled systematic processes for measuring and evaluating the laboratory’s quality across the path of work flow. For a laboratory, these measurements may include both internal and external processes. Assessments are used to determine whether the laboratory’s goals are met and if the laboratory is in compliance with regulatory and accreditation agencies.

BOX 21.3 HOW TO CHANGE AN INCORRECT ENTRY ON A RECORD

Step Action

1. Draw a straight line through the incorrect information.
Example: 73C
2. Write the correct information proximate to the line-through.
Example: 73C 37C
3. Write your initials and the date of correction proximate to the corrected entry.
Example: 73C 37C PJS 01/18/01
4. Write the reason for the correction, when appropriate. Do not use the word “error” in the explanation. For this example, the explanation would be that the numbers were reversed when they were entered.

Table 21.3 Quality plan

Quality plan for (year) form (example)		
Planned quality indicators	CI teams/projects	Audits
Turnaround time for blood, amniotic fluid, and bone marrow specimens	Decrease the number of HER2 specimen resets	CAP on-site in 2nd quarter
Success rate for bone marrow specimens		
Number of revised reports		
Others		
A quality plan does not have to be a formal document. The plan outlined here makes it very easy for the laboratory management and staff to see what is planned for a given year. Reproduced with permission from Mayo Clinic Rochester.		

The laboratory should develop an assessment plan yearly, and monitor how well it conforms to the plan. The plan does not need to be elaborate in design, but it should cover all aspects of assessments (Table 21.3).

21.4.1 Internal assessments

Internal assessments consist of internal audits, ongoing monitoring of quality indicators, and regular participation in competency programs. These evaluations are critical for ensuring the laboratory's quality. A laboratory can pass regulatory and accreditation requirements and still have quality deficiencies.

Internal audits

Internal audits should be performed on a regular basis by individuals who are not directly involved with the functions being audited. It should be clear to the laboratory being audited what type of method will be used to gather information and how the analysis will be performed. Data can be collected physically by observing people, property or events; by verbal questioning; by reviewing written materials; by comparing information and looking for patterns; or by a combination of these methods. Auditors are trained to look for evidence of compliance, and often follow the path of work flow. Auditors require laboratory personnel to show them what they are looking for rather than ask questions that have a direct answer. Shortly after completion of the audit, the laboratory management, or designee, should receive a written report indicating what, if anything, did not meet the laboratory or institutional goals. The laboratory personnel/management will need to develop a written plan to correct any deficiencies.

Quality indicators

Quality indicators should be monitored to identify variation in the process. Variation sometimes results from commonly occurring causes, such as random loss (chromosome analysis), difficulty of the case (turnaround time), and an overlap of signals (FISH analysis). Sometimes, however, variation results from a specific cause, such as increased chromosome breakage

due to environmental changes, increased turnaround time due to an unexpected increase in patient samples, or changes in FISH reporting ranges due to a new lot of probe (or other changes in the process). Routine checks of the procedure, such as recordings of temperatures, gas levels, equipment maintenance and use of control probes and specimens, provide laboratory personnel with a timely opportunity to identify and correct variation. Control probes and specimens serve as effective indicators to ensure that the process is working appropriately, that the correct probe(s) was applied (FISH), and that the appropriate amount of reagent was used (breakage studies). Their use in FISH also helps interpret performance values of the assay, and verifies that the scoring criteria were used correctly [10]. Methods for using control probes/specimens and for monitoring our processes with them provide information for both the current test and the test performance over time. Control data should be assessed before reporting patient results, and out of control values need to be investigated.

Monitoring quality indicators ensures that day-to-day operations remain in control over time. Quality indicators should be monitored across the path of work flow. Common examples in cytogenetics are the quality of specimens when they arrive, time from collection to delivery, control specimens, time from receipt to final report, band level achieved, number of culture/analysis failures and reporting errors.

Most laboratories generally report quality indicators as the number not meeting criteria divided by the number of total samples. Using that method makes it hard to track whether or not the process is “in control” over time. To monitor quality indicators over time, a control chart is very useful. Control charts provide a pictorial representation of the measurement over time. In addition, they provide an opportunity to identify if a special cause of variation is affecting the laboratory’s process or system, thereby providing an opportunity to improve the system. Control charts should also be used to verify that changes made to the process reflect improved performance, and that the improvement is maintained over time [11].

Control charts are classified as variable or attribute. Variable control charts are used for data that are measurable. Examples of measurable data are mean turnaround times, mean success rates, and amount of chromosome breakage. Attribute control charts are used for data that are countable. Examples of countable data are the number of specimens received, the percent of inadequate specimens received, and the percent of reports that had a clerical error.

The control chart consists of a center line (usually the mathematical average of all the samples plotted), upper and lower statistical control limits (which define the constraints of common cause variations) and performance data plotted over time. Special cause variation can be identified by using several criteria: 1 data point falling outside the control limits, 6 or more points in a row steadily increasing or decreasing, or 8 or more points in a row on one side of the center line. When one of these criteria is met, the special cause variation needs to be identified. In some cases, a process improvement team is needed to establish the root cause(s) of the variation and establish the corrective action. (See the Process improvement section of this chapter for more information.)

Competency program

Evaluation of an employee’s competency is a requirement of CLIA ’88 Sec. 493.1451 [1]. The laboratory technical supervisor is responsible for assessing competency of laboratory technical staff. The performance of individuals responsible for high complexity testing are to be evaluated and documented at least semiannually during the first year the individual tests patient specimens. Thereafter, evaluations must be performed at least annually unless test methodology or instrumentation changes, in which case, prior to reporting patient test results, the individual’s performance must be reevaluated to include the use of the new test methodology or instrumentation.

CLIA ’88 indicates that procedures for evaluation of the competency of the staff must include, but are not limited to: direct observations of routine patient test performance, including patient preparation, if applicable, specimen handling, processing and testing; monitoring the recording and reporting of test results; review of intermediate test results or worksheets, quality control records, proficiency testing results, and preventive maintenance records; direct observation of performance of instrument maintenance and function checks; assessment of test performance through testing previously analyzed specimens, internal blind testing samples or external proficiency testing samples; and assessment of problem solving skills. Some laboratories have developed written tests, checklists or internal proficiency tests to comply with this regulation. Actual case images can be imported to a secure web site (password protected), and a test can be developed to assess competency based on these images.

Training and education in healthcare has adopted outcome-based competencies in the United States. This means that clearly defined and published learning outcomes are developed that the students/employees must achieve. The training curriculum, learning strategies and opportunities need to be designed to ensure the achievement of the outcome, and the assessment process should be matched to the learning objectives. Furthermore, there needs to be a provision of remediation and enrichment for employees, as appropriate [12]. Carefully planned outcome-based training is dependent on objectives that are measurable and includes ongoing training.

21.4.2 External assessments

External audits/accreditation

There are several groups in the United States that accredit or regulate genetic laboratories. These include, but are not limited to:

- Food and Drug Administration (FDA) [13]
- College of American Pathologists (CAP) [2]
- Centers for Medicare and Medicaid Services (CMS) [14]
- Occupational Safety and Health Administration (OSHA) [15]
- The Joint Commission (TJC, formerly JCAH and JCAHO) [3]

In most cases, the on-site assessment of the laboratory includes evidence of compliance with a given standard or regulation. The laboratory should prepare for on-site assessments by having appropriate documentation, records, laboratory procedures, etc. ready for the assessor. In some cases, a failure to comply with the standard or regulation may result in a fine (e.g., OSHA, FDA). In other cases, the laboratory will need to provide an improvement plan and follow-up with evidence of implementation of the plan (e.g., CAP, TJC).

Specific requirements for these groups are available on the Internet. See the reference section of this chapter for the website locations of these groups.

Proficiency testing

CLIA '88 Section 493.801 [1] indicates that each laboratory must enroll in a proficiency testing (PT) program that meets the criteria outlined in that section and is approved by the US Department of Health and Human Services. The laboratory must enroll in an approved program or programs for each of the specialties and subspecialties for which it seeks certification and must test the samples in the same manner as they would test patient specimens.

Laboratories that perform tests on proficiency testing samples must not engage in any inter-laboratory communications pertaining to the results of proficiency testing sample(s) until after the date by which the laboratory must report their results to the program. Likewise, laboratories with multiple testing sites or separate locations must not participate in any communication or discussion across sites/locations concerning proficiency testing sample results until after the date by which the laboratory must report proficiency testing results to the program. The laboratory must not send PT samples, or portions of samples, to another laboratory for any analysis which it is certified to perform in its own laboratory. If CMS (Centers for Medicare and Medicare Services) determines that a laboratory has intentionally referred its proficiency testing samples to another laboratory for analysis, its certification will be revoked for at least one year. Any laboratory that receives proficiency testing samples from another laboratory for testing must notify CMS of the receipt of those samples.

The laboratory must document each processing step for all proficiency test samples - from handling, preparation, processing, examination, and testing to reporting of results. A copy of all records must be maintained for a minimum of two years from the date of the proficiency testing event. Included in this file should be a copy of the PT form that is used by the laboratory to record proficiency testing results and the attestation statement provided by the PT program, which is signed by the analyst and laboratory director, documenting that proficiency testing samples were tested in the same manner as patient specimens are tested. PT is only required for the test system, assay, or examination that is being used by the laboratory as its primary method for patient testing during the PT event.

Each US laboratory performing nonwaived testing must successfully participate in a proficiency testing program approved by CMS, if applicable, for each specialty, subspecialty, analyte or test in which the laboratory is certified to perform under CMS. If a laboratory fails to participate successfully in proficiency testing for a given specialty, subspecialty, analyte or test, or if a laboratory fails to perform successfully in a CMS-approved proficiency testing program, CMS may direct the laboratory to undertake training of its personnel or to obtain technical assistance, or both, rather than impose alternative or principle sanctions for the initial unsuccessful performance. Exceptions, however, may occur when one or more of the following conditions exist:

1. There is immediate jeopardy to patient health and safety;
2. The laboratory fails to provide CMS or a CMS agent with satisfactory evidence that it has taken steps to correct the problem identified by the unsuccessful proficiency testing performance;
3. The laboratory has a poor compliance history.

21.5 Continual improvement

Laboratories will experience situations when, despite our best efforts, things will go wrong. Some will call these errors, but we will refer to them as events. Laboratory events such as unacceptable quality control values, unexpected test result, patient label mismatches, inadequate samples, incorrectly reported results, safety incidents, proficiency testing failure and customer service issues should be documented, categorized and monitored for trends. There are several tools that can be used for quality improvement initiatives. Two common approaches are the Plan-Do-Study-Act (PDSA) and Six Sigma's DMAIC (Define-Measure-Analyze-Improve-Control) business management strategies [16].

When an event occurs, respond to it by taking remedial action and reporting the event to the appropriate authority. In order to encourage reporting, the laboratory should establish a “no fault” reporting atmosphere, that is, no adverse consequences to employees or the work unit. Most problems that exist are system problems [17].

Sometimes processes need to be changed due to the event findings. Sometimes there are new and better ways to do our work. Employees may have ideas that provide effective changes to the process. These are opportunities for improvement.

The goal of process improvement is to analyze the current process, make improvements and maintain the results after it was changed. Prioritize these process improvement endeavors. The problem that occurs most frequently may not be the most critical. Consider the following when prioritizing improvement opportunities: regulatory (effect on license or accreditation), financial (magnitude of failure costs on budget), or customer satisfaction (what makes the most customers the most dissatisfied).

There are many process improvement tools that can be used [18], some of which will be discussed in this chapter. It is important for a process improvement team to use at least some of these tools to effectively improve the process. Often people think they know what will correct the problem, but it may be only part of the story. Failure to identify all possible causes leaves the process vulnerable to errors in the future.

Flowcharts (Figure 21.3) are a valuable process improvement tool. The purpose of the flowchart is to document the sequence of steps in a process. It will identify where hand-offs, points of unnecessary complexity, and redundancies occur. Thus, it will identify sources of potential problems, including, but not limited to, inadequate training and lack of appropriate laboratory process and/or procedure documents.

Brainstorming will generate lots of ideas very quickly. Generally it is done with a group of people, and should involve everyone involved with the process(es). There should be no evaluating or judging of ideas, and no conclusion for an idea should be made during the brainstorming exercise. Once the ideas have been gathered, the team can decide and prioritize which ideas should be incorporated into the improvement process.

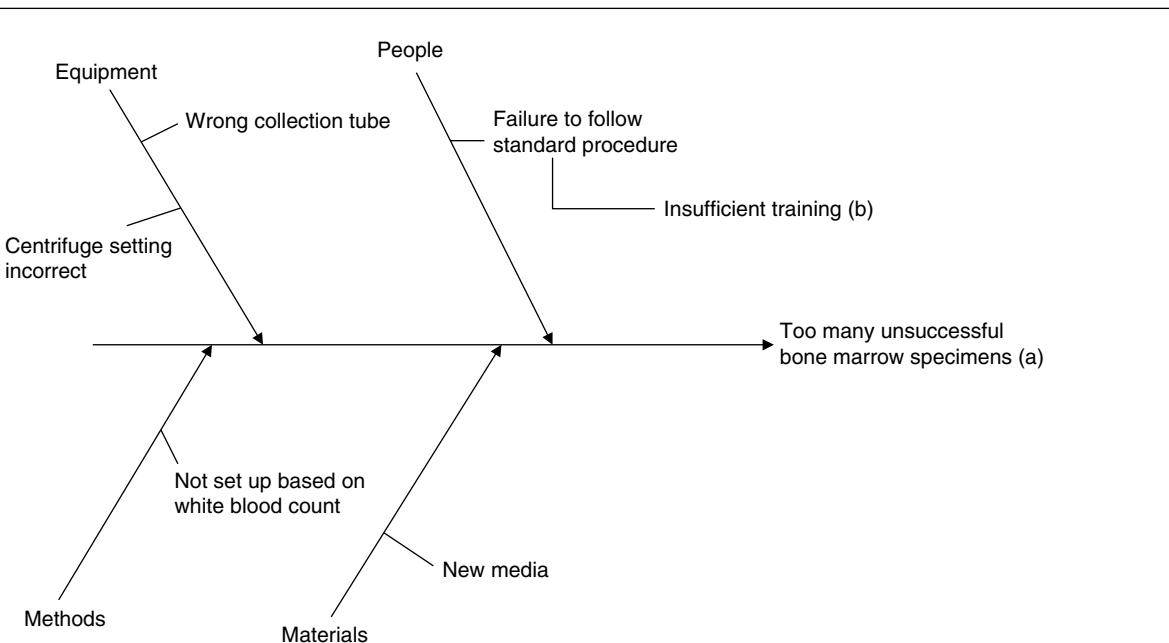


Figure 21.3 Fishbone diagram. A cause and effect fishbone diagram allows a team to identify and graphically display possible causes of a problem. (a) The problem the team is trying to solve. In this case, the team opted to see if equipment, people, methods or materials may be a root cause of the problem. (b) Demonstrates how some ideas can further detail a specific cause.

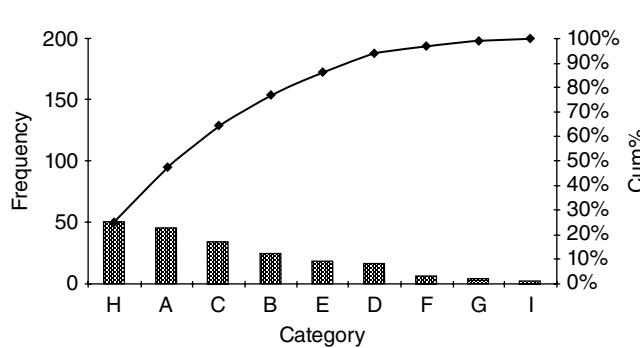


Figure 21.4 Pareto chart. Often there are several causes for a problem, but by resolving a few, a high percentage of the defects or condition will be resolved. It may not be practical in some cases to pursue the issues that account for a small percentage of the problem. In this example, little would be gained by resolving F, G and I, unless patient safety was compromised or a regulation would be violated by not correcting them.

Cause and effect diagrams (Figure 21.3) look at the relationship between the problem and the factors that are causing it. To use the cause and effect diagram, sort the list of causes into major categories (who, what, when, and why or methods, materials, people, and equipment, for example) and sort all the cause suggestions into the appropriate category to decide which theories to test.

Pareto charts (Figure 21.4) display the data in order of relative importance, using a descending bar graph, thus allowing the team to focus on those problems with the greatest impact. In most cases, two or three categories will account for the bulk of the problem and will be the high impact points on which to focus. Look for a break in the cumulative percentage line (the point where the slope begins to flatten out). If there is not a fairly clear change in the slope, look for factors that make up at least sixty percent of the problem.

Regardless of what tools are used, the process of problem resolution is the same. The problem is first identified and prioritized. There is an analysis of the current process and data collection to verify that a process problem exists. Tools are used to identify the root cause of the problem, and ideas are generated for improvement. Once a solution has been selected and implemented, evaluation and follow up data should be analyzed to ensure that the problem has been resolved and performance is sustained over time.

There are other ways to improve processes. Recently, laboratories have adopted Lean thinking [19], a strategy that focuses on the elimination of waste, process variation and imbalance. Each step must create value for the customer. Waste, then, is anything that consumes resources but does not add value. In Lean thinking, waste includes, but is not limited to: waiting (specimen and patient wait times are both included), amount of technologist/specimen movement to perform the testing, overprocessing (such as unnecessary re-testing or performing a test that is not requested by the client), excessive inventory, laboratory results awaiting confirmation and release, failure to get the proper result/consultation for the proper patient to the proper physician, retesting, redrawing or result revision that could have been avoided. Problems are identified by mapping the current processes, identifying problem areas, and using process improvement tools such as those discussed in this section to improve the laboratory's process flow.

21.6 Summary

Quality should be a key component of how a laboratory practices and should be part of everyone's daily work. Good laboratory practices are woven from quality practices. Defining and implementing a quality system is one method to achieve the laboratory's quality goals. Although it may take time to incorporate some of the concepts discussed in this chapter into practice, it will save the time spent when a process fails.

References

1. <http://www.cdc.gov/clia/regs/toc.aspx>.
2. College of American Pathologists. <http://www.cap.org/apps/cap.portal>
3. Joint Commission. <http://www.jointcommission.org>.

4. Berte LM, Nevalainen DE. *Quality Systems for the Laboratory*. American Society of Clinical Pathologists, Chicago, 2000.
5. Clinical and Laboratory Standards Institute. *Quality Management System: A Model for Laboratory Services; Approved Guideline*, 4th ed. CLSI document QMS01-A4. Wayne, PA: Clinical and Laboratory Standards Institute, 2011.
6. American College of Medical Genetics. http://www.acmg.net/AM/Template.cfm?Section=Laboratory_Standards_and-Guidelines&Template=/CM/HTMLDisplay.cfm&ContentID=3173
7. Clinical and Laboratory Standards Institute. *Defining, Establishing and Verifying Reference Intervals in the Clinical Laboratory; Proposed Guideline*, 3rd ed. CLSI document C28-P3. Wayne, PA: Clinical and Laboratory Standards Institute, 2008.
8. Wiktor AE, Van Dyke DL, Stupca PJ, Ketterling RP, Thorland EC, Shearer BM, Fink SR, Stockero KJ, Majorowicz JR, Dewald GW. Preclinical validation of fluorescence in situ hybridization assays for clinical practice. *Genetics in Medicine* 2006; 8:16–23.
9. Clinical and Laboratory Standards Institute. *Laboratory Documents: Development and Control; Approved Guideline*, 5th ed. CLSI document GP2-A5. Clinical and Laboratory Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2006.
10. Stupca PJ, Meyer RG, Dewald GW. Using controls for molecular cytogenetic testing in clinical practice. *J Assoc Genet Technol* 2005; 31: 4–8.
11. Kelley DL. *How to Use Control Charts for Healthcare*. American Society for Quality, Quality Press, 611 East Wisconsin Avenue, Milwaukee, WI 53202, 1999.
12. Harden RM. *Developments in Outcome-based Education*. Institute for International Medical Education, 1999. www.iime.org/documents/harden.htm
13. <http://www.fda.gov>.
14. <http://www.cms.hhs.gov>.
15. <http://www.osha.gov>.
16. Juran Institute, Inc. <http://www.juran.com>.
17. Nutting PA, Main DS, Fischer PM, Stull TM, Pontious M, Seifert M Jr, Boone DJ, Holcomb S. Toward optimal laboratory use. Problems in laboratory testing in primary care. *JAMA* 1996;275:635–639.
18. Brassard M, Ritter D. *The Memory Jogger II: A Pocket Guide of Tools for Continuous Improvement and Effective Planning*. GOAL/QPC, 2 Manor Parkway, Salem, NH 03079, 1994.
19. Womack JP, Jones DT. Lean consumption. *Harv Bus Rev* 2005;83(3):58–68.

Contributed protocols section

IMPORTANT: No protocol included in this manual should be used for clinical testing unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements for all chemicals used in the laboratory.

Protocol 21.1 Quality control overview document

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Logging and processing of samples

Purpose

To maintain quality control of the logging and processing of specimens in the Cytogenetic Laboratory.

Procedure

Specimens received on weekdays are unpacked and logged in by the Front Office personnel. Those received on weekends and holidays are unpacked and logged in by technologists or laboratory assistants. All samples are immediately quick accessioned into the OHSU patient order database, Tamtron.

Using nitrile gloves for protection, unpack specimen carefully, to be sure that all paperwork and specimen containers are found in the packaging materials. Be sure all containers are labeled with the patient name. Save a copy of the FedEx or other shipping company forms from the box, in case it has information not provided inside. Make a note on the paperwork if container is leaking, specimen is clotted, or if any other unusual conditions are found. For amniotic fluid specimens, record color and presence of blood for AF/ACHE test purposes. Initial and date all notes made.

Stamp the date on the paperwork and fill in the time received, type of sample and type of media/containers the sample was collected in, what method of transport was used, and initials of login person.

Logbook: Enter the patient name, birthdate if applicable (for prenatal specimens, enter mother's name, first and last, instead of a birthdate), physician name, type of specimen, and type of study under the notation of the date received. For prenatal studies, enter the last name and "fetus," "abortus," "stillborn," or "neonatal death," depending upon circumstances, for the patient name. "Fetus" refers to a viable unborn baby; "abortus" is a deceased fetus up to 20 weeks; "stillborn" is a deceased fetus after 20 weeks; and neonatal death is a child born alive which then expires. All studies, prenatal or other, are given the date of receipt in the laboratory, and the next consecutive GL number available in the Logbook is assigned to the specimen being logged in. Double check all GL numbers to be sure no error has been made.

Place a GL number sticker bearing the patient's number on the logbook page, the specimen tube(s), and any other pertinent materials. **All tubes** must be marked with the GL number.

Enter the patient information in the computer (front office staff) including:

- Patient name, account number, MR number
- Date of specimen procurement
- Date and time specimen was received in the laboratory
- Physician's name and location as well as phone number/pager
- Reason for referral
- Clinical information
- Cross reference to other cytogenetic studies in the Cytogenetics Laboratory
- Type of specimen
- Type(s) of studies required (full/high resolution/FISH, etc.)

Note any comments about the specimen (STAT results required) and note priority if the physician has assigned one.

For PGD/PGT studies, note number of slides received. Patient name is mother's last name and Embryo for the first name. No matter how many slides/embryos received, only one is logged in per patient cycle. All results will be put on a single report/GL number.

Reconcile the specimen paperwork with the appropriate telephone message slip on the Specimens Expected clipboard and save it with the paperwork.

Specimen flow sheet: Each specimen type has a working flow sheet to record what stage the specimen is at in the workflow. Enter the specimen on the appropriate flow sheet; each has patient name, GL number, reason for referral, and physician. In addition, the amniotic fluid, CVS, and fibroblast flow sheets have GA, specimen size or type, and culturing information. The bone marrow flow sheet has previous studies listed, and urgency of results. The blood flow sheet has culture durations. The tumor flow sheet has a description of the specimen. All types of flow sheets will have the culture durations on them; either at sign-in or after specimen has been cultured and harvested.

II. Tissue culture

For solid tissue culture and prenatal setups, **set up only one patient at a time**, and **only have only the specimen from that patient in the hood during setup**. For blood and bone marrow setups, **have only one patient sample open at a time, while all other patient samples are held separate**.

After setting up the culture, double-check the tubes for name and GL number before going to the next patient. Remove all materials from the first setup before going to the next patient.

For attached cultures, two different kinds/lots of media are used for each specimen whenever possible; cultures in centrifuge tubes with multiple types of media, stimulants, and culture durations.

For prenatal studies, place each patient's culture in two different incubators with separate temperature controls. Place all other samples cultures in one incubator whose temperature controls are monitored daily. Incubators have emergency backup power (generators) for power outages.

All cultures are closed system cultures so if incubator gas supply is interrupted, there is a much greater chance of culture survival than with an open system. There is also much less chance of culture contamination than with an open system.

Set up duplicate cultures and carry them through as independent cultures (separate processing, pipettes, slides) for all specimen types unless insufficient sample is received; if less than two cultures are established, the report will reflect this.

Check the temperatures of incubators each day; they should be from 36.5 to 38 °C.

For CO₂ incubators, take Fyrite readings at least twice per week, usually Monday and Friday; they should be between 4.5–5.5%.

Check temperature of refrigerators and freezers each workday; they should be between 3.5 and 5.0 °C and –25 to –15 °C, respectively.

Test for sterility all media and saline components for tissue culture before use using tryptose phosphate broth. Discard media if any contaminants appear to be present.

Change water in water baths weekly, and wipe the bath out with alcohol.

Calibrate thermometers yearly.

III. Harvesting

Check pipetters daily to be sure they read correctly on fixative component bottles.

Centrifuges are calibrated and checked by clinical engineering on a yearly basis.

Blood, bone marrow and solid tumor harvesting pipettes are stored in tubes at the back of the tube rack, directly behind the corresponding centrifuge tube, and are labeled with the patient GL number.

Plastic coated absorbent paper is changed routinely.

IV. Slide-making

Make slides on only one patient at a time, and have only that patient's tubes in the working rack. All others are in another rack out of the work area.

Label slides with a GL number, slide number, date, and at least the first three initials of the last name (preferably the whole name if feasible). Slide labels are available that include the patient's name and GL number.

Place slides in the aluminum tray with a space between different patients, or with the labels turned in opposite directions.

V. G-banding

Change the trypsin and rinse jars each morning, and check the first slide to be stained for adequate banding quality. If the trypsin is not performing adequately, make new solutions. If this does not solve the problem, inform the lead technologist.

VI. FISH

All FISH orders are confirmed with the referring physician if there is any question about the order. For example, “FISH for chromosome 13” could mean ploidy level or deletions of 13q. Samples sent with “reflex FISH” orders are confirmed with the referring physician, unless part of our laboratory’s standard of care testing. For example, any constitutional blood sample with a clinical diagnosis of a heard defect will automatically have FISH testing for a potential TUPLE1 deletion.

Each slide is labeled before hybridization with the patient name, GL number, probe and which end of the slide has which probe if more than one is used on more than one location. Slide labels are available that include the patient’s name and GL number for the study.

Use a new pipette tip to withdraw probe from the original vial each time to prevent cross contamination of probes.

When adding probe to the slide, double check the vial of probe against the slide label before adding it to the slide.

Have only one vial of probe in the immediate FISH work area at a time to prevent the use of a wrong probe. All others are in a separate rack, removed from the immediate work area.

Check expiration dates on all reagents before use, and discard expired reagents.

The UV bulb is changed on the FISH microscope when it reaches ~250 hours. The X-Cite Fluorescent Illuminator System bulbs are changed when they reach ~1,500 hours.

The pH meter is calibrated each workday that it is used, and the technologists’ initials written on the pH meter QC sheet above the meter.

The Hybrite is checked each day that it is used to confirm that all programs are correctly entered and used.

Check the FISH water baths each time they are used for temperature and water level. Temperature should be at that required for the specific protocol. Water level should be adequate to cover the liquid in the Coplin jars.

Check each slide before scoring and after scoring for the information on the slide label to be sure that the correct patient and probe have been scored.

All new probes are checked for specificity and sensitivity before use as well as cutoff values for normal and abnormal ranges.

Images of signal patterns of each probe in metaphase and/or interphase are stored in the FISH Quality Control book.

If a probe is ordered on a sample from a treated neoplasm to detect residual disease, a baseline study should be run on the original study, whenever possible, to be sure that the probe is informative and that there is not a variant signal pattern in the patient.

Metaphases are checked on FISH slides whenever possible, to be sure that the interpretation of the interphase signals is correct.

When writing preliminary reports on the computer, each report will be discarded before starting the next one to be sure not to carry over any information from the previous report.

All written preliminary reports will be checked by another technologist or by a Laboratory Director if complex or unusual results are reported.

VII. Microscopy, imaging, and photography

When a slide is chosen and stained, the GL number and name on the slide is double checked against the name and GL number on the patient folder, and checked again at the microscope against the count sheet. All images are captured digitally using Leica Microsystems software. The images are captured into a patient file which is created in the computer program and patient data (name, birth date, GL number) is automatically attached to each printed image. Only one patient’s file should be open at a time to protect against mixing images between two different patients. Images are routinely stored weekly onto a DVD disk and a backup disk. The disks are stored in separate fireproof boxes.

Chromosome analysis: See QA/QC notebook for required number of cells analyzed, karyotyped, counted, etc.

Karyotyping: Images are captured and karyotyped using Leica Microsystems software. The patient information will automatically be placed on the karyotype. The ISCN may be entered in the computer or added by hand later.

See Preliminary Result Protocol for details about calling results before the final report is completed. In certain cases requiring prompt patient management decisions, it is acceptable to give a preliminary report. These cases include: prenatal specimens such as amniotic fluid, CVS, PUBS; blood on newborns with life threatening conditions or sex determination questions; FISH in neoplasms such as APL, CML, neuroblastoma; post BMT bone marrow samples; etc.

VIII. Case review and sign out

Cases are reviewed in the following pathway.

After all karyotypes and other materials (FISH, panels, etc.) are completed, the case is reviewed and checked by the technologists who did the microscopy, and all errors found are corrected (e.g., clerical, clinical indications, referrals, mistakes

in cutting karyotypes, etc.). Studies performed by technologists who are new or in training will be double checked by one of the Lead Technologists. The study is summarized by the technologist(s) on the worksheet, and the billing sheet is filled out. The blank report form is also filled out and signed by all technologists involved. Then the study is entered in the computer patient database from "case study" to "director review", and the folder is given to the Laboratory Director.

The Laboratory Director will re-check all karyotypes and FISH images, all clinical information, and correct any errors. Billing may be altered at this point. The Director will also check for appropriateness of study, completeness of study, band levels, typographical errors, missed abnormalities or variants, etc. If study is complete and acceptable, the report form is filled out with: normal/abnormal result, with ISCN nomenclature; number of cells examined, with chromosome counts; number of cells karyotyped; additional cells counted or further studies done (e.g., FISH information); written interpretation of the findings, if appropriate; suggestions for further work to be done on the patient or his/her family; electronic signature of the director; date preliminary results were given (see separate document on preliminary results); date of the final report. If band levels are insufficient for the type of study, a repeat sample will be requested, if possible. The appropriate band level for each study is at the discretion of the Laboratory Director.

After the report has been typed by the Administrative Assistant or the Laboratory Director, it is re-checked by the Director and then re-checked by a lead technologist before returning to the Administrative Assistant to be mailed to the requesting physician. It is given a final check for clerical and billing errors by the A.A. and is then mailed to the client.

Results are only given to the patients' physician, her/his nurse, or the laboratory sendouts department of the referring institution. Any other requests must be accompanied by a patient release form. The name of the person to whom the result is given is recorded in the patients' chart on the worksheet, along with the date, what was said to the doctor, and the caller's name. If a result is faxed, a copy of the FAX is kept for a record.

IX. Turnaround times

The following turnaround times are to be used for cytogenetic samples.

Amniotic fluid/ CVS: 90% of studies should have a final report by 14 days from specimen receipt. (CAP guideline is 90% of cases final-reported by 14 calendar days).

Bone marrow/unstimulated blood: 90% of studies should have a final report by 21 days from specimen receipt. (CAP guideline is 90% of cases final-reported by 21 calendar days).

Constitutional blood studies: 90% of studies should have a final report by 28 days from specimen receipt. (CAP guideline is 90% of cases final-reported by 28 days).

STAT peripheral blood (constitutional): 90% of cases should have a preliminary report within 3 calendar days for stat results (CAP guideline is the same) and 90% of studies should have a final report by 7 days from specimen receipt. (CAP guideline is 90% of cases prelim by 72 hours of specimen receipt, and 90% final-reported within 7 days).

Fibroblast cultures (constitutional): 90% of studies should have a final report by 6 weeks from specimen receipt. (CAP guideline is 90% of cases final-reported within 6 weeks of specimen receipt.)

Solid tumor cultures: 95% of studies should have a final report by 35 days from specimen receipt. (No CAP guideline at present).

Her-2/neu slides: 95% of studies should have a final report by 2 weeks from specimen receipt. (No CAP guideline at present).

Glioma slides: 95% of studies should have a final report by 3 weeks from specimen receipt. (No CAP guideline at present).

X. Delays in test results

In cases where delays in turnaround times would affect patient care (e.g., STAT newborn blood samples, bone marrow transplantation studies, prenatal tests, etc.), when appropriate, notification of such delays shall be given to the referring physician by phone, FAX, or other rapid means, and an estimate shall be made about when the result will be available. Preliminary results may be given to assist the physician with decisions until such time as a final report is available.

XI. Critical limits

Stat prenatal tests (such as interphase FISH for aneuploidy, or percutaneous blood sampling, PUBS), stat newborn tests (such as blood for aneuploidy or DiGeorge/Velocardiofacial syndrome), and certain neoplastic disorders (such as acute promyelocytic leukemia) require immediate results for prompt patient care, and thus are considered critical samples with special turnaround

time requirements. These tests should have preliminary results as soon as technically feasible, and final reports should be available immediately thereafter, especially on prenatal samples where sometimes action cannot be taken without written results.

Amniotic fluid interphase FISH for aneuploidy: Preliminary results should be available within 24 hours after receipt of specimen if received in the laboratory before 3:00 PM, or within 3 days if received on a Friday, and the physician does not want a weekend result. Final report will go out when the chromosome study is complete.

PUBS: Preliminary chromosome results should be available within 24–72 hours after receipt of specimen, depending upon mitotic index and blood volume. Final reports should be available within 7 calendar days.

STAT bone marrow samples for interphase FISH: Preliminary results should be available within 24 (if sample is received by 3:00 p.m.) to 48 hours after receipt of specimen.

STAT bone marrow samples for metaphase analysis: Preliminary results should be available within 24 hours after receipt of specimen, and the final report should be available after 7 calendar days.

Preimplantation FISH on blastomeres for aneuploidy should be available within 24 hours of receipt, and for translocation plus aneuploidy analysis, within 36 hours. Final report is sent out immediately after the preliminary report.

XII. Monitoring analytic performance

1. **Chromosome studies:** If there are insufficient metaphase cells available to complete a study (see Guidelines for Chromosomes Analysis chart for specific requirements), the technologist will fill out either a Culture Failure Report (for no cells found), or a Suboptimal Study Report (for less than 20 metaphases) both of which list remedial actions to be taken if appropriate, progress the study through the Tamtron steps to Director Review, add the Failure/Suboptimal Report to the front, and put in the Director Review basket. The Director will review the reason(s) for the suboptimal results, determine if the study is of clinical value as it is, and whether further work, such as interphase FISH studies, would be of help. The Report is filed by one of the Lead Technologists in either the Suboptimal Result Book or the Culture Failure Book. These Reports are formally reviewed quarterly by the Lead Technologists, Director, and Manager to determine if there is any significant trend; however, as they are filed in the book(s), trends should become evident.
2. **FISH Studies:** Each probe is tested for analytical specificity and sensitivity, and a cutoff value for normal results is calculated (see FISH QA/QC). Specificity and sensitivity should be 98% or greater when the probe is tested on peripheral blood culture preparations. If the probe does not satisfy these criteria, another probe should be found from another source that does meet the above criteria. For FISH studies in which no metaphase cells will be present, such as preimplantation testing, metaphases from a normal blood culture will be run as a control against the possible use of a wrong probe. If the control slides do not hybridize as expected, the hybridization should be repeated on both the test and control slides using freshly prepared probes. For follow-up on bone marrow studies from patients previously studied in this laboratory, the patient's original study may be recruited as a positive control for the current study, since treatment may have made the original cell line disappear. This assures that the probe being used to test for residual disease is appropriate for the purpose. If the baseline control does not hybridize as expected, then the new FISH study requested is not reported, and if possible, another FISH test is investigated for detecting residual disease in the patient.
3. **Fanconi anemia:** There are several parts involved in the quality control of the breakage analysis done to test for Fanconi anemia (FA). The first involves the patient blood sample, which needs to come with a WBC, HCT, and % Lymphocytes. Many of the patients that we routinely study for FA present with low WBC and Hct. To control the number of lymphocytes introduced into the culture we adjust the Hct to 50% and inoculate enough whole blood to allow for adequate cell growth, usually 0.5 cc of this adjusted blood sample. If the counts and Hct are within normal range no adjustment is needed. The second involves the clastogen components, Mitomycin C (MMC) and Diepoxybutane (DEB). Lots are tested when received and tested against established control values. The stock DEB is very stable but has a very short half-life once diluted. Therefore it has to be made fresh daily. MMC, however, is very stable in the working solution concentration and is made every six months. Each new lot and working solution is run in conjunction with the previously tested lot to insure its ability to perform properly. Control values have been historically generated with normal controls periodically run along with known FA positive patients who have samples sent in for on going evaluations. There are also cell lines available for positive control testing if the need arises. The third QC component involves the training of technicians to score for breaks and radials. In the beginning of their training, their work is checked by the Lead Technologist, and breaks and radials marked captured images by the tech are reviewed by the Director before each study is signed out. Finally, we receive slides from another lab, which have been made from cultures treated with DEB, MMC or no clastogen. These slides are sent blinded with the key sent only to the Director. Each tech in the blood breakage section scores and reports their results to the lead worker and to the Director. This exercise is done twice a year.

Protocol 21.2 Monitoring specimen quality from off-hill sites

Contributed by Oregon Health & Science University (OHSU) Knight Diagnostic Laboratories, Portland, OR

I. Purpose

To track suboptimal results from specimens or clusters of specimens due to specimen handling errors at certain non-OHSU locations.

II. Procedure

1. Specimens that arrive from outside of the OHSU system are occasionally exposed to adverse conditions or improper handling that compromises the success of the tests. In order to monitor and ensure the quality of specimens received from remote sites, documentation of any unusual appearance or performance of the specimen is made by the technologist(s) culturing, harvesting, and analyzing the specimen, both on the pertinent flow sheet and in the GL folder. For example, if a bone marrow or blood arrives hemolyzed, this would be written on the Culture Sheet, and on the Worksheet in the GL folder, as well as on the flow sheet. If an amniotic fluid of advanced gestational age is received with no cells in the fluid, this would be documented on the amnio flowsheet and on the Worksheet in the GL folder. In addition, the referring physician and any intermediate laboratories will be notified of the problem in an attempt to find out why the problem occurred. If the specimen culture is unsuccessful, it will be documented both in the Culture Failure Book and, because it was suspicious at arrival for some problem, in the Incident Book.
2. Suboptimal specimens are noted in the GL file. If the specimen condition compromises the study, and it is possible to get another sample, a new sample is requested immediately, documented in the original and subsequent file and the final reports are cross-referenced in the interpretations. If a suboptimal sample must be used and the final product is adequate, there is no mention in the interpretation. However, if the quality of the study is deemed to have been impacted by the sample condition, a statement to that effect is made in the report interpretation. A new sample may be requested through this path.
3. All specimens that result in growth failures are documented in the Culture Failures book (reviewed by the Lead Technologists when putting a new document in the book) and would show trends in failures. Any trends observed from specimens from a common source would result in follow-up by the Director(s) of the laboratory.
4. All specimens that result in significant problems are documented in the Incident book, (reviewed by the Lead Technologists when putting a new document in the book) and would show trends if several came from the same center. Any trends observed from specimens from a common source would result in follow-up by the Director(s) of the laboratory.

CHAPTER 22

Laboratory management

Mervat S. Ayad and Adam Sbeiti

Quest Diagnostics Nichols Institute, San Juan Capistrano, CA, USA

22.1 Introduction

It is a commonly conceived notion that Management is no more than common sense in action. Actually, management is truly a combination of art and science. The art of management lies within motivating your team to give their best on a constant basis; the science is to provide the tools to accomplish that. This is definitely true in the complex and ever-changing field of healthcare management.

One important challenge for the clinical laboratory environment, especially in genetic testing, is keeping current within a rapidly evolving field. A couple decades ago the average cytogenetics laboratory was a small hospital-based practice. Today, a major portion of testing is performed in large reference laboratories. Needless to say, the approach a manager takes in running a small cytogenetics laboratory that is part of a pathology department in a hospital will differ drastically from the manager running a large reference laboratory. Likewise, a shift in the sample mix toward an increased volume of oncological samples, and the introduction of genomic microarray assays, will also affect a manager's focus. Balancing both the needs of internal staff with the overall vision of the institution, while still remaining profitable, are all key elements to a successful operation. This may sound simple, but in practice, change is not always predictable, as demonstrated by the 1990s. After decades of operational profitability as the result of low direct costs and favorable insurance return, clinical laboratories were suddenly faced with augmented regulatory requirements and expanded managed care. To circumvent revenue loss, commercial laboratories engaged in fierce competitive bidding for these managed care accounts. These changes became the driving forces behind many of the new trends in the healthcare industry.

The levels of managerial hierarchy will depend upon the size of the clinical laboratory and its operational affiliation. Most laboratories have a director, manager and/or supervisor, and staff, with each position utilizing its own set of expected skills, whether technical, human, or conceptual. In the end, however, an efficient laboratory requires the contribution of all its staff members; therefore, each staff member in the laboratory, whether running daily operations or researching a small project, will benefit from the managerial techniques provided in this chapter.

Laboratory supervisors and managers need not be financial experts, but they must understand the basic principles of financial planning. They must be able to read and utilize financial reports and assess the overall financial situation of their departments or institutions, an ability that is indispensable in this competitive market. They must also keep abreast of recent trends in laboratory management, including the changing nature of work, work force diversity, competition, collective bargaining, the Six Sigma approach in quality management, and the ethical and social responsibilities of business.

Managing a project or a team is not performed in a vacuum. Several internal and external factors, such as, the nature of the organization, size of the laboratory, location, and a whole set of laws and regulation, all influence the way we manage. Today, reimbursement for health care services is declining and operational costs are rising, making a favorable bottom line more and more difficult to achieve. The development of complex and esoteric tests also adds to the rising cost of laboratory testing and overall medical care expenses. With the restraints that society has placed on the budget for medical care and medical services, including laboratory test ordering, institutions are now being monitored and scrutinized for overuse and misuse.

Finding a moderate means to control utilization and ensure cost-effective, error-free laboratory services, while maintaining the highest quality of patient care, are the challenges facing today's managers. These inherent changes and continuous adjustments may pose a new set of challenges to overcome, but with a carefully designed strategic plan, and the constant communication and involvement of the staff, a positive change can and will be accomplished.

22.2 Management concepts and functions

In order to maintain efficiency and economic savings, laboratories must master a systematic approach that will address upcoming changes in healthcare initiatives. This is accomplished by four managerial disciplines: planning, organizing, directing, and monitoring. These fundamental functions of management have remained the same since their inception at the beginning of the twentieth century; however, the details required to accomplish each initiative depend upon industry-specific influences and challenges.

22.2.1 Planning (goal setting)

Good management starts with planning, that is, establishing an agenda and stating goals and objectives, in other words, the mission and purpose of the project. This function is commonly referred to as planning/budgeting to emphasize the importance of allocating the proper resources for the project. The planning process includes all of the following:

1. Taking into consideration the resources you have at hand and those you will be able to acquire;
2. Exploring all possible scenarios and evaluating all viable plans;
3. Assessing the current situation and evaluating the data available;
4. Selecting the plan that will most likely take you where you want to go;
5. Developing a realistic timetable for events to come; and
6. Having a contingency plan in case the proposed one does not work.

Another important part of the planning process that is often overlooked is soliciting opinions and feedback from those staff members who will be involved in the process. It is extremely critical to have your staff on board at all times. The more they feel they "own" the process, the greater chance for success. The best way to achieve this is to have an effective and dynamic two-way communication between manager and staff. This two-way communication should continue throughout the process.

22.2.2 Organizing/staffing needs

The next step for the manager is to select his/her team and make the proper job placements. It is essential that the staff be made aware of the plan, and that they have appropriate information and training. The most important role of the manager during this phase, therefore, is to coordinate and synchronize the efforts of all team members who are involved in executing the plan, and to provide all necessary tools and resources in a timely fashion. A visual illustration of the role and responsibility of each staff member is also helpful, along with a well-defined job description. Special consideration should be given to the logistic elements of a laboratory setting that might affect the outcome of the project, such as, sample flow within the department, interdepartmental interaction, and the department's layout.

22.2.3 Directing (leading)

During this phase of the process, managers are required to step up their efforts and become leaders. With well-defined goals and sound organization, the manager now must play the role of a coach, giving the team-signal to go ahead. Instructions to the team must be clear and specific, and team efforts must be synchronized in a way that will best accomplish the mission.

Effective communication and interpersonal skills is vital to the success of this stage. Each team member has a unique role to play and a distinctive talent to contribute to the overall success of the project. A good manager is able to communicate effectively with each staff member, while directing the efforts of all, in order to realize the goal at hand. After all, great managers are great leaders.

22.2.4 Monitoring (controlling)

One of the most crucial elements in management is monitoring progress. Shortcomings and problems no doubt will happen. A successful manager makes sure things are going according to the plan, and when they are not, will make the required adjustments. An ideal plan should provide the manager with options and alternative plans so that proper modifications can be made or efforts can be redirected and monitored.

During this controlling stage, it is also important to have effective monitoring tools that will provide both quantitative and qualitative indicators to follow the process. Some of these indices should cover staff performance and contribution. Remember that the staff “buy-in” is vital to the success of the process.

22.3 Personnel management

With the unfortunate decrease in cytogenetics training programs, and ultimately their graduate pool in the workplace, there is growing concern over the shortage of trained cytogenetics laboratory personnel, especially certified/licensed technologists. This situation will probably result in intense competition for qualified candidates. It is essential that laboratories sustain a pool of experienced and skilled staff to meet the high demands of analyzing oncological and highly complex cases, as well as perform advanced FISH and microarray assays. Appropriate levels of staffing with qualified personnel will enhance the laboratory’s ability to handle changes in specimen mix and the development of new assays at the same time, and still maintain a competitive turnaround time (TAT). To attract new talents, and retain and motivate the experienced pool of staff, management must provide an attractive working environment that includes training, continuing education and career development opportunities.

22.3.1 Staff level assessment

Remaining competitive is a challenge that faces all managers, but bringing in new technology also has its drawbacks, because it requires extensive time and personnel training to complete the validation process. Managers must periodically ask, “Is our laboratory adequately staffed to get the job done according to quality standards?” Likewise, when a position opens, they must carefully assess whether the position can be eliminated, redefined, downgraded or upgraded. Most laboratories use some form of workload and productivity measurement to determine the proper level of staffing and the need for replacing or adding more staff. The assessment should include delineation of duties, skills, and education. Other factors, such as volume increase, specimen mix changes, and TAT improvements can also be used for the justification of additional staffing needs.

22.3.2 Job descriptions

The recruitment process starts with a well-defined job description. This document includes position title, position code or number, exempt/or nonexempt status, reporting relationship, job summary, job duties and responsibilities, educational level requirement, minimum qualifications, experiences and skills, certifications/licenses if required, and the effective date for that job description. These job descriptions should be updated periodically and as needed. Providing staff with a copy of their job descriptions during the hiring process, promotions, corrective actions, and the annual performance appraisal will also remind current employees of their job responsibilities and hold them accountable for their performance.

22.3.3 Selecting your team

Selecting the right people to be a part of the team is not an easy task, and yet it is the most critical factor to the success of the cytogenetics laboratory. Choosing the right team members will foster a cohesive, harmonious, and productive work force. Finding qualified candidates starts with a review of the job description for that particular position and then establishing the hiring criteria needed to meet those expectations. The manager is responsible for a clear vision of tasks and behaviors needed in order to be successful in that position.

Hiring the right person with appropriate education, specific technical skills, and experience is only one side of the equation; often, what is more difficult is identifying the personality traits that contribute to being a team player. Traits and qualities, such as reliability, initiative, consistency, efficiency, and a positive attitude towards the job and people, are subjective and thus difficult to quantify. While there is no set formula to select the perfect employee, there are effective behavioral-based interviewing skills that can help provide a more objective assessment of the candidate.

Behavioral questions are those that solicit a situation, action taken, and the outcome. An example is to ask the candidate about a time that he or she had to deal with a stressful situation or a quality problem and how it was handled. There are several tools and training materials available that might be useful in enhancing the interview. Background, work history, and reference checks can also assist in making the right decision. It is important to follow the law and your institution’s written policy that prevents discrimination in the workplace and provides guidance in appropriate hiring practices.

A second method is the group or panel interview, when two or more individuals interview a candidate at one time. This approach provides a stronger candidate assessment and hiring decision; however, juggling time commitments and schedules can become a challenge when more than one interviewer is involved. When hiring at the supervisor level or above, however,

it is strongly advised to use a panel interview that consists of an appropriate mix of individuals. For example, the committee should remain small, but it should include key representatives of the department, such as the director, manager, supervisor and/or group lead/senior technologist. This type of cohesiveness will create a sense of ownership within the work environment, and will also offer training opportunities for individuals who wish to gain interviewing skills.

As mentioned earlier, developing effective interviewing skills is critical in hiring, retaining, and building a strong work team. The amount of preparation required prior to the interview depends on a manager's style and comfort level within the process. **Structured interviews** are when questions are prepared in advance; **unstructured interviews** are purely ad hoc, without questions prepared; and **semi-structured formats** incorporate a combination of both structured and unstructured. This latter approach tends to work well for most interviews.

There are many different ways to ask a question, with some more effective than others. The **leading question** can be used to direct a discussion towards a specific topic, but it may provide minimal information about the interviewer unless it is followed up with more targeted questioning by the interviewer. Leading questions like "Are you a quality-minded person?" which solicit subjective self-evaluation of an abstract quality should be avoided.

Direct questions require a short or straightforward response, usually with yes or no. These questions also do not provide much information, for example, "Are you a licensed supervisor?"

Open-ended questions are more difficult to create, but will provide a greater insight into the interviewee's character. An example for a behavior-based/open-ended question is: "Think of a situation where you completed an assignment with a team. Tell us about some of the challenges you faced." By leading with a more general situation or characteristic, the interviewer not only gets an answer, but will also have a chance to observe the candidate's style, skills, and level of details presented in the response. As a follow up question, you can ask the candidate to elaborate on how s/he handled or resolved these challenges.

"**Probing**" **questions** are often used as follow-up questions; therefore, they are contingent upon the interviewer also being an excellent listener. For example, to explore how a person can handle conflict in the workplace, the interviewer may begin with an open-ended question like, "Everyone at some point has worked with a co-worker that they did not get along with. Tell me about a time that you were not getting along with a co-worker." Upon hearing a specific detail, the interviewer might follow-up with probing questions like: "How did you handle the situation?" or "What was the result?" or if not satisfied with the resolution, "How do you think it could have been handled (or avoided)?"

Finally, **hypothetical questions** are often used to evaluate how a candidate can think spontaneously. They are mainly problem-solving questions that require the candidate to use training and previous experience to answer the question. For example: "If you were hired, how would you identify the major roles and responsibilities of your new position?" Candidates should be given time to formulate their answers. They also should be allowed to ask any questions for further clarifications.

22.3.4 Training

The laboratory should establish a documented training system for all different sections of the Cytogenetics department. The manager will then develop or oversee the formation of a **training plan** for each job assignment, which must include all preanalytic, analytic, and post-analytic procedures. In addition, the manager will make sure that revisions of the training plan are warranted, and that any new procedure will be properly documented.

Now that the new hire is on board, management will ensure that proper training procedures are in place. Evaluating the training of new employees allows the trainer to assess the new hire's ability to perform the job's responsibilities accurately and according to written standard operating procedures. By direct observation, a qualified trainer will ensure and document that all learning objectives are met and that the trainee is capable of performing procedures correctly. The trainer will also determine and document areas of re-training, if needed. A signed training record by a qualified trainer, the new employee, and the supervisor should be dated, filed and retained according to regulatory retention period guidelines. Staff training is required at all levels to ensure quality and consistency within the cytogenetics laboratory.

22.3.5 Competency

Competency is defined as the determination of an individual's capability to perform set expectations, to do a job accurately and safely, and to recognize and solve problems without needing assistance. Competency assessment is an objective evaluation that assures that staff continues to perform their assignments accurately and according to established standards. It is the responsibility of the laboratory manager to ensure that policies and procedures are established for monitoring individuals who conduct the different phases of testing, to assure that they are competent to process specimens, perform test procedures, and to report test results promptly and proficiently, and whenever necessary, to identify needs for remedial training or continuing education to improve skills.

Competency assessments must be performed semiannually during the first year of employment, and on an annual basis for all employees. The assessment should follow CLIA '88 (Clinical Laboratory Improvement Amendments of 1988) regulatory requirements.

22.3.6 Performance evaluation and appraisals

Performance appraisals are an excellent communication tool, which allows the manager/supervisor, as well as employee, to provide constructive feedback on work performance. The manager is responsible for developing an effective appraisal system that should include a formal (written) segment once or twice a year, as well as informal and ongoing feedback (verbal) throughout the year. This system is part of a planned periodic management function to evaluate the employee's performance, competency, behavior on the job, skills, and progress towards completing goals and objectives. It also allows for outlining needs for improvements, additional training, and future plans. It is a key tool for salary increases, promotions, and career planning.

The frequency of the appraisal process is up to management. Some laboratories have established a mid-year performance evaluation, as well as the end of the year performance review process. These managers want the employees to be appraised of their performance so it does not come as a surprise at the end of the year. Also, this gives an opportunity for the staff to correct areas of development if needed. Mid-year performance can be short and specific.

Performance evaluations are most effective when they are a two-way process. Allowing the employee the opportunity to provide feedback on his/her performance in a form of self-appraisal, joined with the supervisor's performance evaluation, is a powerful communication opportunity. Both parties are able to express their opinions, and the manager is able to learn what the other party has observed. Furthermore, the supervisor and employee meet to discuss, agree, and finalize the performance appraisal. This step is critical as it provides the staff ownership and accountability for their job performance and future goals.

The annual performance evaluation can be scheduled at a set time of the year or at the anniversary of the employee's hire date. Most large laboratories opt to conduct the appraisals during the same time-period every year, to allow for consistency and timely delivery to all staff. In addition, an effective program should also include management's commitment to follow through performance throughout the year (e.g., monitor quality and productivity for each staff member).

Successful managers should establish a fair, objective, specific, and measurable evaluation guideline for all to follow. This guideline should be clear, concise, and aligned with job expectations, such as quality, productivity, and specific plans for improvements. Some laboratories will use a weighing system to evaluate and grade different job duties and functions, which can lead to a fair and consistent evaluation, as well.

22.3.7 Staff motivation and retention

Attrition can be costly in terms of lost productivity, knowledge, and experience, not to mention the financial cost of recruiting, hiring, and training replacements. There is no magical formula for retaining staff; it takes competent managers who actively listen to their employees' concerns and act upon those issues that are within their control. General tools that may boost morale and job satisfaction are:

- hold frequent informative meetings,
- empower staff members to make decisions and be accountable,
- encourage teamwork,
- offer instant recognition, and
- address problems and concerns as they arise.

Some laboratories conduct annual employee satisfaction surveys, which will highlight the areas that are working well and identify improvement opportunities. The manager's role is to share the results of such surveys with each employee and design an action plan to work and improve the areas of concern.

Not all employees will leave because of work-related dissatisfaction. Some reasons may be personal (thus beyond management control), for example, going back to school, spouse job relocation, family reasons; others may be work-related (e.g., within management control), such as job dissatisfaction, workload and conditions, schedule, salary and compensation, career development, etc. Establishing exit interviews with departing staff will help understand the reasons behind their departure and also potentially identify areas that could be improved.

Effective managers will listen to their employees and be sensitive to their needs, as well as be mindful of workplace diversity and general morale. Options like raising pay scales, enhancing health benefits, offering continuing educational

opportunities, and providing career advancement, are universal; some may be more accessible to change than others, but acknowledging and exploring these options demonstrates to the employee that management has heard their concerns and has acted on them. Whether outcomes are successful or not, most employees will feel a sense of accomplishment that action had been taken on their behalf. The manager's role in recognizing, rewarding, motivating, and celebrating big accomplishments and small victories is critical to the success of the department and in building an energetic, committed, and enthusiastic workforce.

22.3.8 Policy manual

It is usually the responsibility of the Human Resource department to provide supervisors and managers with a policy manual. This manual includes all pertaining policies, such as recruiting, compensation, benefits, and payroll. The Policy Manual should also include policies regarding attendance, overtime, leave of absence, corrective counseling/disciplinary action, and the exit process, among other employee relations' issues. It is the manager's obligation to remain current on these issues and to apply all policies in a fair and consistent fashion. The manager's role in sharing and communicating pertinent policies with staff is important and will provide for a fair and equitable workforce, as well as a disciplined work environment.

22.4 Quality management and control

It is the manager's ultimate responsibility to make sure that his/her department is complying with all regulations and, more importantly, that accurate and timely results are being reported at all times. Laboratory management should set up and sustain policies and procedures for a comprehensive quality assurance and quality control plan. The quality assurance plan should be clearly defined, documented and designed to address the entire process, including pre-analytical, analytical, and post-analytical aspects of testing. The plan should also meet or exceed the CLIA '88 guidelines, along with the other regulatory and accrediting agencies [1]. Attention should be given to problem resolution by identifying opportunities for improvement and establishing any corrective actions, if needed. The goal of a well-run laboratory is to provide the highest quality of patient care by constantly seeking ways for improvement.

The debate regarding which phase of testing is more important, pre-analytical, analytical or post-analytical, is counter-productive. Running the most accurate assay on the wrong sample does not help the patient; neither does reporting the wrong result due to clerical error. The manager should establish and implement a system to insure proper patient identification and preparation. The plan should address specimen collection, specimen identification, preservation, and transportation. Special attention should be given to the requisition form in use and handling of verbal orders (**pre-analytic**). A program should be in place to ensure that all staff members are trained and competent to perform their duties. Other policies regarding safety procedures, handling of complaints, PT (proficiency testing), QC (quality control), and calibration ought to be in place, as well (**analytic**). The manager is responsible for the establishment of a system that will produce accurate and timely results, including appropriate reference ranges/cut-off values and critical values (**post-analytic**).

Quality assurance regulations are evolving constantly and are placing an increased demand on the manager's time. Depending on the laboratory's size and level of staffing, it might be advantageous to designate a qualified staff member as the Quality Assurance Officer on a full or part-time basis. This designee would perform some or most of the quality assurance responsibilities, and would report directly to the manager.

22.4.1 Technology assessment and implementation

Continuous improvement and innovation in laboratory operations is a high priority in the Cytogenetics laboratory today, so that tests that provide the most recent advances can be offered. The manager, therefore, must continuously investigate new technologies, equipment, and automation that are available in the marketplace, and with the director, evaluate the feasibility of integrating these new technologies in the laboratory. This demand for state-of-the-art testing is often associated with acquiring advanced automation tools, which will allow the laboratory to remain competitive, and still operate efficiently and with cost savings. At the same time, these developments will enhance workplace pride. Careful evaluation and planning will ensure a timely delivery of new products and procedures.

Developing an implementation plan should begin a year before a test's projected implementation, first by identifying any new equipment and procedures that would improve the test process, followed with devising timelines and responsibilities to ensure accountability and timely implementation. Larger laboratories dedicate experienced research and development (R&D) staff to handle such projects. The R&D staff will focus on testing and properly documenting the validation of new operating

procedures and equipment that meet the standards of CLIA '88 and other regulatory guidelines. This approach is becoming more and more important, especially for those laboratories that are seeking approval from state or other regulatory agencies.

For many institutions, the project summary, which includes the selection of new technology, is reviewed by a voting committee. In order to aid this committee in making a collective decision, this summary should address the impact on patient care, cost analysis, return on investment, feasibility study, competitive gains, benefit and risk assessments, and additional resources required (labor, IT support/interface). If approved, the traditional procedure for capital budgeting and purchasing of new or previously owned equipment should be followed.

Extra precaution should be taken when considering used equipment. It is equally important when purchasing new or previously owned equipment to evaluate the vendor's standing in the marketplace and to poll current users of the same equipment. It is also imperative to look very carefully into the terms of purchasing, service contracts, and availability of local vendor technical support. Some vendors allow the laboratory to test costly new equipment or automation platforms for an agreed period of time on a trial basis, which can help the laboratory make a more informed decision before purchasing.

Once the validation process for a new test or equipment is successfully completed, the results and standard operating procedures (SOP) are reviewed and approved by the Director. R&D staff, in turn, will properly train operational personnel on using new assays and equipment; will work hand in hand to assure a smooth and successful transition to operations; and will remain available for technical consultation, as needed. In some laboratory settings, the staff may serve dual functions – R&D and operations. Nevertheless, it is advisable that the process follows similar steps.

After the equipment and its standard operating procedure(s) have been transferred to operations, the hand-off process from R&D to operations is completed with appropriate signatures and approvals. The manager will then be responsible for documenting the training and competency of the newly trained staff. A quality monitor of the new assay and/or equipment performance should be implemented and documented.

22.4.2 Financial management

Terms and tools

Asset: Any possession owned by an individual or a business that has commercial or exchange value, such as, properties or claims against others.

Audit: An inspection and examination of a business' accounting records and procedures performed by trained professionals, who will verify the accuracy and completeness of the financial records. It could be internally conducted by a staff member of the organization or independently by an outside professional.

Amortization: In general terms, the repayment of a loan by periodic payments. In business, it generally refers to the process of spreading the cost of an intangible asset over the expected useful life of the asset. It helps spread the cost of capital expenses over a specific period of time.

Balance sheet: An itemized statement that details the total assets and liabilities of a business in order to depict its net worth at a specified date. It is also called the statement of financial condition.

Capital: Money and/or property invested in a firm and are used to conduct business.

Capital expenditure (CAPEX): The amount of money used during a particular period of time to acquire or improve long-term assets, such as, equipment or property.

Depreciate: Distributing the purchase cost of an asset over its depreciable life, as long as it meets IRS standards. In other words, it is the amount of expense charged against earnings (write off) each year of an asset's useful life, as long as the property is deemed acceptable for depreciation by IRS standards [2].

Equity: Ownership or percentage of ownership in a business or item(s) of value.

Expense: The amount of assets or services used during a period of time.

Financial plan: A blueprint relating to the financial future of a firm.

Financial analysis: Analysis of a company's financial statement, often by financial analysts.

Liability: Financial obligations that arise during the course of business operations, for example, a loan, expense, or any other form of claim that must be paid at a specific time to satisfy the contractual terms of such an obligation. It is recorded on the right side of the balance sheet.

Net income: Business total earnings, reflecting revenues, minus the costs of doing business. In other words, it is the difference between business revenue and its expenses. It is also known as net profit.

Revenue: The total amount of assets or money received by a business for goods or services provided during a certain time period.

Income statement: Statement of business revenue and expenses for a specific period of time. The difference between total revenue and total expense is the business net income. It is also known as a profit and loss statement.

Financial statement: A report that quantitatively describes the financial well-being of a business. It includes the income statement, the balance sheet and often the cash flow statement. The statement is compiled on a quarterly and annual basis.

Fixed cost: A cost that is set or fixed for a specific period of time and for a given level of operation.

Variable cost: A cost that is directly related to and comparable with the level of operation or volume of products produced.

Direct costs: Costs that can be directly identified with a particular product, function or service. Direct costs include labor and materials, such as salaries, other employee fringe benefits, supplies and equipment purchased. Direct costs may be fixed or variable.

Indirect costs: Those costs that are not classified as direct. Indirect costs represent the expenses of doing business that are not readily identified with a particular activity or with the manufacture of a product, but are necessary for the general operation of the company.

Operating margin ratio: The difference between the revenues received from providing a service and the expenses needed to support the operation.

22.4.3 Cost per test

The qualifications and number of personnel needed to adequately staff a clinical laboratory, coupled with the amount of work expected from each worker in a specified time period, all influence the cost per test for that specific assay. All clinical laboratories, including cytogenetics, need to be precise and at the same time practical in their pricing practices, if they are to remain viable in this increasingly competitive market. Pricing and cost analysis are thus important laboratory management tools. Several methods can be used to calculate the cost per test in the laboratory, some being more complicated than others. Software programs are also available for this purpose, but you should carefully review the cost accounting methods that are applicable to your institution before purchasing a specific program.

Costs are divided into four different categories - direct, indirect, variable, and fixed (see 22.4.2, Terms and tools for clarification). To ensure an appropriate cost per test, all expenses must be captured, including pre-analytical, analytical, and post-analytical components.

Direct costs are those items that are readily measurable. Included in this group are salaries with fringe benefits, costs for reagents, consumable supplies, capital for testing equipment, and equipment maintenance expenses.

Indirect costs (or overhead) can be more difficult to quantify than direct costs. Indirect costs include administrative oversight, logistics (couriers and specimen transportation), facilities (real estate, building maintenance, utilities and furnishings), quality assurance, marketing, sales, malpractice, legal expenses and information technology. Indirect costs also vary depending on the practice setting, such as, hospitals versus independent laboratories. Allocation of indirect costs to the laboratory can be accomplished by various methods, one being to allocate the cost to different departments according to their size or number of staff employed.

Variable costs are affected by specimen volume and market variation for suppliers' costs.

Fixed costs, however, are not affected by change in volume.

Price is regularly guided by what the marketplace will accept or tolerate, and ought to be above the cost for that particular test. To determine a competitive price for a laboratory test, it is recommended that cost analysis be performed on a biannual basis or whenever a new test or methodology is introduced. For most cytogenetics laboratories, the manager's involvement in this process is to determine the direct cost, such as the cost of labor and reagents used, including controls and repeat testing, along with the amount of time spent performing the assay by nonlicensed personnel, a licensed technologist, and the laboratory director.

22.4.4 Productivity and workload

Cytogenetic testing is still predominately a manual process and thus labor-intensive, in spite of the successful introduction of automation in recent years. In addition, a wide range of variable factors can negatively impact workflow and productivity, for example, adverse sample conditions, poor preparation quality, complex analytical rearrangements, and extra analytical time for mosaic cases. To complicate matters even further, staff member responsibilities vary widely between different laboratories. Some laboratories may have specialized technologists, such as full-time microscopists or a full-time tissue culturist; others may divide responsibilities among all staff members who will carry out different duties at different sections on a daily basis. Nevertheless, the number of qualified personnel needed to staff a clinical laboratory, coupled with the amount of work expected from each worker in a specified time period, will determine a laboratory's productivity rate, and in turn, will influence the cost per test for that specific assay.

Daily productivity (DP) for the entire laboratory is measured by dividing the daily-units-tested (DUT) volume by total FTE count.

$$DP = DUT \div FTE$$

The daily-units-tested volume is the total monthly-units-tested (MUT) volume divided by the total revenue days (TRD) for the month.

$$DUT = MUT \div TRD$$

Total FTE count includes nonexempt FTEs, overtime FTEs, and exempt FTEs. The laboratory productivity could also be expressed as the number of units per month. A cytogenetics laboratory with efficient operations will maintain daily productivity at a value of two or better.

Productivity expectations can be plainly stated as the number of cases analyzed per day per one FTE. Our experience has found, on average, that a dedicated and experienced technologist should be capable of analyzing a minimum of three oncology cases/day or five constitutional cases/day. It is reasonable to expect a higher productivity level if the Metaphase Finder automated platform is used.

Cytogenetics laboratories may also consider adopting a unit system by assigning a specific unit to each assay or sample type. For this strategy, management should take into account the nature of the sample-mix at their laboratory, the level of staff experience, and the simplicity of data collection for monitoring purposes. The unit will reflect the difficulty level and the time needed to finish the case for that specific assay or sample type, which will also include FISH testing, as well. These unit numbers will keep the technical staff informed of management's expectations per day. Any productivity assessment system must guarantee that the laboratory will comply with all quality standards and guidelines.

A routine chromosome study on blood could be assigned to one unit, high resolution studies 1.5 units, hematological study on a bone marrow 2–2.5 units, amniotic fluid 0.8 unit, CVS 1.2–2 units (will depend on whether a direct culture is involved) and so on. FISH assays could be divided into several categories, depending on the level of complexity and time needed to perform. FISH testing can be assigned a range of units between 0.8 for the simple ones to 4 for the complex cases or panels. A different and most probably higher level of expectations should be set if a computer-assisted, automated platform is used. Evaluating the workload for FISH assays should also consider the extra testing performed as follow-up or confirmation of abnormalities, among other factors. The proposed unit values are not rigid or preset; each laboratory should develop a unit system that fits their operation and reflects their vision. Other factors to consider include the cost per test, staff expertise, and case mix.

A unit system that fits the need of a large regional lab might not be the right one for a small-specialized laboratory. Some laboratories, for example, will assign a higher unit value for prenatal assays. The system should be flexible and also allow for adjustments as needed to factor in additional work that needs to be done to finalize the study, or any other technical difficulties.

22.5 Budget development and monitoring

22.5.1 Forecast

The cytogenetics laboratory requires a demanding management of human and financial resources. Each year, and usually with the help of the finance department, the laboratory goes through a planning process of forecast that provides financial targets based on anticipated (forecasted/targeted) volumes for the coming year. The manager's responsibility is to assess the needs for staffing and capital, according to the assumptions of the forecasted volume and specimen mix. This process includes a planning calendar, volume projection, revenue projection, and expense projection.

Planning calendar sets target and due dates of deliverables throughout the planning process. In most laboratories, the planning process takes place during the third quarter or the beginning of the fourth quarter of the year. Usually, the finance department prepares the planning assumptions and/or templates and sets due dates for the laboratory.

Volume projection forecasts the volume at the test code level or a summary of the estimated total volume on a monthly basis for the planning period required. Volume projection should include a baseline volume calculation that reflects current business levels for a period of time, as defined by the institute, for example, the actual volume for the last 3–6 months. In addition, any assumption of future volume growth or loss will be factored in. Projections of additional volume should take into consideration the organic growth of existing assays and the new tests and platforms that will be added during that period.

Revenue projection can be calculated at a test code level, or as a summary of the projected test volume revenue. This is based on the current pricing of the tests for the same period used for volume ($\text{Revenue} = \text{Volume} \times \text{Price}$). In addition, consideration should be given to any pricing increase assumptions in the coming year, such as, new test offerings or changes in volume mix.

Expense projection should include the current spending and capital expenditure plan for the new fiscal year. A baseline expense projection would be established, based on current business, using the same time period that was used for the volume. Also incorporated into the baseline would be any expenses due to projected changes in volume, supplies, staffing and equipment (capital and noncapital cost). The projection should address any additions or reductions that might be associated with specific initiatives, such as, automation or process improvement projects.

22.5.2 Budgeting

It is through budgeting that a laboratory turns its strategic plan into daily operations. A budget expresses planned revenues and expenses, as well as the volume of services and amounts of resources, for example, FTEs (full-time employees) and capital, which are required for realizing them. Through the budget process, the laboratory establishes priorities, allocates its resources, and controls its costs. It authorizes new programs and services and sets performance standards for existing ones. A budget thus serves as a tool and a benchmark for monitoring the performance of the organization (Trends and measures).

There are two key concepts that are integral to healthcare budgeting - full-time equivalents (FTEs) and units of service (UOSs). An FTE is one employee paid for a total of 2,080 hours/year ($8 \text{ hours/day} \times 5 \text{ days/week} \times 52 \text{ weeks/year}$). This is the standard labor measure. Labor is divided into productive time spent working on job-related activities, and no-productive time, which includes vacation days, holidays, and sick time. UOS is the measurement of work for a given area; it often represents the number of billed tests.

Three major types of budgets are developed during the budget process. The one that laboratory managers are most familiar with is the **operating budget**. Usually, the manager has direct responsibility for managing his/her cost center and operational expenses. The **capital budget** can be developed simultaneously with the operating budget. The manager will input the projected needs for the coming year, e.g., which equipment (new or replacements) should be purchased or repaired, which facility needs additional space, etc. Most organizations have a system in place to collect information on these needs on a departmental level and then arrange priorities centrally with respect to organizational goals and available funding.

The third type is the **cash budget**. This is fully prepared by the finance department and predicts the cash flow in and out of the organization and the resultant cash availability. While obscure to most, it is the most critical of the three budgets. In order to remain solvent and thus stay in business, the organization must carefully plan its cash reserves, cash disbursements, any borrowing needs, and its investment activities.

The budget process

Creating a budget entails a set of steps, some of which occur at the organizational level and some at the departmental cost center level. The steps include the following:

1. Establish organizational goals and objectives, for example, business growth/increase by 10%.
2. Review key environmental factors, such as regulatory issues, competition, technology, and the economy. This function is usually done at the organizational level, although the laboratory may have unique information about its particular niche, such as, marketing activities or new technology or methodologies.
3. Determine starting assumptions, for example, inflation, payment levels, and other key volumes. This function is usually performed at an organizational level.
4. Develop the statistical budget of each UOS in the laboratory. The laboratory manager will need to consider projections of the number of tests and forecasted volumes, using the information from steps 1–3.
5. Devise the revenue budget, based on the statistical volume's forecast, multiplied by the applicable charge per UOS; this results in the **gross revenue budget**. In most organizations, consideration of contractual allowances, write offs, and bad debt are factored in to estimate the **net revenue budget**.
6. Prepare the expense budget on the basis of data gathered in steps 1–4. Because labor often constitutes roughly 70% of an organization's budget, the expense budget is frequently broken down separately into a staffing budget and a nonpersonnel expense budget. The staffing budget takes into account the numbers and types of employees, pay rates, and FTEs required in each cost center.

7. Assuming the capital budget was produced in parallel with the previous steps, the manager will provide input on equipment needs. Once all department budgets are rolled into one organizational budget, a process of negotiation and revision between department manager and financial staff usually takes place. The operational income is then calculated.
8. Develop the cash budget; this is usually prepared by the finance department.
9. Continue negotiations and revisions across the organization.
10. Submit the budget to the organization's leaders for approval.

22.5.3 Capital expenditure

Capital expenditure (CapEx) is money spent to acquire/purchase physical assets, such as, a building or new equipment. CapEx also includes money spent in payment for lease commitments/arrangements that are associated with each physical asset. A CapEx proposal must be submitted for approval if the physical asset meets two criteria: it is projected to have a useful life equal to or greater than three years, and it has a unit cost in excess of a set amount of spending. Some companies, for example, set \$1,000 as the value at which a cost becomes a capital expense. Examples of assets that may require a CapEx proposal are the following:

- purchase, construction, renovation, expansion, leasing and disposition of real estate, buildings, machines, IT, and other tangible equipment;
- purchase of patents, technology, software, and other intangible assets;
- purchase or replacement of lab equipment;
- repairs to existing fixed assets.

Some organizations provide managers with a standard CapEx template. This template will provide a framework and guide for processing a CapEx request. The CapEx request should include the following information: amount of funds requested, project description, timing of the investment, alternatives, and risk assessment if the project were not to be approved. It might also include the economic/financial impact of the CapEx request, including the cost benefit analysis, and an assessment of the free cash flow that would be potentially generated by the project. A financial evaluation of alternatives should also be performed whenever possible.

The following capital budgeting tools/methods are always taken into consideration: weighted average cost of capital (WACC), net present value (NPV), internal rate of return (IRR), depreciation and payback period. These will be discussed later.

Weighted average cost of capital

The WACC in finance, expressed as a percentage, is the discount rate (time-value of money) used to convert expected future cash flow into present value. WACC is based on the cost of capital of the business, which reflects the impact of financing decisions. As a rule, future cash flows generated are discounted by WACC. The discounted cash flow approach (DCF) describes a method to value a project using the concepts of the time value of money. The DCF methods determine the present value of future cash flows by discounting them using the appropriate cost of capital. This is necessary because cash flows in different time periods cannot be directly compared.

WACC and interest rates should be evaluated on a regular basis. Planning and analysis will ensure a periodic update of the latest WACC and interest rates.

Net present value

NPV is a standard method for the financial assessment of long-term projects and is also used for capital budgeting. NPV compares the value of a dollar today to the value of the same dollar in the future, taking inflation and returns, among other factors, into account. Positive NPV implies that the return on the project is greater than the cost of capital; investing in such projects creates a value or profit. Negative NPV projects should be avoided unless there are compelling reasons to support the spending (reasons may include strategic importance, required infrastructure, etc.).

The NPV is obtained by discounting the cash flows for each year of its relevant, useful, lifetime period at a specified discount rate. For purposes of this guideline, the WACC is used as the discount rate. The NPV shows the discounted cash surplus or cash shortfall of the project and gives an indication of the value created or destroyed. Managers, however, should keep in mind that the amount of capital available for investment is usually limited.

Internal rate of return

Often used in capital budgeting, IRR (internal rate of return) is the discount rate at which the present value of all future cash flows from a particular project equals to zero. Generally speaking, the higher a project's internal rate of return, the more attractive the project is. Assuming all other factors are equal among the various projects, the project with the highest IRR would probably be considered the best to initiate first. IRR also represents the return on the CapEx project and gives an indication of the project's profitability. The IRR must be in excess of the company's cost of capital in order to create value.

Depreciation

Depreciation is associated with capital investment. It is the allocation of the cost to acquire an asset, which has a useful life of more than one year, into each year of the asset's life [2]. For example, if a piece of equipment costs \$100,000 and has a useful life of 10 years, after which it is worth nothing, an annual depreciation value of \$10,000/year would be recorded as an expense for each of the 10 years.

Payback period

Payback period is the length of time required to recover the cost of an investment, based on the project's projected after-tax free cash flow. It is not an indication of profitability, nor does it consider the time-value of money.

Inventory

The simple definition of inventory is the supply of reagents, media, and consumable goods on hand in the laboratory at the time. It appears as part of the current assets on the balance statement and contributes to the bottom-line of a company's performance.

A successful business will maintain a well-balanced inventory at all times, which means on-hand supplies that are adequate to carry out laboratory operations until the next order is received. Excess inventory may have a negative effect to the overall operation by tying up resources that might serve more useful purposes elsewhere; therefore, managing and keeping the right amount of supplies is becoming an important aspect of running any business, including the clinical laboratory. Some primary factors that may limit inventory storage include space availability, product shelf life, vendors' past performance, product reliability, and projected rate of usage.

A manager's involvement in inventory tracking will depend on the size and nature of the laboratory or respective institution. There are also generally accepted practices and standards, as well as, industry-specific policies and procedures, which govern the way an inventory is conducted. Most managers are involved to some degree in conducting an inventory on consumables, such as media and reagents. All managers, however, must promote and sanction these regular inventory maintenances. They also must be familiar with their institution's policies and practices in order to select the best staff members to conduct these counts, who in turn must be properly trained with regard to both the process and subsequent documentation. Having a dedicated person to perform the task will improve consistency. Some businesses apply the double-count approach, where two counters perform their duties independently to improve the accuracy of the count.

Some products carry their own unique set of inventory challenges. For example, the volume within FISH probe vials is usually small, making its measurement a tedious task; yet, these reagents represent some of the most costly and critical items for the cytogenetics laboratory. One alternative method that may eliminate having to measure the volume of each probe on hand, and yet still be able to document the current inventory for that reagent, is to maintain an accurate usage log for all FISH probes.

Numerous software programs can be implemented that will help an organization effectively manage its inventory – for example, some technologies have implemented barcoding, which has been shown to reduce errors. If you are not familiar with the business software, however, it can be a difficult task to undertake. Software selection and implementation is thus better left for the professionals. Ultimately, an effective inventory control system requires good communication between different sections within the laboratory and also between the laboratory and staff involved in purchasing.

22.5.4 Trends and measures

Having established a budget, management must regularly monitor the extent to which actual experience differs from the budget, called a **variance**. Monthly reports are used to communicate these actual versus budget performances throughout the organization, so that timely decisions can be made and corrective action, if necessary, can be taken. These reports contain detailed line items that are critical to the control functions of management.

Controllable variances can be resolved by management actions – for example, vendors may be substituted, contracts may be re-negotiated, or alternative methodologies may be pursued. Uncontrollable variances, such as factors that may lead to an increase in test volumes, may necessitate management to make changes to more discretionary expenditures in order to offset these uncontrollable factors.

Variance analysis commonly focuses on the company's actual results versus budgeted expectations on a line-by-line basis for each cost center. The job of the manager is to monitor and report/explain the variances to superiors, and to make any necessary changes. Three of the major factors creating a variance are volume, price, or quantity.

Volume variances result from a change in volume, either higher or lower. A flexible budget takes into account these variations.

Managers will compare projected versus actual and check any variances.

Price variances are due to changes in the price of a supply or service versus what was anticipated in the budget.

Quantity or efficiency variances represent differences in the amount of input (labor and/or supplies) that was used to produce each UOS, e.g., units/FTE and/or cost/test. Variations in labor are important, because labor costs constitute such a large proportion of expenses. Both labor costs and hours can also be subdivided into worked and paid categories. Laboratory productivity is thus defined as the UOS produced, divided by the laboratory's input that was required to produce them (in either hours or cost).

22.6 Conclusion

The role of the manager will continue to evolve in the coming years to accommodate the accelerating changes in the field. The laboratory manager will be facing many challenges in a healthcare environment that is constantly shaped by financial constraints, strict federal regulations, and a workforce that is more diverse and mobile. Unfortunately, the political, economic, and social volatility of our current environment characterizes healthcare in the 21st century. Funds and support for health services are uncertain. In addition, the economic future of healthcare is dependent on the following trends and concerns:

- an aging population that will increase demands of laboratory testing;
- an increasing federal deficit and concern about the future viability of Medicare;
- the expansion of costly oncology- and molecular-based testing and technologies;
- a healthcare insurance system unable to provide coverage and proper care for millions of Americans;
- the shortage of trained laboratory personnel, especially certified/licensed technologists.

Efforts put forth for the success of laboratories in the 21st century are dependent upon a strategic and systematic redesign of laboratory operations. First, laboratory management must differentiate their business from their competitors' by adding extra value for their products or services. They must be versed in the areas of cost discipline, economies of scale, automation, and favorable reimbursement of insurance companies. At the same time, the manager and staff must review the operation to eliminate steps with no added value or ones that are simply unnecessary. Process redesign involves the essential rethinking and redesign of business practices to achieve improvements in key performance measures, like quality indicators, process cycle times, process cost, and patient outcomes. Managers must develop a structured process to create an environment that embraces change, such as, expanding on-site test menus, reducing TAT, and acquiring the latest technologies and automation at all levels.

It is critical to the success of the manager to be able to predict the upcoming needs of the laboratory. The manager must, therefore, seek out new technology and automation opportunities and be prepared to take the necessary action. The management system of the future should be able to accurately predict customer demand and allocate resources by anticipating the needs for care rather than reacting to them.

References

1. Centers for Disease Control and Prevention. Clinical Laboratory Improvement Amendments (CLIA). <http://www.cdc.gov/clia/default.aspx>
2. U.S. Department of the Treasury – Internal Revenue Service. *How to Depreciate Property*. Publication 946, 2012. <http://www.irs.gov/pub/irs-pdf/p946.pdf>

Suggested reading

Thompson CB. *Interviewing Techniques for Managers*. NY: McGraw-Hill; 2002.

CHAPTER 23

Laboratory information system

Peining Li¹ and Richard Van Rheeden²

¹Clinical Cytogenetics Laboratory, Yale University School of Medicine, New Haven, CT, USA

²Clinical Cytogenetics Laboratory, University of Iowa Healthcare, Iowa City, IA, USA

23.1 Historical perspective

From 1960 to the 1980s, clinical cytogenetics laboratories mainly applied cell culture and differential staining techniques to recognize numerical and structural chromosomal abnormalities. All laboratories had a manual procedure to manage data collection and report writing. This procedure included, but was not limited to, inputting patient demographics into a log book, recording case processing and analytical results on a worksheet, and typing cytogenetic reports and index cards. This method of data management was time-consuming, error-prone, and difficult for data search and cross-reference. The necessity of using a computerized database to manage these information-rich records and high complexity tests in a cytogenetics laboratory was recognized by Loughman WD et al. in 1980 [1]. Since then, advances in computer technology, especially in hardware functions, software development and networking capacity, have enabled many progresses in laboratory information management. Consequently, three major changes have taken place in most cytogenetic laboratories: (1) a computer-assisted imaging system has become the current standard for karyotyping and fluorescence in situ hybridization (FISH) imaging (see Chapter 16, Fluorescence in situ hybridization (FISH)); (2) automated laboratory information systems (LIS) have replaced many manual procedures; and (3) the world wide web has provided a wealth of online reference resources and facilitated inter-lab communications (see Chapter 25, Online genetic resources and references).

Today, a full service cytogenetic laboratory processes multiple types of specimens and performs high complexity tests such as chromosome analysis, FISH, and possibly chromosomal microarray (CMA) by either array comparative genomic hybridization (aCGH) or single nucleotide polymorphism array (SNP array) for prenatal, postnatal, and cancer patients. In addition to the increasing amount of testing and the widening spectrum of diseases, increasingly stringent government regulations and higher standards of quality management for laboratory operations necessitate the use of an automated system for management of laboratory information. A specifically designed LIS for a cytogenetic laboratory can streamline data processing and result reporting, improve efficiency in managing quality control and quality assurance data, and insure accuracy in billing practices.

23.2 General description of LIS

23.2.1 LIS concept

The development of LIS or LIMS (laboratory information management system) has been a new specialty focusing on using computer software for data management of various laboratory operations [2, 3]. At the most basic level, an LIS is defined as the functions built into a class of software for a laboratory to manage patient data and issue results. A comprehensive LIS specifically designed for a cytogenetic laboratory may include the ability to collect and store data, analyze and report results, interface with lab instrumentation and outside clinical facilities, organize workflow, evaluate personnel performance, monitor quality assurance (QA) and quality improvement (QI) parameters, and ensure accurate billing practice. LIS applications exist on a variety of platforms such as dBase, Oracle, Meditech, and Microsoft Access in addition to proprietary systems. Although

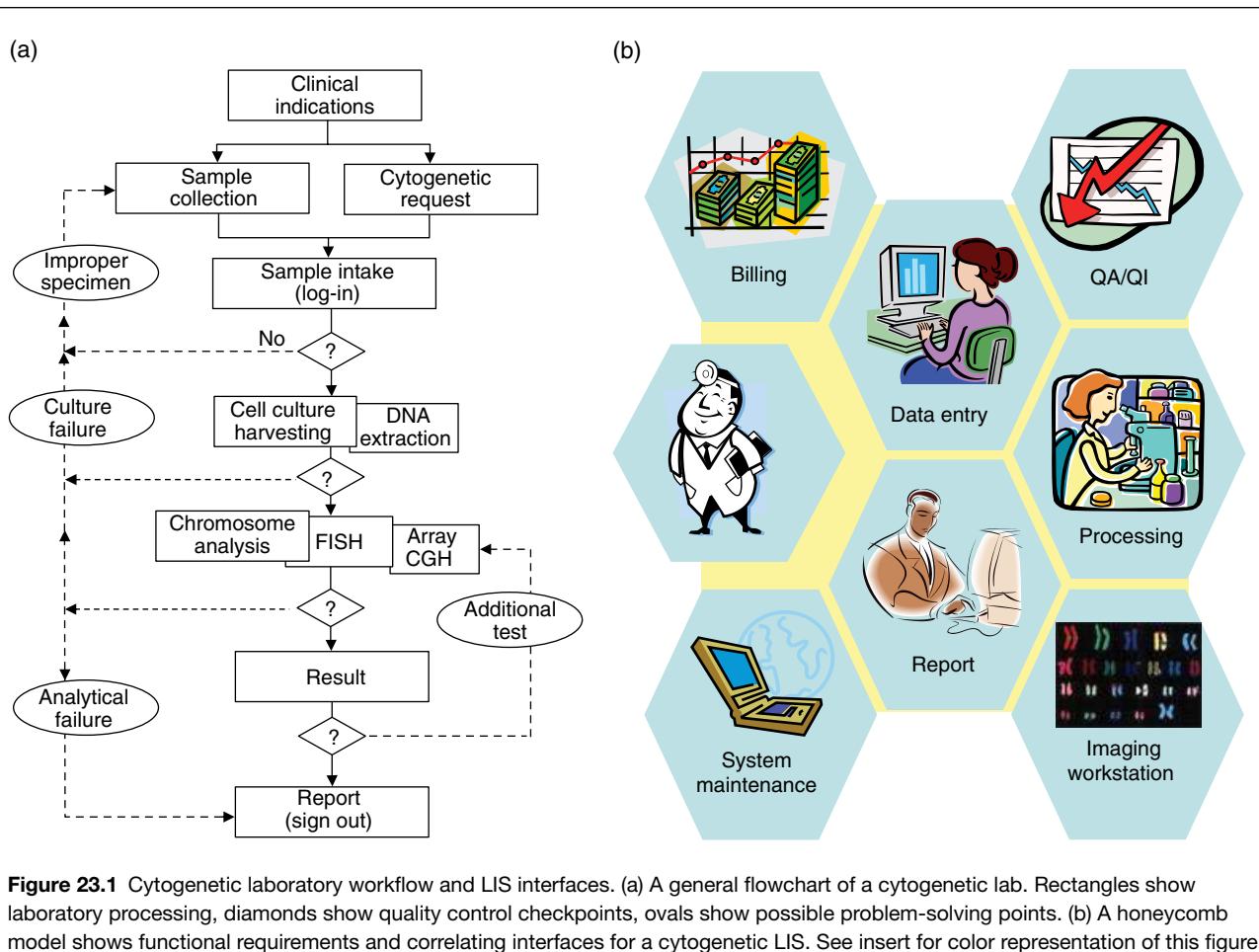
LIS have been widely used, for many cytogenetic laboratories a manual system using a simple notebook or logbook is still valuable as a direct daily reviewing method and a supplemental hardcopy backup.

23.2.2 Software architecture and hardware considerations

An LIS, consisting of specifically designed software and properly installed hardware and peripherals, could be customized to facilitate a wide variety of laboratory activities. An early version of a computerized database was designed to perform storage, search, and correlation tasks [1]. More recent systems have integrated functions to monitor laboratory performance, to review quality assurance and quality improvement parameters, and to ensure prompt billing processing [4–6]. For example, a report writing function may be designed to have an interface with a hospital information system for online reporting to physicians, to have a connection to internet reference resources, and to have a link to QA/QI for real time monitoring. The demands of a multi-task LIS require a software architecture consisting of several functional modules and multi-faceted interfaces. Figure 23.1 illustrates the workflow of a general cytogenetic laboratory and a honeycomb model for the functional requirements and correlating interfaces of a cytogenetics LIS.

Corresponding to a cytogenetic laboratory's routine operations, the LIS software architecture could be organized into four functional modules: (1) the data entry module for patient demographics and sample tracking; (2) the report module for analyzing results and writing reports; (3) the laboratory management module for evaluating personnel performance, monitoring QA/QI parameters, and ensuring prompt and accurate billing; and (4) the system maintenance module for updating software codes, file links, result templates, reference tables, user records, and other system functions.

The data entry module usually has user-defined fields for inputting demographic data, operational information, and financial/billing data. All specimens that come in to a laboratory will be assigned at least two unique identifiers such as a lab



case number and the patient name. The demographic data usually includes patient information, specimen type, date of service (collection and receiving time), clinical indications, and referring physicians. Operational information includes methods of specimen processing and the type of test that should be performed. Financial/billing data include insurance information, ICD-10 codes, CPT codes, and related test prices. Frequently encountered data such as specimen type, clinical indications, and referring physicians could be organized into reference tables and presented as a searchable list in a drop-down menu. To maintain data integrity, the searchable information should be “limited to list”, i.e., no free text entry allowed. Intra-lab networking between the LIS computer and major laboratory instruments such as karyotyping/FISH imaging workstations will allow instant transfer of demographic data to instruments. This will cut down the time for repeat entry and reduce the chance of typographical errors.

The report module is mainly designed for laboratory directors and administrators to analyze results and to generate reports. For the convenience and necessity of reviewing demographic data, this module should include the major portion of the data entry module. In addition, fields for summarizing test results; for entry of ISCN nomenclature [7], and for date and signature should be included. Most LIS will have a template table to store commonly used interpretations. The report content may be typed in manually or be imported from a drop-down menu linked to a list of templates. The report format should contain all elements required by the College of American Pathologists (CAP) and recommended by American College of Medical Genetics (ACMG) guidelines [8]. These elements include: name and address of testing laboratory, patient name, unique identifying number, patient date of birth, name of physician or authorized person ordering the test, specimen source, date specimen received in the laboratory, date of report, clinical indication(s) for the test, number of cells counted, analyzed, and karyotyped, band resolution (required only for constitutional cases), banding methods, comment on adequacy of specimen, and signature slot for a qualified cytogeneticist. It is also a CAP requirement that the original report should be retrievable when amendments were added. This could be done by storing the original report in an uneditable format such as.pdf or other noneditable file. Other functions such as previewing the final report, printing index cards or other documents retained in the laboratory, and online reporting to the institutional information system or referring physicians could also be included.

The laboratory management module provides methods to generate a variety of QA/QI and administrative reports. For example, QA/QI reports of abnormal cases, abnormal detection rate, average G-band resolution, and turn-around time (TAT) could be preprogrammed. Administrative reports include technician productivity reports, a billing form with proper ICD-10 and CPT codes, and tickler reports for prioritizing unfinished cases and monitoring unbilled cases. Additional data mining functions can also be incorporated to facilitate requests for specific abnormal results to be utilized in retrospective studies.

The system maintenance module is used to update the file links and to edit and add new content for all reference tables and report templates. The reference tables may include commonly used demographic fields and all-coded fields such as specimen types, clinical indications, referring physicians, ICD-10 codes, and CPT codes. Report templates could be organized by types of tests such as chromosome, FISH and CMA, and by types of specimens such as bone marrow, peripheral blood, amniotic fluid, etc. As a system security procedure, user activity report documenting the log-on and log-off time of each user could be considered.

A specifically designed LIS software requires properly installed and functionally matched hardware. Considering the rapid progress of computer hardware and peripherals, it is strongly recommended that the laboratory staff work together with the institutional computer facility manager to determine the optimal specifications of computer hardware, peripherals, operating system, and networking components. This is extremely important for laboratories that need to establish an intra-lab network from front desk data entry to back end reporting, and to link laboratory LIS to the institutional or hospital information system.

23.2.3 Validation and implementation

The objectives and functional requirements determine the criteria in selecting an LIS for a cytogenetics laboratory. To make sure the selected LIS could perform all designated functions in a smooth and user-friendly fashion, a series of procedures to validate the LIS before implementation is recommended. The first step is to evaluate the software design. All user-defined data fields in the database table, reference tables, and template tables should be organized properly. Interfaces between functional modules should be user-friendly and easy to operate. The second step is to check the system integration, especially the data assignment, file links, report format, and programmed functions. For examples, input data should be assigned to related tables and shown on the reports, all drop-down lists should be linked to designated tables, and all QA/QI parameters should be generated from test cases. The third step is an in-situ trial to test system performance. This could be done in parallel with current data management system in at least two to three weeks of real case workflow. Any deficiencies noted in software design, function integration, and system performance should be documented and corrected by the programmers. System validation is a time-consuming and detail-oriented process, but serves as an initial quality assurance step to ensure a functional LIS.

The implementation of a validated LIS into clinical services requires the development of a standard operating protocol (SOP) and proper training of laboratory users. The SOP provides step-by-step instructions for using the system, and it should be included in the laboratory manual. An LIS downtime procedure should be included in the SOP. Training for users should begin upon LIS installation. A user-friendly system usually minimizes training efforts. A data entry user may need minimal training, and high-end users such as the laboratory director and administrators may need extensive training on basic computer and networking skills. As with most software, LIS exists in a life cycle from software design, validation, implementation, to modification or updating. As part of laboratory quality assurance, continuous periodic operational testing of LIS is recommended. Careful documentation of any problems recognized from the operational testing will be helpful in software modification and system updating.

23.2.4 Compliance and security

A cytogenetic LIS must comply with all relevant regulatory agency guidelines. In the United States these include CAP, CLIA (Clinical Laboratory Improvement Amendments), HIPAA (Health Insurance Portability & Accountability Act), ACMG guidelines and some state agencies. If the system (meaning the server stack and archive backup) is housed within the lab, then appropriate space and other physical considerations such as fire extinguishers and wire routing must be properly implemented. All procedures and support protocols must be documented and reviewed annually. Any hardware modifications must be documented and followed up with testing to ensure that the modified system still performs properly. A quick check of the General Laboratory Checklist from the CAP will serve as a useful guide in taking the proper steps to ensure compliance.

Remember, whether or not the system is purchased or developed, the confidentiality and security of the LIS should be assured through at least a two-level procedure. The data entry module is available to designated laboratory staff by issuing a first-level logon password. Entry to the other modules requires second-level passwords only available to the lab director, administrator, and programmers. Each user should have a unique password which needs to be renewed in a 3–6-month interval. User activity such as log-on and log-off time may be documented. All data should be backed up manually in the lab and automatically in the institute's information system on a daily basis.

23.3 LIS in cytogenetics laboratories

A basic understanding of software architecture, hardware requirements, as well as validation and implementation procedures will be of great help for the decision-making process in selecting an LIS for the cytogenetics laboratory. A recent CAP survey [9] recommended a 7Fs model for LIS evaluation. In general, the laboratory seeking to implement an LIS should get answers to seven questions: What “function” does the system do? What are the “features” for performing the functions? How well does the system “fit” with the existing or planned operations? How do the users “feel” about it, is it easy to learn and easy to use? What kind of “follow-up” service and support will be available? What is the real “financial” cost with regard to purchasing and maintenance? What are the “future” prospects for this system?

Many cytogenetic laboratories use a “home-made” LIS or a modified LIS from the institutional or hospital information system. Some imaging workstations, such as those from Metasystem and Applied Spectral Imaging, have built-in LIS functions for case log-in, processing monitoring, and report writing in their imaging software. Some commercial LIS have been developed for clinical cytogenetics. For example, the Genial Passport by GenialGenetics (http://www.genialgenetics.com/genetic_database_software.htm) and the Softgene by Softcomputer (<http://www.softcomputer.com/products/softgene.php>) all have a cytogenetic module in their genetics software package.

Although cytogenetic LISs share similar principles and function requirements, the software architecture, hardware equipment, and system integration may be different from laboratory to laboratory. In the following section, we will present the CytoGen system from Washington University and summarize features of other LIS. The readers, however, should not assume that the following discussion exhausts the list of LIS available to the genetics laboratory. The intention of this review is to foster an understanding for the capabilities and advantages of an LIS and to introduce specific features that could improve workflow processes. The omission of a software package is not indicative of a product's inferiority, but is more likely due to unfamiliarity with the package at the time this chapter was being prepared.

23.3.1 The CytoGen system from Washington University in St. Louis

CytoGen is a cytogenetic LIS developed and maintained by the Pediatric Computer Facility (PCF) in the Department of Pediatrics at Washington University in St. Louis. It was designed to the specifications of clinical cytogenetic operations. The system was validated and implemented in 1998 and replaced a system that was not Y2K compliant. The data from the previous system, which goes back to 1994, was imported into the new data repository.

What the user actually sees on the screen (the front end) is written in a 4GL language called Uniface. The 4GL language is a high-level computer language (such as IBM's Structured Query Language or SQL) that allows nonprogrammer users to write programs that query databases and create custom reports. This is a model-driven component-based development environment. The data repository (where the data is stored) is Microsoft SQL Server. The data repository was migrated from Oracle to SQL Server in 2005. The table structure, screen interfaces, reports and codes are strictly under the control of the PCF programming staff. No part of this is accessible to the end user. Any modifications made to the program are discussed by the Laboratory Director and the Lead Programmer in PCF. All changes are tested and then documented when completed. Previous versions of the program are stored on a secure server, in the event there is a need to back out a change.

The security that is used to access the application and the data is HIPAA compliant. The application resides on a secure server behind a firewall. In order to gain access to the application, the user must have a password-protected account that has access to the Citrix (application) server and be set up as a Uniface user and a cytogenetics user. In addition, they must be given access to the SQL (data) server. A final layer of protection is that the username must be set up in a table within the Uniface application. In order to gain access, validation must occur in all of these places. All access and changes are written into SQL server logs with all pertinent data being captured. When an employee terminates, it is the responsibility of the Department Administrator to insure a User Account Script is filled out to terminate all privileges. This is transferred electronically to PCF User Support, where the account is inactivated. They in turn transfer the information to the programming staff to remove the terminated employee from the Cytogenetics Security table.

The application and the data reside on two different servers. All of these files are backed up on a nightly basis. The backups are then stored at an offsite location.

Computer screen snapshots of CytoGen interfaces are shown in Figure 23.2. In general, the CytoGen application has the following specifications.

Case log-in and specimen processing (Figure 23.2a)

- New case numbers are generated sequentially and designated with a prefix equal to the calendar year. Patient demographics, operational information, and billing data are logged-in and stored in tables or text files at the discretion of the lab technologists.
- The patient's previous tests are available to insure that no duplication of testing is performed and previously detected abnormalities are documented.
- Culture types are displayed based on the specimen. Worksheets are printed out.
- Specimen labels can be generated from the system.
- A text file with patient and test data can be transferred to a secured folder that is shared by the users of the application. This file is then read in by the CytoVision® system.

Report writing (Figure 23.2b)

- Test-specific screens that accommodate unique diagnosis and observations are provided by the report module.
- Cell counts for chromosome and FISH analysis can be stored and reported in a table format.
- Multiple diagnoses and associated results for a patient can be included in the report.
- Report can be generated from a dropdown list of interpreting templates or by directly type in the result.
- FISH and chromosome reports can be either printed or faxed directly from the system.
- Supplemental and revised reports can be created for each of the above.
- A read-only version is available which can allow physicians to query results based on a patient's name or date of birth.

Laboratory management (Figure 23.2c)

- A billing application and a QA module are included.
- Multiple diagnostic ICD codes and billing CPT codes can be typed in or selected from a dropdown menu.
- Over a dozen standardized queries including abnormal cases, band resolution, turn-around time, processed studies, case output by tech, cancelled studies, and unacceptable specimens can be run by preprogrammed applications.

System maintenance

- The system is maintained and updated by PCF programmers.

(a)

(b)

(c)

Figure 23.2 Screen snapshots of CytoGen LIS. (a) Case log-in module shows fields for patient information, referring physicians, clinical indications, specimen types, etc. (b) Reporting module shows fields for diagnosis and test results (left panel) and linked templates (right panel). (c) Laboratory management module shows billing functions (left panel) and links to QA/QC queries (right panel).

Table 23.1 An overview of various cytogenetics LIS

Cytogenetic laboratory	University of Iowa	Yale University	Orchard Harvest
LIS system	Cyto-LIS	CytoAccess (version 1.02)	Cytogenetics LIS
Software			
Programming		Visual Basic	Proprietary
Operating system	Windows XP	Window XP	Windows XP
Database/tool used	Microsoft Access 2001	Microsoft Access	4th Dimension
Hardware			
Computer unit	Mainframe Server	University Server	PC based server
Intra-lab links	Dell PC	Dell Optiplex GX620	PC
Outreach connections	N/A	Link to LabMed LIS for online reporting	Available
Installation			
System validation	Ongoing development of lab database	Three rounds of testing/modifying	On site during installation
Implementation	In use	In use	One week training
Follow up service	Continuous support from the computing facility	Revisions with changes in links and report format	90–120 days for final install Annual updates
Features			
Data entry	Drop down menus for patients demographics and other test data	Drop-down menus for case log-in, print out function for chromosome, FISH, aCGH worksheet, patient history, etc.	Drop down menus. Printed forms.
Result reporting	Drop-down menu to list of results, also ability to enter free text.	Drop-down menu linked to lists of karyotype, FISH, aCGH templates, functions for printing index card, fax cover, online reporting, etc.	Automated data download and fax on signature of final.
QC/QA parameters	TAT, abnormality rate, band level, failure rate, tech productivity	G-band resolution, TAT, abnormal detection rate, failure cases, etc.	As determined by lab.
Billing tracking	NA	Billing form, list of unbilled cases.	Full tracking of accounts.
System security and maintenance	Password protected Maintained by hospital facility	Password needed. Easy to update drop-down menus and report templates, programmer needed for change coding and links.	Password required. Onsite trained admin, yearly updates. System maintained by host institution (hardware, backups).
Estimated annual cost for hardware/software	System maintained by hospital facility.	Estimated annual budget \$3700 ~ \$4800.	Depends on complexity of the installation.

23.3.2 Examples of other cytogenetic LIS systems

Table 23.1 summarizes features of LISs for cytogenetic laboratories of The University of Iowa Healthcare, Yale University [6], and Orchard Harvest Group.

23.4 Trends for the future LIS

As demonstrated in the above examples, current cytogenetic LIS are multi-task tools for data gathering, report writing, QA/QI checking, and billing tracking. An ideal comprehensive LIS would be seamless, performing the integration of patient, specimen and results with minimal input. The patient would be registered by assigning a clinic ID number or a unique

barcode. Every test would be ordered electronically following a scan of the patient number. All of the required information would be entered into the electronic order before the specimen could be drawn. The lab would scan the sample tube upon arrival and check the patient name and test information. The LIS would initiate QC checks, label printing, and result validations for each specimen. The date and time the culture was set up would be available when the culture technologist scanned the pre-printed culture labels as the specimen went into the incubator. Daily harvest lists would be generated and could be cleared only after the harvest label was scanned. When the slide label is scanned following banding, the case is assigned to a technologist. The patient data would be scanned from the slide into the imaging system and all the demographic information would appear on the karyotype, FISH and CMA results. The supervisor or director would enter the results and the final report would be entered into the patient electronic file immediately after the director signed the final case report. Images and statistical data of karyotype, FISH and CMA could be attached into the report. Outside clients would have a fax sent with the electronic signature the instant the report was available. All turn-around time, abnormal results and tech caseload would be instantly available for review. In this world, the only data entered manually by the lab would be the report and the final signature. This fully automated LIS is achievable in the near future.

The LIS in a cytogenetic laboratory is usually an integral unit of a large institutional or hospital information system. Starting 2004, the Department of Health and Human Service (HHS) has supported development of national health information technology to accelerate patient access to electronic medical records (EMRs) (<http://www.hhs.gov/healthit/>). This measure could potentially transform the current reporting system and require cytogenetic laboratory's LIS to communicate with health care providers and the physician community. To transfer cytogenetic results into the EMR system, rules and standards dictating the format and type of cytogenetic results flowing through the EMR need to be established.

The application of bioinformatic approaches to extract knowledge from collections of data to form or to test new scientific hypotheses has gained increasing interest in the field of cytogenetics. The use of a cytogenetic database for data mining is of clinical and scientific significance. Several related issues such as multi-center cytogenetic data management [10]; a database system for CGH analysis [11]; and integration of cytomorphology, cytogenetics, molecular, and expression profiling [12] have been explored. With the completion of the Human Genome Project, there is an increasing demand for interrelating chromosome findings with genomic mapping and gene functions. Fortunately, the International System for Cytogenetic Nomenclature (ISCN, 2009) [7] has been the standard in the documentation of chromosome abnormalities, which makes it possible to translate an ISCN karyotype into a genomic "karyotype." Recently, a web-based data analysis system with an ISCN converter [13], and an online database and bioinformatic toolbox for cancer cytogenetics using an ISCN reformatter [14] have been reported. Many cytogenetics laboratories have developed their own database or LIS. A common challenge for all LIS users is to have a standardized automation system for classifying disease category, recording patient history per HIPAA regulations, integrating other test results, and converting ISCN descriptions into genomic "karyotypes". These systems will enable large-scale data mining using multi-lab or multi-center databases. Eventually, novel data-driven knowledge will be applied to improve diagnostic accuracy and therapeutic efficacy for the patients.

Acknowledgments

We thank Shashikant Kulkarni and Stephen Lamp of the Cytogenetic Laboratory at Department of Pediatrics, School of Medicine, Washington University in St. Louis for their contribution on the CytoGen system (23.3.1). We also thank Joan Samulson, Audrey Meusel, Susan Hemingway, and Bixia Xiang of Yale Cytogenetic Laboratory for their effort in developing the CytoAccess System.

References

1. Loughman WD, Mosher DC, Epstein CJ. GENFILES: a computerized medical genetics information network. III. Chromo: the cytogenetics database. *Am J Med Genet.* 1980; 7: 267–278.
2. Hinton MD. *Laboratory Information Management System: Development and Implementation for a Quality Assurance Laboratory*. New York: Marcel Dekker, 1994.
3. Paszko C, Turner E. *Laboratory Information Management Systems*, 2nd ed. New York: Marcel Dekker, 2002.
4. Asare AL, Klimczak JC, Caldwell CW. A cytogenetics information system for automating quality assurance and quality improvement documentation in the clinical laboratory. *Proc AMIA Annu Fall Symp* 1997: 840.
5. Asare AL, Caldwell CW. An information system for improving clinical laboratory outcomes. *Proc AMIA Symp* 2000: 22–26.

6. Xiang BX, Hemingway SS, Qumsiyeh M, Li PN. CytoAccess, a relational laboratory information management system for a clinical cytogenetics laboratory. *J Assoc Genet Technol* 2006; 32: 168–170.
7. Shaffer LG, Slovak ML, Campbell LJ, eds. *ISCN (2009): An International System for Human Cytogenetic Nomenclature*. Basel: S. Karger, 2009.
8. American College of Medical Genetics *Standards and Guidelines for Clinical Genetics Laboratories* (2006 Edition). http://www.acmg.net/Pages/ACMG_Activities/stds-2002/stdsmenu-n.htm
9. College of American Pathologists 2005 Survey of LISs (archived, http://www.cap.org//apps/docs/cap_today/survey/11_05_24-56_LISsurvey.pdf)
10. Shuster JJ, Carroll AJ, Look TA, Pui CH, Land VJ, Jackson J, Pullen DJ, Steuber CP, Crist WM. Management of cytogenetic data in multi-center leukemia trials. *Comput Methods Programs Biomed* 1993; 40: 269–277.
11. Berrar D, Dubitzky W, Solinas-Toldo S, Bulashevskaya S, Granzow M, Conrad C, Kalla J, Lichter P, Eils R. A database system for comparative genomic hybridization analysis. *IEEE Eng Med Biol* 2001; 75–83.
12. Dugas M, Schoch C, Schnittger S, Kohlmann A, Kern W, Haferlach T, Uberla K. Impact of integrating clinical and genetics information. *In Silico Biol* 2002; 2: 34.
13. Hiller B, Bradtke J, Balz H, Rieder H. CyDAS: a cytogenetic data analysis system. *Bioinformatics* 2005; 21: 1282–1283.
14. Baudis M. Online database and bioinformatics toolbox to support data mining in cancer cytogenetics. *Biotechniques* 2006; 40: 269–272.

CHAPTER 24

Animal cytogenetics

Marlys L. Houck¹, Teri L. Lear² and Suellen J. Charter¹

¹San Diego Zoo Institute for Conservation Research, San Diego, CA, USA

²(deceased)* formerly, Department of Veterinary Science, University of Kentucky, Lexington, KY, USA

24.1 Introduction

Interest in animal cytogenetics began in the late 1800s with Walther Flemmings' first recorded observation of animal chromosomes [1]. Early analyses were limited by the quantity of samples and quality of preparations, but as techniques improved, geneticists began to document the diploid number and morphology of hundreds of species of vertebrates and invertebrates. As in human cytogenetics, the use of colchicine and hypotonic treatment enabled better visibility of the individual chromosomes and more accurate counts. Several comprehensive accounts detailing the history of and techniques used for animal cytogenetics have been published [2–5].

Prior to advances in banding methodologies, most animal karyotypes were derived from conventional Giemsa-stained chromosomes. This process facilitated description of the size and centromere location of the chromosome, and in many cases, also identified the sex chromosomes. The *Atlas of Mammalian Chromosomes* [6] is an excellent compilation of the nonbanded karyogram samples of over 500 species, and in most cases includes a representative male and female of each species presented. Early sources of both vertebrate and invertebrate chromosomes include a compilation by Makino [7] and the *Mammalian Chromosomes Newsletter* (1960–1986). More recently, a collection of G-banded karyograms from approximately 850 mammalian species was published [8]. Information on chromosome numbers and karyotypes in mammals can also be obtained from online resources such as Benirschke's Comparative Placentation (<http://placentation.ucsd.edu/homefs.html>).

To date, the known diploid numbers for mammals range from $2n=6$ in female Indian muntjacs (*Muntiacus muntjak*) [9] to $2n=102$ in *Tymanoctomys barrerae*, the red viscacha rat [10]. There are still many species such as the rare Javan rhinoceros (*Rhinoceros sondaicus*), tree pangolin (*Manis tricuspis*), mountain nyala (*Tragelaphus buxtoni*), mountain gorilla (*Gorilla gorilla beringei*), Cat Ba langur (*Trachypithecus p. poliocephalus*), and giant muntjac (*Megamuntiacus vuquangensis*) for which the diploid number has not yet been determined. Animal cytogenetic research has focused primarily on mammalian species, reflecting the broader interest in this group, although there are several smaller compilations of avian, reptilian, and amphibian karyotypes [11–13]. Banded karyotype standards have been established for the most prevalent domestic species and common laboratory species, but are lacking for most exotics.

* Editors' Note: Teri L. Lear, PhD, October 11, 1951 to May 14, 2016. Dr. Teri Lear left behind a rich legacy within the equine community. Her expertise on the horse's karyotype inspired numerous studies on equine genetics. She shared her passion for the field with both master's and doctoral students she trained, and she frequently lectured to practitioners and horse owners about how to recognize equine genetic disorders. One of her most exciting accomplishments was the role she played as one of the leaders in the Horse Genome Project, which sequenced the horse genome. This may be Teri's last chapter, but it is only the beginning of the legacy she has left us. We only wish she could have seen this chapter. She would have been proud.

24.2 Domestic animal fertility

24.2.1 Cattle

Cytogenetic analysis has proven valuable for detection of fertility problems among domestic/agricultural species. For example, the standard cattle (*Bos taurus*) karyotype was established as $2n=60$, consisting entirely of acrocentric chromosomes [14–17]. Reduced fecundity in $2n=59$ individuals with a 1;29 Robertsonian translocation has been reported and inadvertently selected for because the cows carrying the fusion element exhibited superior milk production [18–21]. Identifying the translocation carriers can have positive implications on the cattle breeding industry.

24.2.2 Horses

Normal domestic horses (*Equus caballus*) have a diploid chromosome number of 64. The karyogram shown in Figure 24.1 is of a normal female horse (mare), 64,XX, and was derived from “Twilight,” the individual from the Baker Institute, Cornell University, whose DNA was sequenced for the Horse Genome Sequencing Project <http://www.genome.gov/20519480>. Most equine samples submitted for chromosome analysis come from breeding stallions and mares with fertility problems. The most common abnormalities are those affecting mares, such as: X monosomy, often referred to as XO syndrome or equine Turner syndrome; sex chromosome mosaicism; and sex reversal syndrome. Other chromosome abnormalities affecting mare fertility, such as partial sex chromosome deletions, like Xp deletion and sex chromosome-autosome translocations, are rare [22].

Autosomal abnormalities, such as deletions, duplications, or rearrangements, appear to be less common than sex-chromosome abnormalities. However, some autosomal rearrangements may have been missed, because most samples are only Giemsa stained to rule out sex chromosome abnormalities and are not treated with trypsin prior to staining (GTG), which would bring out the identifying bands within each chromosome. Autosomal trisomy causes mild to severe congenital abnormalities in horses and has been identified for the smaller acrocentric chromosomes 23, 26, 27, 28, 30, and 31 [23, 24, 25, 22, 24, 26, respectively]. Autosomal translocations cause a reduction in equine fertility due to early embryonic loss, usually prior to day 45 of pregnancy.

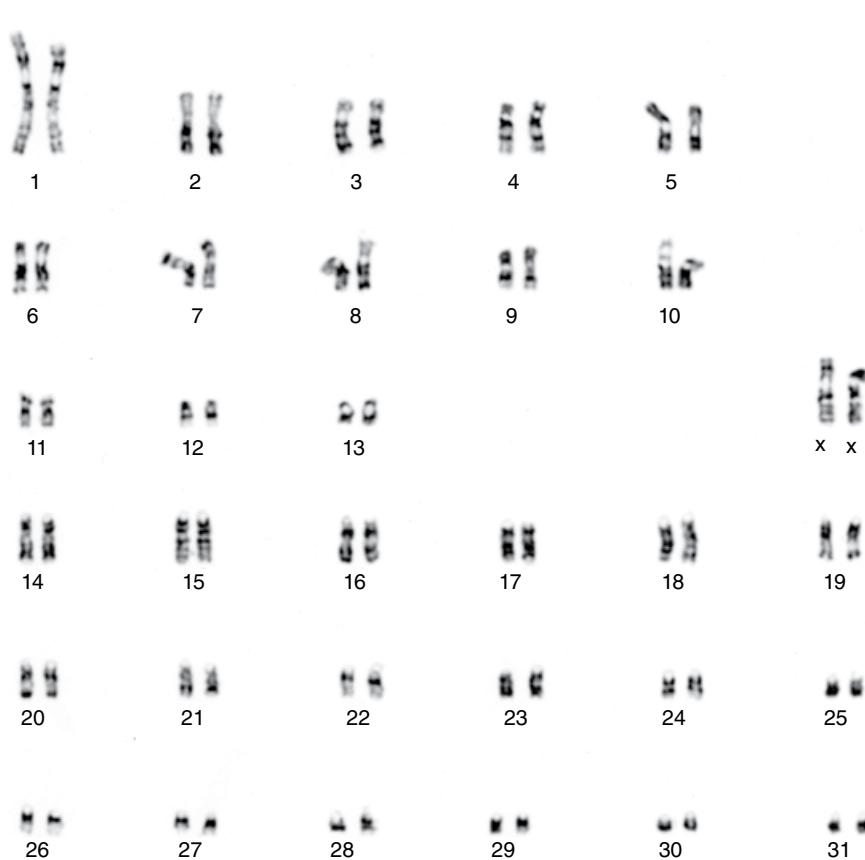


Figure 24.1 Domestic Horse. GTG-banded karyogram of a normal female horse, *Equus caballus*, 64,XX, derived from “Twilight,” the individual sequenced for the Horse Genome Sequencing Project.

A translocation in a stallion was found to cause early embryonic loss in the mares carrying his foals [27]. To date, only two other autosomal translocations, both in mares, have been published [28,29], both causing early embryonic loss. However, recent studies have identified three more autosomal translocations, suggesting that translocations may be more common in the equine population than previously believed [30].

24.3 Captive management

Cytogenetic research was first proposed as an essential management tool for zoo populations in 1969 [31]. Since that time, a large number of mammalian taxa commonly bred in zoological gardens have been karyotyped, leading to the discovery of many unexpected chromosome complements [32–35]. Knowledge of the diploid number, chromosome morphology, and sex chromosome structure for a species, whether domestic or exotic, provides insight regarding relationships among diverse taxa and can reveal reproductive isolation among phenotypically similar groups of animals.

24.3.1 Species integrity

Cryptic species are populations that are reproductively isolated, but not easily distinguishable morphologically [36]; they may have cryptic chromosomal variation where geographic chromosome differences are present, but not noticeable by phenotype [35]. Establishing the standard karyotype for a species, especially those obtained from documented locations in the wild, provides a reference for future confirmation of sex and species once animals have been bred in captivity. Although hybridization does occur naturally in the wild, there is a higher incidence among captive animals resulting from the intermingling of populations that would not normally encounter one another due to geographical barriers or behavioral traits. Owl monkeys (*Aotus* species), for example, were brought to the USA from different parts of South America for use as experimental animals in laboratories and unknowingly allowed to hybridize. Later, comprehensive cytogenetic analyses by Ma and others documented diploid chromosome numbers from $2n=46$ to $2n=58$ among *Aotus* species and determined that hybridization within these groups likely contributes to their poor reproductive performance in captivity (reviewed in [37]).

Inadvertent hybridization of species with such chromosome variation often results in sterile offspring. A landmark case of hybridization detected through karyotyping was documented in Kirk's dik-diks (*Madoqua kirkii*), small African antelopes. Wildborn individuals were successfully bred in captivity for several years, but fertility among this genus declined dramatically in subsequent generations. Although the phenotypes of the North American zoo population derived from Kenya and Tanzania were indistinguishable, cytogenetic analyses revealed two distinct karyotypes as a result of six chromosomal rearrangements including inversions, additions or deletions, a translocation, and a tandem fusion [38,39]. Inadvertent hybridization of the two genetically distinct groups resulted in fertile females, but sterile males. After analysis of all individuals was complete, the animals were paired accordingly, hybrids were removed from the breeding pool, and the captive population rebounded.

Another early finding was a pericentric inversion that distinguishes Bornean (*Pongo pygmaeus*) from Sumatran (*P. abelii*) orangutans [40]. These two species were often misidentified based on physical appearance, especially females and young animals. Routine chromosome analysis provided a reliable method of species differentiation in wildborn and first generation individuals. This observation has been of great importance in the captive management of orangutans by preventing unwanted hybridization.

More recently, karyotype analyses in the Nile crocodile (*Crocodylus niloticus*) contributed to a study that revealed a cryptic evolutionary lineage within this species that was not discernable by phenotype. Although the eastern ($2n=32$) and western ($2n=34$) lineages of this group were found to be distinct in chromosome number and morphology, they were previously not recognized as distinct groups which could lead to loss of crocodilian biodiversity [41].

Failure to ascertain karyotype information prior to captive propagation can lead to a situation years later where it is no longer possible to determine the original chromosome complement of a species. For example, by the time cytogenetic analyses were undertaken for Soemmerring's gazelles (*Nanger soemmeringii*) after many years of captive breeding in zoological parks, the diploid numbers of the 28 animals available for study ranged from 34 to 39. The wide variety of chromosome numbers resulted from combinations of three Robertsonian translocations, and two sex to autosome translocations [42] (Figure 24.2). It is presumed that the difficulty in establishing self-sustaining populations of Soemmerring's gazelles is related to the chromosome complements of the founding stock. Unfortunately, there is no clear record of the diploid number for this species before captive propagation.

24.3.2 Chromosome abnormalities

In other cases, routine karyotyping can reveal unexpected findings, such as Klinefelter syndrome in a phenotypically normal Siberian tiger, *Panthera tigris altaica* [43] (Figure 24.3), Turner syndrome in a sable antelope (*Hippotragus n. niger*) with stunted growth [44], or XY chromosomes in a "female" Siberian lynx, *Lynx L. wrangeli* (unpublished SD Zoo), leading to further examination and discovery that the animal was actually a unilateral cryptorchid male. In each of these cases, karyotyping led to the unexpected findings that the animals were sterile and therefore should not be included in captive breeding programs.

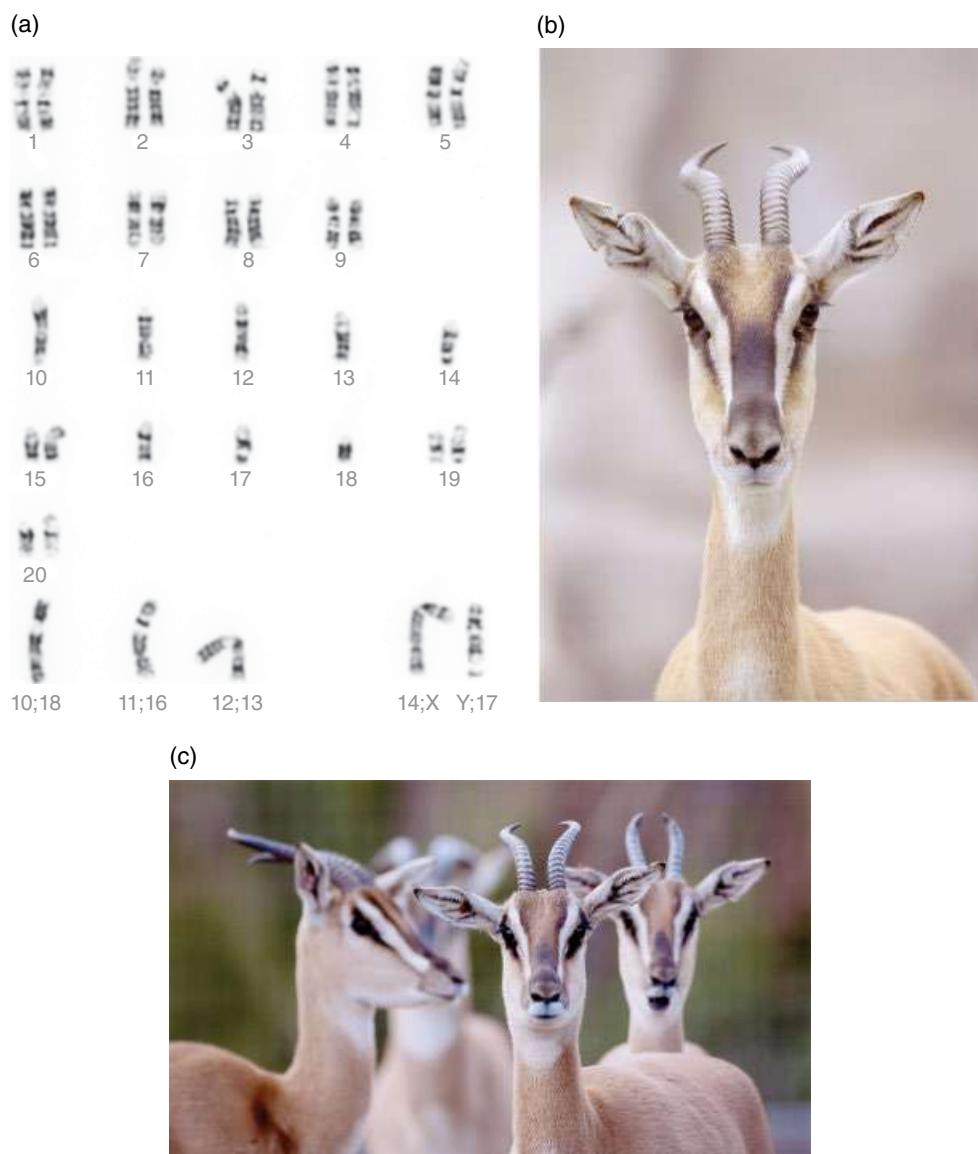


Figure 24.2 Soemmerring's Gazelle. (a) Soemmerring's gazelle, *Nanger soemmerringii*, GTG-banded karyogram with three autosomal Robertsonian translocations and two sex to autosome translocations, $2n=37,XY$. Diploid numbers in Soemmerring's gazelle have been reported between $2n=34\sim39$. (b,c) Photos courtesy of Zoological Society of San Diego. See insert for color representation of this figure.

As awareness of cytogenetics as a tool for veterinary medicine and captive management increases, more samples are provided for analysis. Early assumptions that the more prevalent abnormalities found in humans should also occur in other species, especially primates, have been confirmed by such findings as the Klinefelter and Turner syndrome examples above, trisomy X in a black rhinoceros (*Diceros bicornis*) [45], trisomy 22 in an orangutan [46], trisomy 13 in a crab-eating macaque (*Macaca fascicularis*) [47], trisomy 15 in a gorilla's product of conception (unpublished Woodland Park Zoo), trisomy 18 in a bonobo (Figure 24.4), and a de novo deletion of the distal portion of 4q in a growth-retarded gorilla [48] (Figure 24.5). It is expected that this list will continue to grow as more individuals are studied.

24.3.3 Studbooks

Pertinent karyotype information is sometimes tracked in history records called studbooks, which include pedigrees and listings of the various locations where members of a captive population have been held. Examples include the species distinction in orangutans and karyotype distinction in dik-diks mentioned above. Studbooks are primarily used for monitoring and

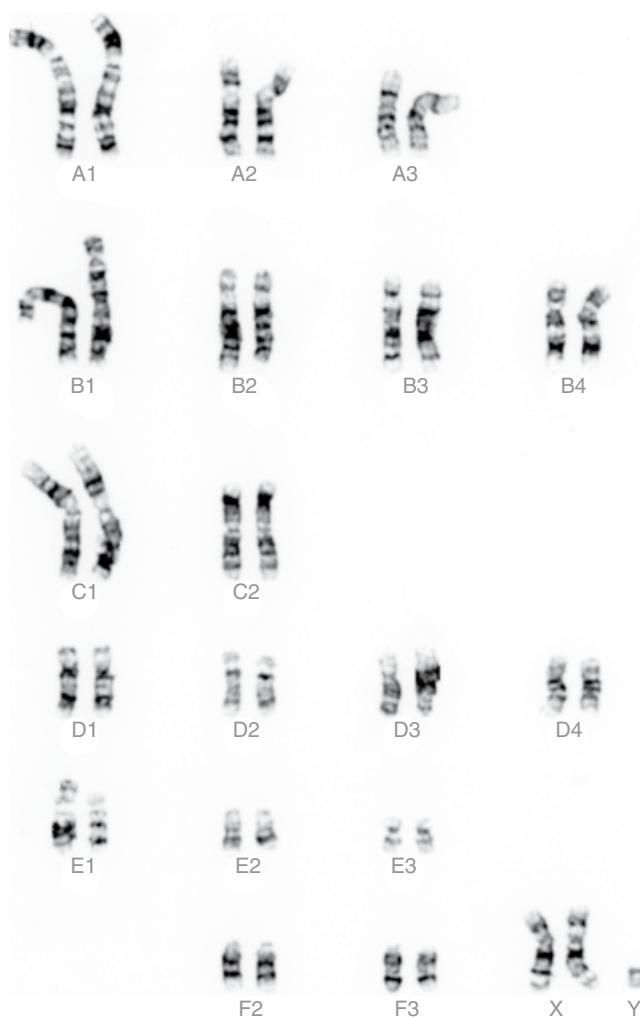


Figure 24.3 Siberian tiger karyogram with Klinefelter syndrome. GTG-banded karyogram of a male Siberian tiger, *Panthera tigris altaica*, with Klinefelter syndrome, $2n=39$, XYY. Numbering follows the standardized feline karyotype. Source: Suedmeyer, J Zoo Wildl Med 2003. Reproduced with permission of Elsevier.

managing populations in zoos and aquariums and are a valuable source of information for making breeding decisions to retain genetic variation and avoid inbreeding or hybridizing.

24.4 Wildlife conservation

Cytogenetic analyses of exotic species populations is especially valuable for wildlife conservation and management and can be applied to questions involving species identification, hybridization, phylogeny, gene evolution, and the structure and variation of a population. Detecting numerical and structural chromosomal variation between and within populations is an important tool in assessing reproductive fitness. The rapid decline of animals in the wild due to loss of habitat, poaching, disease, and predation by introduced species has mandated the need for captive propagation of many species. The entire population of some endangered species, such as the California condor (*Gymnogyps californianus*), Arabian oryx (*Oryx leucoryx*), and Przewalski's horse (*Equus przewalskii*), once existed only in captivity before carefully managed breeding programs made it possible for them to be reintroduced into their native habitat. The success of these breeding programs is dependent in part on knowledge of their karyotypes.

Early efforts to establish breeding pairs among monomorphic California condors would not have been possible without sex determination by karyotyping. Currently, knowledge of the chicken genome and condor karyotype is being used to map the condor genome and identify the chondrodystrophy gene responsible for early embryonic death in the highly endangered condor [49].

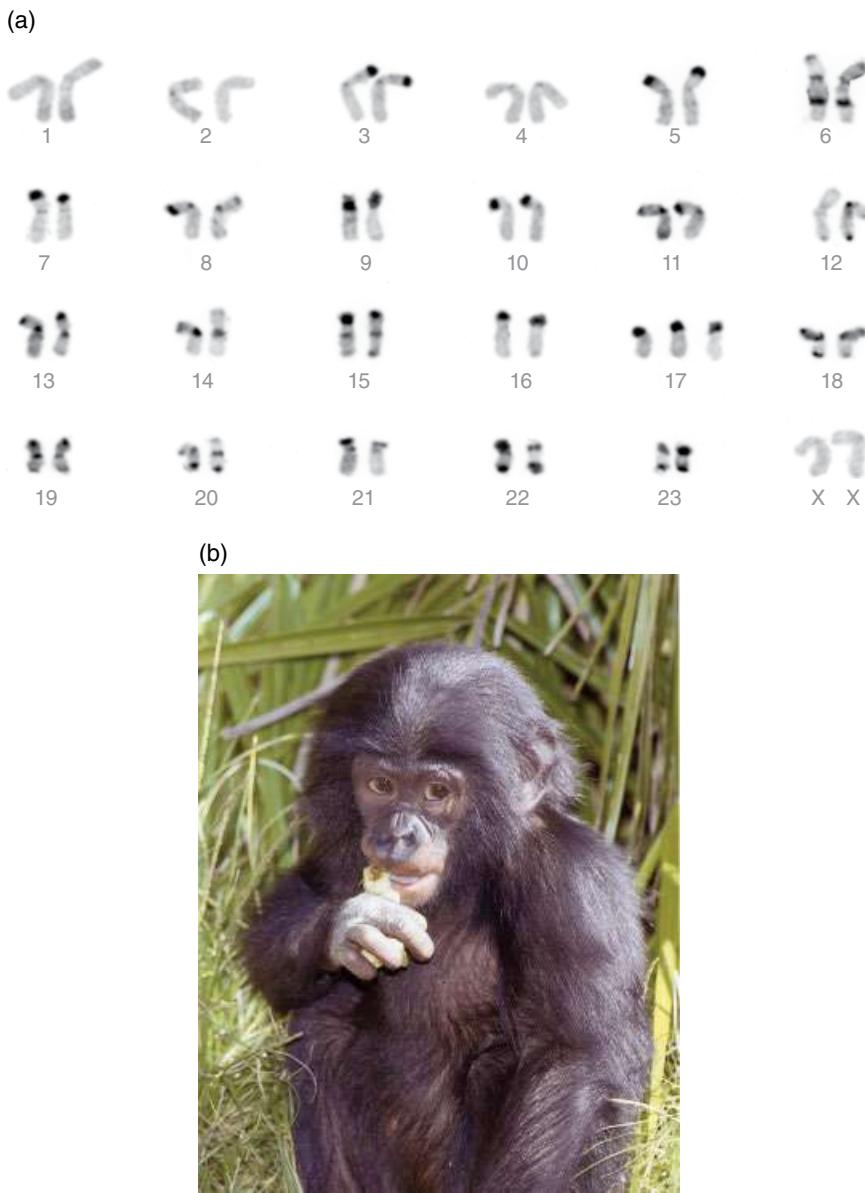


Figure 24.4 Bonobo. (a) C-banded karyogram of a bonobo, *Pan paniscus*, with a trisomy, $2n=49, XX,+17$. Chromosome 17 in the bonobo is homologous to chromosome 18 in humans. (b) Photograph of a bonobo, *Pan paniscus*. Photos courtesy of Zoological Society of San Diego. See insert for color representation of this figure.

24.5 General sample collection considerations

24.5.1 Regulations

Collection of samples from exotic (nondomestic) animals should be opportunistic and follow all applicable regulations. Institutional Animal Care and Use Committees (IACUC) are required to oversee and evaluate all aspects of animal care and use in institutions that have animals for research or instructional purposes. IACUC approval must be granted prior to sample collection. Import and export of animal samples (including DNA products) from other countries must meet all restrictions and requirements mandated by the United States Fish and Wildlife Service (USFWS) and the Convention on International Trade of Endangered Species (CITES) <http://www.cites.org/>.

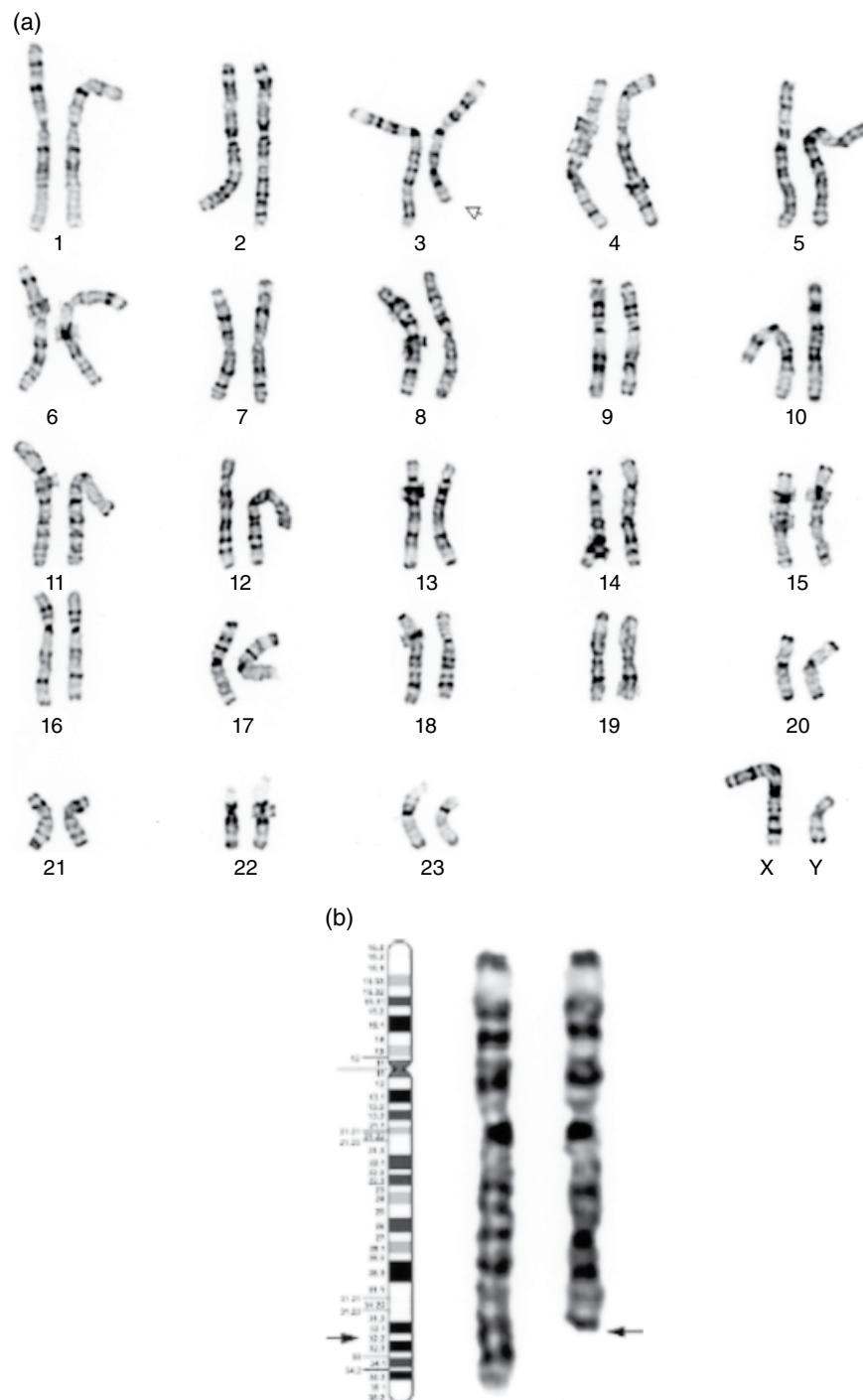


Figure 24.5 Gorilla karyogram showing a deletion. (a) GTG-banded karyogram of a gorilla, *Gorilla gorilla*, with a deletion, $2n=48, \text{XY}, \text{del}(3)(\text{q}22)$. (b) Chromosome 3 deletion compared with human chromosome 4 idiogram. (Lear et al., *Cytogenet Cell Genet* 2001;95:228–233). Reproduced with permission from S. Karger AG, Basel.

24.5.2 Record keeping

It is important to record relevant information for each sample collected, including taxonomic name, common name, sex, sample date, and identification number for captive species or GPS location for wild animals. The current proper taxonomic name (to subspecies level when possible) should be obtained using reputable resources [50,51], keeping in mind that the nomenclature changes as knowledge about animals increases. When collecting tissue, it is also important to note the exact anatomical site, because gene expression patterns vary among different cell populations [52].

24.6 Fibroblast cell culture

24.6.1 Bioresource banking

General principles of animal cell culture can be found in manuals, such as those by Freshney [53] and Masters [54]. Tissue, feather, and organ (in post-mortem cases) samples are preferable to blood samples so that fibroblast cell cultures can be established and cryopreserved in genetic resource collections such as San Diego Zoo's Frozen Zoo® (<http://www.sandiegozoo.org/>). Cell lines banked in liquid nitrogen at -196 °C can be maintained indefinitely and provide a continual source of genetic material for a wide variety of studies. The living cells can be utilized to obtain chromosome preparations, expanded to generate large quantities of DNA and RNA, and potentially be used for somatic cell nuclear transfer. Induced pluripotent stem cells (iPSCs) capable of differentiation into multiple cell types have been generated from skin cells of humans [55,56], mice [57], and rhesus monkeys [58] by direct molecular reprogramming and more recently this technology was adapted to reprogram cryopreserved adult fibroblasts of endangered species in an effort to preserve their genetic material [59]. It is especially critical to establish and cryopreserve cell lines on endangered species of known origin that might not be available in the future. However, collecting blood samples in addition to biopsies can be advantageous in cases where cytogenetic analysis indicates a mosaic condition in the fibroblasts. Normal results from the lymphocyte harvest could suggest that the abnormal fibroblast cells derived as artifact during the lengthy cell culture process.

24.6.2 Fibroblast sample sources

Skin biopsies can be obtained opportunistically when animals are immobilized for other purposes such as veterinary exams, radio-collaring, or shipment between locations. Ear notches generated from identification procedures, routinely done at many zoos, are a good sample source for ungulates (hoofed animals) including exotics. Primary cell lines can be successfully established from tissue samples collected post-mortem (at necropsy), as long as the tissue has not been frozen, become necrotic, or begun decomposing; this usually translates to a window of 1–3 days, depending on those factors that can accelerate tissue decomposition, such as temperature and body condition. Once the sample is placed in a biopsy vial containing growth media and antibiotics, it can be held for several days (or even weeks) before processing, provided it was collected aseptically. Storing the biopsy vial containing the tissue at 4 °C extends the length of time it can be held.

The preferred tissues for cell culture from mammals, birds, and reptiles include skin (including ear notches from mammals), eye, gonads, trachea, lung, and kidney. Placental biopsies are a noninvasive source of mammalian cells, especially valuable in cases where it would not otherwise be possible to sample a newborn animal or when tissue from stillborn animals will not grow.

Blood feathers are an additional excellent source of fibroblasts from avian species. They should be collected when new feathers are regenerating after loss from molt, while there is abundant feather pulp. Once the feather is mature, it no longer contains sufficient living cells for establishing a cell line. If no blood feathers are found, a few tail and/or wing primary feathers can be gently pulled, causing a new feather to regenerate within one to three weeks, depending on the size and species of the bird. After a mature feather is removed, blood feathers generally begin to emerge one to two weeks later in small birds, and after two to three weeks in larger species.

In reptiles, skin can also be collected from the tail tip or areas of skin with fewer scales, such as toe webbing on large species like crocodilians. Choosing the sample type is dependent in part on availability, but cleanliness of the location is also an important consideration. The skin of many marine mammals and other species that are frequently in the water (e.g., hippopotamuses, crocodilians, amphibians) often contains a wide array of microbes that cause cell culture contamination. In these cases it is preferable to culture the internal organs when possible, to reduce the chance of external contaminants. However, other amphibian samples, such as skin, foot, tongue, and eye have shown promise when properly disinfected.

Table 24.1 Recommended growth temperatures. This table shows the incubation temperatures for optimal cell growth for each class

Species	Temperatures	Comments
Amphibians	20–30 °C	
Birds	40 °C	
Fish	15–27 °C	
Mammals	37 °C	Exceptions: koala, echidna, and cetaceans 32–33 °C
Reptiles	32–33 °C	

24.6.3 Fibroblast culture conditions

Temperature

Optimal culture conditions for fibroblasts can vary widely, depending on the species. Incubation temperature is a critical factor and can range from 15 °C for fish to 40 °C for birds. Table 24.1 lists the temperatures most commonly used to incubate cells from the major taxonomic groups. The table can be used as a starting guideline, but if the fibroblasts from a particular species are not thriving and there is sufficient material, duplicate flasks should be grown at several temperatures to find the most favorable condition.

Media and CO₂

Various growth media in combination with CO₂ percentages can also have a significant effect on culture quality. In general, most mammalian, avian, and reptilian cells grow well in standard media such as alpha-MEM, RPMI, etc., supplemented with antibiotics and 10% fetal bovine serum (FBS) at 5–6% CO₂. Some exceptions include perissodactyls (rhinoceroses, tapirs, and equids), carnivores, elephants, koala, echidna, and others that exhibit enhanced growth with the addition of fibroblast growth factor (FGF). Increasing the FBS up to 20% is also beneficial in some cases where the cells will not thrive under standard conditions. Table 24.2 lists suggested media for various taxa.

There is less information available on successful amphibian and fish cell culture, but cells from these groups seem to have the same CO₂ requirements, although some protocols suggest a low oxygen environment may be beneficial. Amphibian cultures grow successfully at 20–23 °C (for taxa from cool climates) or 27–30 °C (tropical species), while fish cells require temperatures below typical room temperatures (15–18 °C with some reports up to 27 °C). If it is not possible to maintain the flasks in a CO₂ incubator, the cells should be initiated in nonfilter cap flasks and placed in the lowest temperature CO₂ incubator available for 2–6 hours after each feeding until the pH re-establishes. The flasks can then be capped tightly to retain the CO₂ until the next feeding. Both fish and amphibian cells can be grown successfully in alpha-MEM with 5–10% FBS, although the medium most frequently mentioned in the literature for amphibian cell culture is Leibovitz's L-15. If there is sufficient tissue available for experimental conditions, other media that have shown promising results include RPMI-1640 and Ham's F-12K. For general techniques, see references 4, 12, 53 and, 54.

24.7 Peripheral blood culture

Peripheral blood primarily provides a nonrenewable source of material for obtaining chromosome harvests, although the lymphocytes of some mammalian species, including humans, some great apes, and other primates, have been successfully immortalized using Epstein–Barr virus [60]. Peripheral blood culture provides results more quickly because of short culture duration and also facilitates harvests that often have longer chromosomes and a higher mitotic index than those derived from fibroblasts. However, if optimal blood harvest conditions for a particular species are not known, the results can be substandard. Other disadvantages include the need for fresh samples, the inability to retain some of the sample for additional harvests, and the presence of nucleated red blood cells in avian and reptilian species. In the latter, the RBCs must be removed from the sample, because they significantly outnumber the lymphocytes and do not respond to hypotonic treatment. The nuclei remain so compact that it is unlikely they would be useful even for FISH analysis.

Table 24.2 Suggested media. This table provides the media most commonly used for optimal cell growth for each classification

Class	Subgroup	Tissue type	Media	Alternate media
Amphibians		All	Completed Alpha MEM supplemented with 1% Pen-Strep Fungizone®, Normocin and Gentamicin	HyClone L-15 Leibovitz media 1 : 1 FGM/L-15 supplemented with 5% FBS and 1% Pen-Strep L-Glutamine AmnioMAX™ II supplemented with 5% FBS and 1% Pen-Strep L-Glutamine Adjust amount of FBS 5–10%
Birds		Eye, trachea, skin	Completed Alpha MEM	Supplement with 2% chicken serum and/or Glucose Increase FBS to 20% FGM supplemented with 20% FBS
		Feathers	FGM supplemented with 20% FBS and 1% Pen-Strep Glutamine	
Fish		All	Completed Alpha MEM	
Mammals	Artiodactyla	All	Completed Alpha MEM	Increase FBS to 20%
	Carnivora	All	1 : 1 completed Alpha MEM:FGM	Increase FBS to 20%
	Perissodactyla	All	1 : 1 completed Alpha MEM:FGM	Increase FBS to 20%
	Primates	All	Completed Alpha MEM	
	Diprotodontia	All	1 : 1 completed Alpha MEM:FGM	
	Koalas	All	FGM supplemented with 20% FBS and 1% Pen-Strep L-Glutamine	
	Proboscidea	All	1 : 1 completed Alpha MEM:FGM	
	Cetacea	All	FGM supplemented with 10% FBS and 1% Pen-Strep L-Glutamine	
Reptiles		All	Completed Alpha MEM	

Culture and harvesting of peripheral blood for nonhuman species follows the general procedures described in Chapter 3 (Peripheral blood cytogenetic methods) with some variations (see lymphocyte protocols in this chapter for details). When working with an unfamiliar species, multiple harvests are often necessary to determine the specific requirements for a particular taxon. One advantage of fibroblast cell lines over blood culture is the ability to perpetuate the cells and perform multiple harvests varying the technique. Consequently, after observing the results from the initial harvest, conditions can be adapted during successive attempts until the mitotic index, length, and spreading are optimal.

24.8 Chromosome analysis

24.8.1 Conventional staining

Once an optimal harvest has been obtained, there are several options for levels of analysis depending on what is already known about the species and the amount of information needed. Nondifferential staining directly with Giemsa (also called nonbanded, classical, conventional, routine, solid, or homogeneous staining) provides the basic information about the chromosomes, such as size, diploid number, and centromere location (Figure 24.6). Although this method is now rarely used in human cytogenetics, it is a fundamental step in evaluating nonhuman karyotypes, especially when little is known about the species. Analysis of nonbanded metaphase spreads permits more precise identification of telomere, centromere, and other structural features, by providing a more discernable morphology than banded chromosomes. Often, conventional staining is the best method for identifying the fundamental number, size, and morphology of the sex chromosomes; numerical or large structural abnormalities; presence of satellites, breaks, or gaps; and presence of B chromosomes in species that have not been described previously.

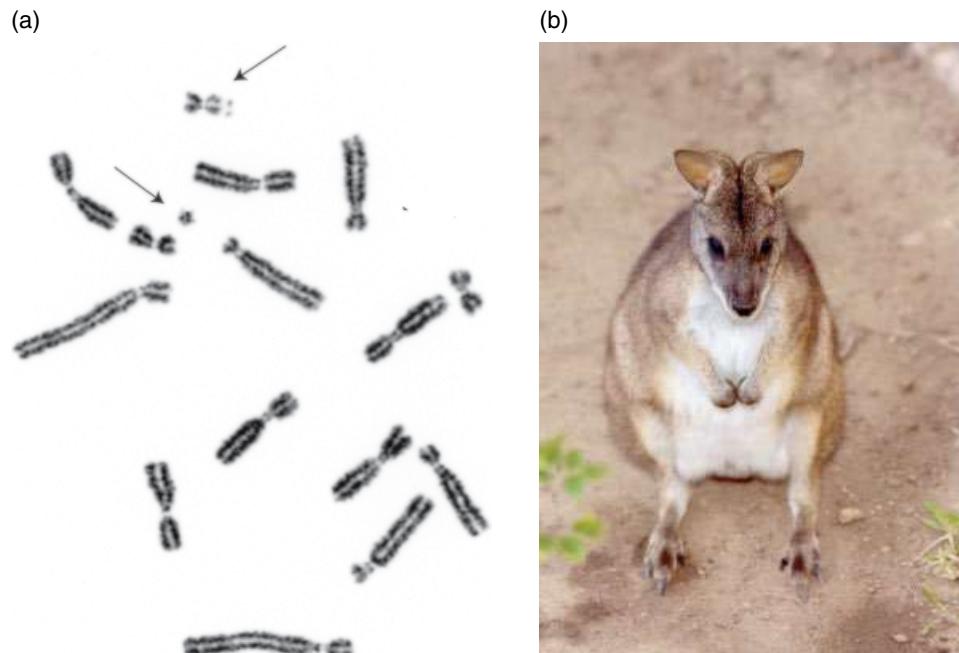


Figure 24.6 Parma wallaby. (a) Metaphase spread of a male parma wallaby, *Macropus parma*, $2n=16, XY$. Arrows indicate the X (larger element) and Y (small element). (b) Photo of a parma wallaby, *Macropus parma*. Photos courtesy of Zoological Society of San Diego. See insert for color representation of this figure.

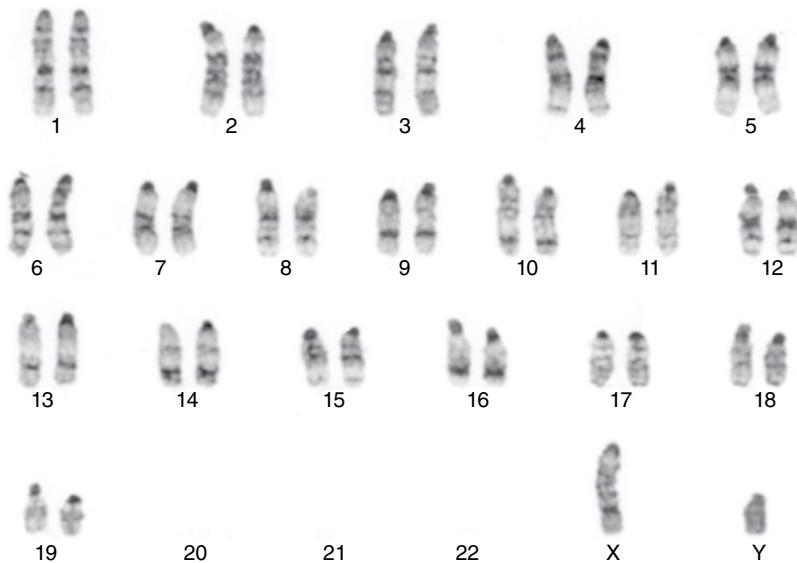


Figure 24.7 Mouse. G-banded karyogram of a male mouse, *Mus musculus*, $2n=40, XY$. Karyogram contributed by Helen Jennings Lawce. Reproduced with the kind permission of Nicole Owen.

24.8.2 Banding

In general, the purpose and methods of banding animal chromosomes are the same as for human chromosomes (see Chapter 6, Chromosome stains). G- and R-banding have traditionally been the most common methods used. Figures 24.7 and 24.8 show representative karyograms of mouse and rhesus macaque, respectively.

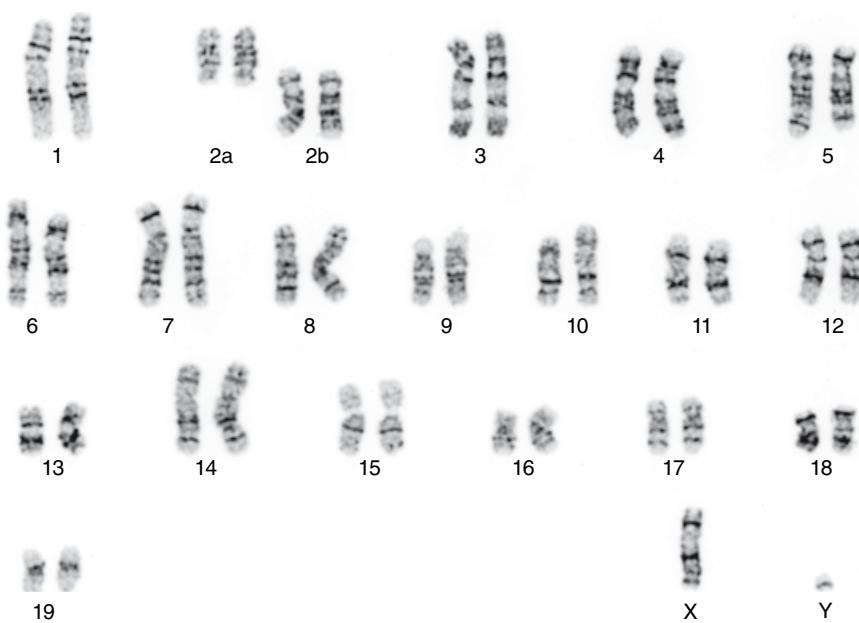


Figure 24.8 Rhesus macaque. G-banded karyogram [119] of a male rhesus macaque, *Macaca mulatta*, $2n=42$, XY, taken from fibroblast cultures. Reproduced with the kind permission of Nicole Owen.

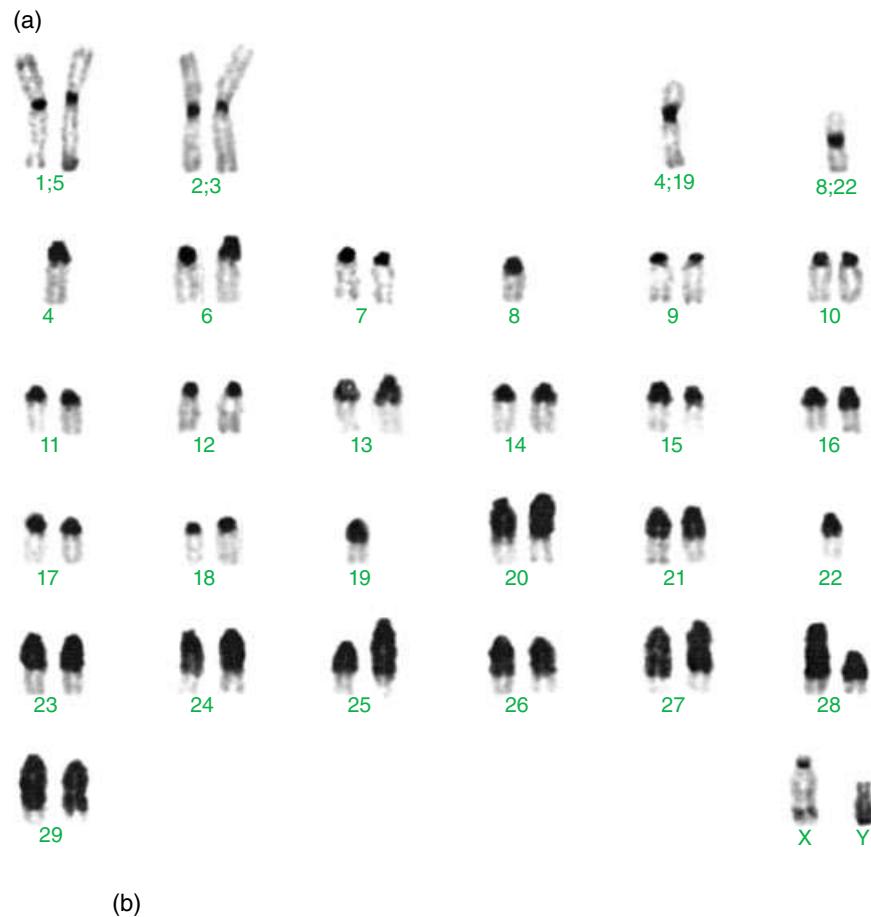
C-banding to visualize constitutive heterochromatin is a useful and informative tool for analysis of animal chromosomes. Heterochromatin variations may accumulate rapidly within taxa, even among populations of a single species, and chromosomal patterns of heterochromatin distribution may vary significantly in different genera of families [61,62]. In mammals, heterochromatin can be found at primary and secondary chromosome constrictions, telomeres, interstitial regions, and on the sex chromosomes. C-banding is entirely absent in the centromeric regions of some species, for example, slow loris (*Nycticebus pygmaeus*) and potto (*Perodicticus potto*) [63], while, in contrast, many bovid species have large heterochromatin blocks frequently occurring below the centromere in acrocentric chromosomes (Figure 24.9) and whales have large blocks of interstitial and terminal heterochromatin [64,65]. Some primate species, including gorillas, bonobos, and woolly monkeys (*Lagothrix lagotricha*), have areas of abundant interstitial heterochromatin on numerous chromosomes of varying morphology (Figure 24.4). In many mammalian species, the Y chromosome can appear partially or entirely heterochromatic, as does the W chromosome in some avian species, which facilitates identification of sex chromosomes in taxa that are monomorphic or have not been previously characterized by cytogenetic methods.

24.8.3 Digital imaging

When choosing an imaging system for working with a wide diversity of animal species, it is important to select one with a software program capable of unlimited flexibility for creating karyogram templates quickly and easily, such as the CytoVision Genus® system by Leica Microsystems. MetaSystems and Digital Scientific also offer versatile template software.

24.8.4 Karyotyping

Chromosome terminology, counting the metaphase spread, and creating the karyotype follow the general principles for human cytogenetics as described in Chapter 7, Human chromosomes: identification and variations. However, there is wide variation in styles for arranging animal karyograms. If there is an internationally accepted, standardized karyotype for a species, the same format should be followed in its arrangement within the karyogram (see 24.8.5, Karyotype standards and precedents). For some species, therefore, chromosomes may be arranged entirely by decreasing size, without taking centromere location into consideration, while for another species they might be arranged by size within groups based on centromere location, if a distinction is evident. Unfortunately, this gradation from metacentric to submetacentric to acrocentric is often so slight that it is difficult to find a point of division between two groups. Typically, morphologically distinct chromosome pairs, such as those with secondary constrictions,



(b)

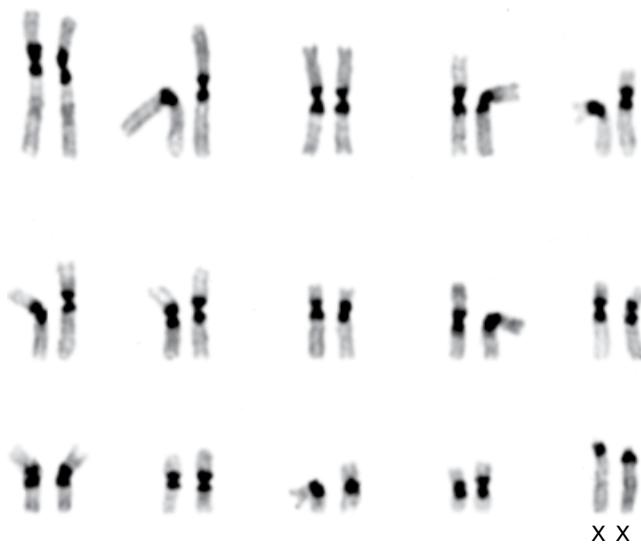


Figure 24.9 Suni antelope. C-banded karyograms of the (a) Suni antelope, *Neotragus moschatus*, $2n=54, XY$. Numbering follows the standardized *Bos taurus* karyotype. (b) Southern steenbok, *Raphiceros campestris*, $2n=30, XX$.



Figure 24.9 (Continued) Photographs are (c) Suni antelope, *Neotragus moschatus*. Photos courtesy of Zoological Society of San Diego. (d) Southern steenbok, *Raphiceros campestris*. See insert for color representation of this figure.

are placed at the end of the karyogram. Metaphase spreads of birds and reptiles often contain numerous microchromosomes, which are generally also placed at the end of the karyogram. A 2004 compilation of B chromosomes (supernumerary chromosomes) in eukaryotes [66] is helpful for avoiding confusion between microchromosomes and accessory chromosomes. Sex chromosomes are usually placed in the lower right corner of the karyogram, if they can be differentiated.

The basic biology of genetic sex determination in vertebrates can be complex [67–69]. In most mammals the sex chromosomes are labeled X and Y, the same as for humans. However, in birds and most reptiles the female is the heterogametic sex and the sex chromosomes are designated Z and W (Figure 24.10). In such cases the W chromosome is female determining and the male is ZZ. Partial karyograms showing only the largest chromosome pairs and omitting the microchromosomes are useful for sex determination purposes. In some cases the Z and W cannot be distinguished using cytogenetic methods (Figure 24.11); for these situations, sex can often be determined by using the polymerase chain reaction (PCR), which will amplify specific regions on the sex chromosomes [70–74], especially if controls are available from confirmed male and female reference animals. One advantage of this method is that it can be done from noninvasively collected samples, like eggshell membrane, feathers, hair, or feces.

24.8.5 Karyotype standards and precedents

Standard karyotype nomenclature and their band-defining idiograms for many species have been derived by consensus of international committees. ISCN (International System for Human Cytogenetic Nomenclature) has proposed karyotype nomenclature standards for chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), gorilla (*Gorilla gorilla*), and orangutan (*Pongo pygmaeus*) [75–80]. ISCNDNA (International System for Cytogenetic Nomenclature of Domestic Animals) and ISCNDNB (International System for Cytogenetic Nomenclature of Domestic Bovids) provide standardization of domestic animal and domestic bovid karyotypes [17,81], while ISSAK (International System for Standardized Avian Karyotypes) has developed karyotype standards for the eight largest autosomes and the sex chromosomes of the chicken (*Gallus domesticus*) [82]. Other internationally accepted karyotype standards include those specific for horse [83,84], donkey (*Equus asinus*) [85], sheep (*Ovis aries*) [86,87], pig (*Sus domesticus*) [88], cat (*Felis catus*) [89], dog (*Canis familiaris*) [90], rabbit (*Oryctolagus*

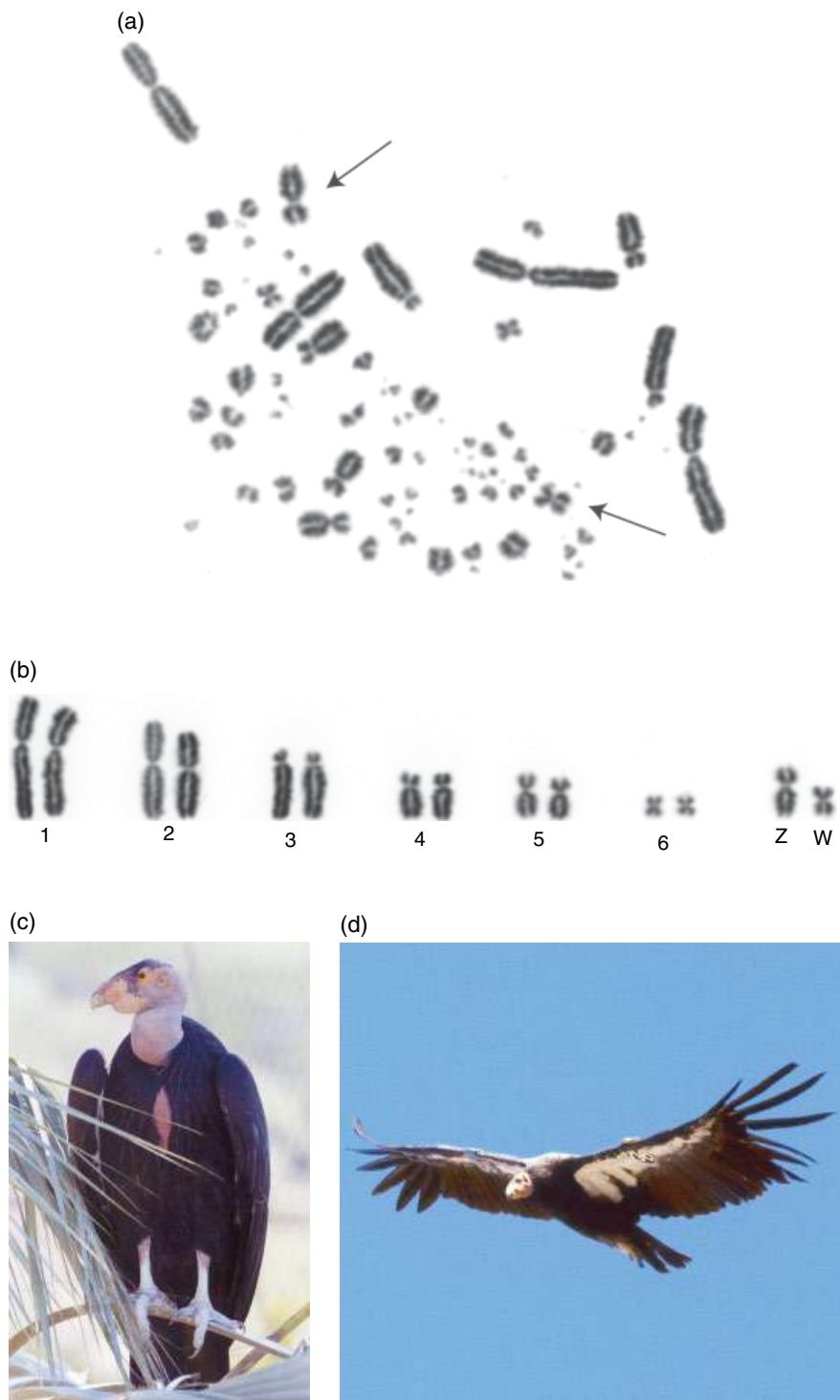


Figure 24.10 California condor, *Gymnogyps californianus*, (a) nonbanded metaphase spread with numerous microchromosomes. Arrows indicate the Z (larger submetacentric element) and W (smaller metacentric element); (b) partial nonbanded karyogram, ZW female; (c) and (d) photographs of the California condor, *Gymnogyps californianus*. (c,d) Photos courtesy of Zoological Society of San Diego. See insert for color representation of this figure.

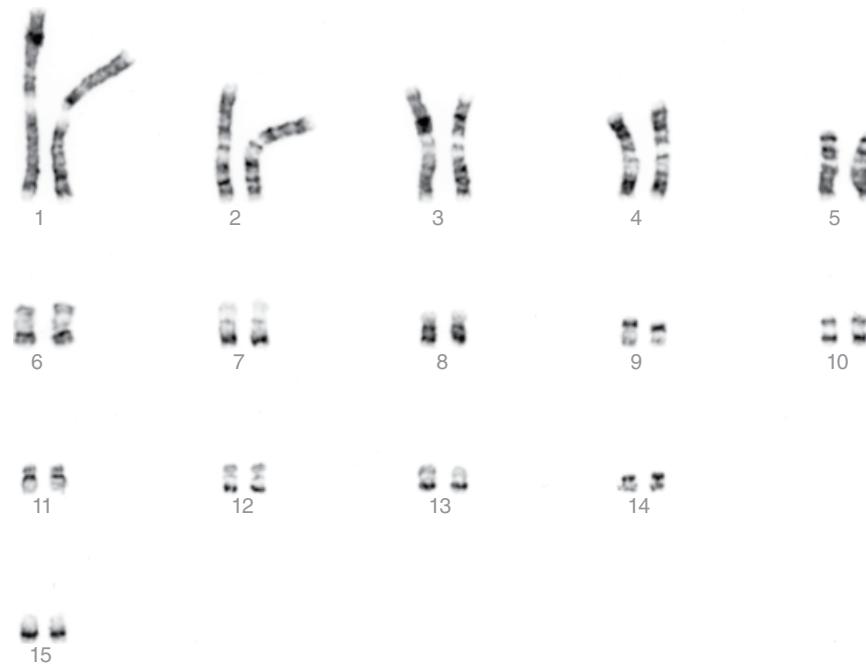


Figure 24.11 Slender-snouted crocodile. GTG-banded karyogram of the slender snouted crocodile, *Crocodylus cataphractus*, $2n=30$. Sex chromosomes are not identifiable.

cuniculus) [91,92], mouse (*Peromyscus*) [93,94], Norway rat (*Rattus norvegicus*) [95], rhesus monkey (*Macaca mulatta*) [96], river buffalo (*Bubalus bubalis*) [97], Arctic or blue fox (*Vulpes lagopus* formerly known as *Alopex lagopus*) [98], and silver fox (*Vulpes fulvus*) [99].

For species that do not have a proposed standard, the karyotype should follow a previous publication, and the karyogram should be arranged accordingly. If a karyotype would benefit by alteration to reflect information obtained after the original publication, a detailed description about the homologous pairs between the two karyotypes should be provided in subsequent publications to prevent confusion.

24.9 Molecular and comparative cytogenetics

24.9.1 Zoo-FISH

Gene mapping has been carried out for a wide variety of species and continues to expand. Cross-species chromosome painting, or Zoo-FISH, can be used to identify genome segments originating from a common ancestor that have been conserved between species for millions of years. This facilitates the transfer of information from species with more developed gene maps such as human, mouse, horse, cow, and chicken, to related species with poorly developed maps. Some interesting Zoo-FISH studies have identified homologous chromosome segments in distantly related mammalian species, provided evidence for tandem fusions of chromosomes in the evolution of muntjac chromosomes, identified multiple sex chromosomes in platypus [100–102], and revealed patterns of chromosome segment conservation (reviewed in [103]).

24.9.2 Reciprocal chromosome painting

Advances in flow-sorting chromosomes have enabled the use of reciprocal chromosome painting for studying chromosome evolution in a variety of endangered species, such as rhinoceroses [104]. It has also been used to identify chromosome segment conservation among ancestral mammalian groups and define a potential eutherian ancestral karyotype [105]. Chowdhary and Raudsepp [106] review the techniques and results of both reciprocal and nonreciprocal Zoo-FISH between closely related nonprimate mammalian species belonging to a myriad of orders.

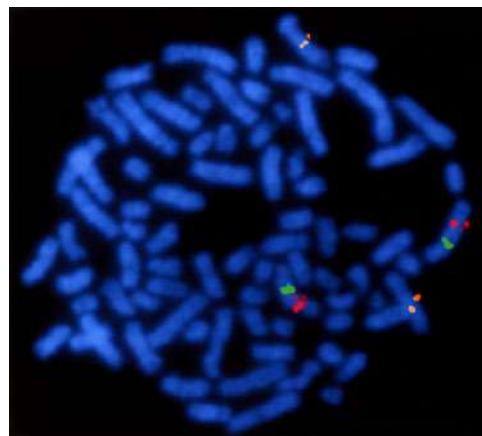


Figure 24.12 BACs from three horse genes. Three horse genes in Bacterial Artificial Chromosomes (BACs) from *Equus caballus* chromosome 1 mapping to two pairs of lowland tapir, *Tapirus terrestris*, chromosomes. The probes are labeled with SpectrumRed®, SpectrumGreen®, and SpectrumOrange®. See insert for color representation of this figure.

24.9.3 BAC maps

While Zoo-FISH and reciprocal chromosome painting provide information on the evolution of synteny groups, they do not provide information on gene order or subtle mechanisms of chromosomal evolution such as centromere movement (neocentromeres, centromere shifts), small inversions, duplications, or translocations. Gene-specific BAC clones can be used to create comparative maps for wildlife species and new model organisms with underrepresented genomes. Currently, more than 200 animal BAC libraries are available from BAC PAC Resources (<http://bacpac.chori.org/libraries.php>) for sixteen mammalian orders and a variety of fish, amphibians, reptiles, and birds. Figure 24.12 demonstrates how well horse genes map to the chromosomes of another perissodactyl, in this case a lowland tapir (*Tapirus terrestris*). The resulting comparative maps are often combined with other mapping approaches to provide tremendous insight into the mechanisms that have contributed to chromosomal evolution, phylogenetic relationships, and ancestral vertebrate karyotypes [107–112]. Both Zoo-FISH and BACs have been used to confirm aneuploidy and define translocations, deletions, and inversions in numerous species, including primates, bovids, horses, dogs, and chickens [28,49,113–118].

24.9.4 Future directions

Whole-genome shotgun (WGS) sequencing is underway for an increasing number of animal species. In addition to the human genome project, whole genome assessment of more than 50 vertebrate species at a level suitable for comparative genomic analyses has now been accomplished. For current updates on genome sequencing projects completed and in progress see <http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi>. DNA sequence information will enhance our understanding of functional nuclear anatomy and chromosomes. This mandates continued investment in comparative cytogenetics and access to dividing cells from a larger variety of species. The complete nucleotide sequence of the African savannah elephant (*Loxodonta africana*) or the nine-banded armadillo (*Dasypus novemcinctus*) will undoubtedly generate further studies of their chromosomes, provided cell cultures are available from sources like the Frozen Zoo®. A new burst of interest in comparative cytogenetics can be predicted as a result of increased genome sequencing efforts. Future research based on comparative cytogenetics will confirm synteny, identify processes involved in genome evolution, and contribute to our understanding of how individual variation in chromosomal structure impacts disease risk and other measures of fitness.

Acknowledgments

We thank Julie Fronczek and Andrea Johnson for reviewing the text and providing expert technical assistance in the preparation of the manuscript and karyograms. We also thank Leona Chemnick, Heidi Davis, and Christie Otten for their contributions regarding PCR. We are grateful to Dr. Kurt Benirschke and Dr. Oliver Ryder for their helpful reviews of the manuscript. This chapter is dedicated to the memory of Arlene T. Kumamoto, a pioneer in the field of exotic animal cytogenetics.

Glossary

Bovid: a mammal of the cattle family (Bovidae).

<http://en.wikipedia.org/wiki/Bovid>. Retrieved July 04, 2011

Chondrodystrophy: a disturbance that affects the development of the cartilage of the long bones and that especially involves the region of the epiphyseal plates, resulting in arrested growth of the long bones.

chondrodystrophy. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved July 04, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/chondrodystrophy>

Crocodilian: any reptile of the order Crocodylia, comprising the true crocodiles and the alligators, caimans, and gavials.

<http://dictionary.reference.com/browse/crocodilian>. Retrieved July 04, 2011.

Cryptorchid: cryptorchidism is the absence of one or both testes from the scrotum.

<http://en.wikipedia.org/wiki/Cryptorchidism>. Retrieved July 04, 2011.

Echidna: also called spiny anteater. Any of several insectivorous monotremes of the genera *Tachyglossus*, of Australia, Tasmania, and New Guinea, and *Zaglossus*, of New Guinea, that have claws and a slender snout and are covered with coarse hair and long spines.

echidna. (n.d.). Dictionary.com Unabridged. Retrieved July 04, 2011, from Dictionary.com website: <http://dict>

Eutherian: of, relating to, or belonging to the Eutheria, a subclass of mammals all of which have a placenta and reach an advanced state of development before birth. The group includes all mammals except monotremes and marsupials.

eutherian. (n.d.). Collins English Dictionary - Complete & Unabridged 10th Edition. Retrieved July 04, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/eutherian>

Monomorphic: having one or the same genotype, form, or structure through a series of developmental changes.

monomorphic. (n.d.). The American Heritage® Stedman's Medical Dictionary. Retrieved July 04, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/monomorphic>

Neocentromere: a functional centromere in a novel location; may lack specific classes of deoxyribonucleic acid usually present in a centromere.

<http://www.answers.com/topic/neocentromere>

Perissodactyl: any of various hoofed mammals of the order Perissodactyla, having one or three hoofed toes on each hind-foot. During the Tertiary Period, perissodactyls were the dominant herbivorous fauna. Horses, tapirs, and rhinoceroses are perissodactyls. Also called odd-toed ungulate.

perissodactyl. (n.d.). The American Heritage® Science Dictionary. Retrieved July 04, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/perissodactyl>

Synteny: the physical co-localization of genetic loci on the same chromosome within an individual or species.

<http://en.wikipedia.org/wiki/Synteny>. Retrieved July 04, 2011.

Taxon/taxa: a taxonomic category, as a species or genus.

taxon. (n.d.). Dictionary.com Unabridged. Retrieved July 04, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/taxon>. Retrieved July 04, 2011

References

1. Flemming W. Beitrage zur Kenntnis der zelle und ihrer Lebenserscheinungen. *Arch für Mikrosk Anat* 1879; 18: 302–436.
2. Halnan CRE, ed. *Cytogenetics of animals*. Wallingford: CAB International, 1989.
3. Hsu TC. *Human and mammalian cytogenetics: an historical perspective*. New York: Springer-Verlag, 1979.
4. Macgregor H, Varley J. *Working with animal chromosomes*. Chichester: John Wiley and Sons, 1983.
5. Popescu P, Hayes H, Dutrillaux B, eds. *Techniques in animal cytogenetics*. Berlin: Springer-Verlag, 2000.
6. Hsu TC, Benirschke K, eds. *An atlas of mammalian chromosomes*. New York: Springer-Verlag, 1967–1977.
7. Makino S. *An atlas of the chromosome numbers in animals*, 2nd ed. Ames, IA: Iowa State College Press, 1951.
8. O'Brien SJ, Menninger JC, Nash WG, eds. *Atlas of mammalian chromosomes*. Hoboken, NJ: Wiley-Liss, 2006.
9. Wurster DH, Benirschke K. Indian muntjac, *Muntiacus muntjac*: a deer with a low diploid chromosome number. *Science* 1970; 168: 1364–1366.
10. Contreras LC, Torres-Mura JC, Spotorno AE. The largest known chromosome number for a mammal, in a South American desert rodent. *Experientia* 1990; 46: 506–508.
11. Hsu TC, Benirschke K, eds. *Chromosome Atlas: Fish, Amphibians, Reptiles and Birds*. New York: Springer-Verlag, 1971.
12. Olmo E, ed. *Cytogenetics of Amphibians and Reptiles*. Basel: Birkhäuser Verlag, 1990.

13. van Brink JM, Kiauta B, eds. Studies in avian karyology. *Genetica*. 1984; 65: 1–118.
14. Eldridge FE. *Cytogenetics of Livestock*. Westport, CT: AVI Publishing Co, 1985.
15. Ford CE, Pollock DL, Gustavsson I. Proceedings of the first international conference for the standardization of banded karyotypes of domestic animals. *Hereditas* 1980; 92: 145–162.
16. Hayes H, Petit E, Dutrillaux B. Comparison of RBG-banded karyotypes of cattle, sheep, and goats. *Cytogenet Cell Genet* 1991; 57: 51–55.
17. ISCNDB (2000): International system for chromosome nomenclature of domestic bovids (ISCNDB). Di Berardino D, Di Meo GP, Gallagher DS, Hayes H, Iannuzzi L, (coordinator)(eds). *Cytogenet Cell Genet* 2001; 92: 283–299.
18. Gustavsson I. Distribution and effects of the 1/29 Robertsonian translocation in cattle. *J Dairy Sci* 1979; 62: 825–835.
19. Gustavsson I. Chromosome aberrations and their influence on the reproductive performance of domestic animals – a review. *Z Tierz Zuchtbiof* 1980; 97: 176–195.
20. Hare WCD, Singh EL. *Cytogenetics in Animal Reproduction*. Slough, UK: Commonwealth Agricultural Bureaux, 1979.
21. Weber AF, Buoen LC, Terhaar BL, Ruth GR, Momont HW. Low fertility related to 1/29 centric fusion anomaly in cattle. *JAVMA* 1989; 195: 643–646.
22. Power MM. Equine half sibs with an unbalanced X;15 translocation or trisomy 28. *Cytogenet Cell Genet* 1987; 45: 163–168.
23. Klunder LR, McFeely RA, Beech J, McClune W. Autosomal trisomy in a Standardbred colt. *Equine Vet J* 1989; 21: 69–70.
24. Bowling AT, Millon LV. Two autosomal trisomies in the horse: 64,XX,-26,+t(26q26q) and 65,XX,+30. *Genome* 1990; 33: 679–682.
25. Buoen LC, Zhang TQ, Weber AF, Turner T, Bellamy J, Ruth GR. Arthrogryposis in the foal and its possible relation to autosomal trisomy. *Equine Vet J* 1997; 29: 60–62.
26. Lear TL, Cox JH, Kennedy GA. Autosomal trisomy in a Thoroughbred colt: 65,XY,+31. *Equine Vet J* 1999; 31: 85–88.
27. Long SE. Tandem 1;30 translocation: a new structural abnormality in the horse (*Equus caballus*). *Cytogenet Cell Genet* 1996; 72: 162–163.
28. Lear TL, Layton G. Use of Zoo-FISH to characterize a reciprocal translocation in a Thoroughbred mare: t(1;16) (q16;q21.3). *Equine Vet J* 2002; 34: 207–209.
29. Power MM. The first description of a balanced reciprocal translocation [t(1q;3q)] and its clinical effects in a mare. *Equine Vet J* 1991; 23: 146–149.
30. Lear TL, Lundquist J, Zent WW, Fishback Jr WD, Clark A. Three autosomal chromosome translocations associated with repeated early embryonic loss (REEL) in the domestic horse (*Equus caballus*). *Cytogenet Genome Res* 2008; 120: 117–122.
31. Benirschke K. Zoos and the pathologist—a two-way street, or Cytogenetics on zoo animals. *Acta Zool Pathol Antv* 1969; 48: 29–41.
32. Benirschke K, ed. *Comparative Mammalian Cytogenetics*. New York: Springer-Verlag, 1969.
33. Benirschke K. Genetic management. *Intl Zoo Yearbk* 1977; 17: 50–60.
34. Benirschke K, Kumamoto AT. Mammalian cytogenetics and conservation of species. *J Heredity* 1991; 82: 187–191.
35. Robinson TJ, Elder FFB. Cytogenetics: its role in wildlife management and the genetic conservation of mammals. *Biol Cons* 1993; 63: 47–51.
36. Mayr E. *Populations, Species, and Evolution*. Cambridge, MA: Harvard University Press, 1970.
37. Torres OM, Enciso S, Ruiz F, Silva E, Yunis I. Chromosome diversity of the genus *Aotus* from Colombia. *Am J Primatol* 1998; 44: 255–275.
38. Kumamoto AT, Kingswood SC, Hugo W. Chromosomal divergence in allopatric populations of Kirk's dik-dik, *Madoqua kirkii* (Artiodactyla, Bovidae). *J Mammalogy* 1994; 75: 357–364.
39. Ryder OA, Kumamoto AT, Durrant BS, Benirschke K. Chromosomal divergence and reproductive isolation in dik-diks. In: Otte D, Endler JA, eds. *Speciation and its Consequences*. Sunderland, MA: Sinauer Associates Inc, 1989: 208–225.
40. Ryder OA, Chemnick LG. Chromosomal and mitochondrial DNA variation in orangutans. *J Heredity* 1993; 84: 405–409.

41. Hekkala E, Shirley MH, Amato G, Austin JD, Charter S, Thorbjarnarson J, Vliet KA, Houck ML, DeSalle R, Blum MJ. An ancient icon reveals new mysteries: mummy DNA resurrects a cryptic species within the Nile crocodile. *Molecular Ecology*, 2011; 20: 4199–4215.
42. Benirschke K, Kumamoto AT, Olsen JH, Williams MM, Oosterhuis J. On the chromosomes of *Gazella soemmeringii* Cretzschmar, 1926. *Z Säugetierk* 1984; 49: 368–373.
43. Suedmeyer WK, Houck ML, Kreeger J. Klinefelter syndrome (47 XXY) in an adult Siberian tiger (*Panthera tigris altaica*). *J Zoo Wildl Med* 2003; 34: 96–99.
44. Mastromonaco GF, Houck ML, Bergfelt DR. Disorders of sexual development in wild and captive exotic animals. *Sex Dev* 2012; 6: 84–95.
45. Houck ML, Ryder OA, Kumamoto AT, Benirschke K. Cytogenetics of the rhinocerotidae. *Sonderdruck aus Verhandlungsbericht des 37 Internationalen Symp über die Erkrankungen der Zootiere* 1995; 37: 25–32.
46. Andrle M, Fiedler W, Rett A, Ambros P, Schweizer D. A case of trisomy 22 in *Pongo pygmaeus*. *Cytogenet Cell Genet* 1979; 24: 1–6.
47. Ward OG, Miller RL, Johnson EH, Lucas JN, Meyne J. Identification of trisomy in *Macaca fascicularis* by fluorescence in situ hybridization with a human chromosome 13 DNA library. *Hum Genet* 1994; 94: 247–51.
48. Lear TL, Houck ML, Zhang YW, Debnar LA, Sutherland-Smith MR, Young L, Jones KL, Benirschke K. Trisomy 17 in a bonobo (*Pan paniscus*) and deletion of 3q in a lowland gorilla (*Gorilla gorilla gorilla*): comparison with human trisomy 18 and deletion 4q syndrome. *Cytogenet Cell Genet* 2001; 95: 228–233.
49. Romanov MN, Koriabine M, Nefedov M, de Jong PJ, Ryder OA. Construction of a California condor BAC library and first-generation chicken-condor comparative physical map as an endangered species conservation genomics resource. *Genomics* 2006; 88: 711–718.
50. Groves, CP. *Primate Taxonomy*. Washington and London: Smithsonian Institution Press, 2001.
51. Wilson DE, Reeder DM, eds. *Mammal Species of the World*, 3rd ed. Baltimore: John Hopkins University Press, 2005.
52. Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, Brown PO. Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci* 2002; 99: 12877–12882.
53. Freshney RI. *Culture of animal cells: a manual of basic technique*, 5th ed. New York: Wiley-Liss, 2005.
54. Masters JRW, ed. *Animal Cell Culture*, 3rd ed. New York: Oxford University Press, 2003.
55. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; 131: 861–72.
56. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007; 318: 1917–20.
57. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; 126: 663–676.
58. Liu H, Zhu F, Yong J, Zhang P, Hou P, Li H, Jiang W, Cai J, Liu M, Cui K, Qu X, Xiang T, Lu D, Chi X, Gao G, Ji W, Ding M, Deng H. Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. *Cell Stem Cell* 2008; 3:587–590.
59. Ben-Nun IF, Montague SC, Houck ML, Tran HT, Garitaonandia I, Leonardo TR, Wang Y-C, Charter SJ, Laurent LC, Ryder OA, Loring JF. Induced pluripotent stem cells from highly endangered species. *Nature Methods* 2011; 8:829–31.
60. Miller G, Shope T, Lisco H, Stitt D, Lipman M. Epstein-Barr Virus: Transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. *Proc Natl Acad Sci* 1972; 69: 383–387.
61. Hsu TC, Arrighi FE. Distribution of constitutive heterochromatin in mammalian chromosomes. *Chromosoma* 1971; 34: 243–253.
62. Ryder OA, Epel NC, Benirschke K. Chromosome banding studies of the Equidae. *Cytogenet Cell Genet* 1978; 20: 323–350.
63. Dutrillaux B, Couturier J, Lombard M, Chauvier G. Cytogenétique de deux Lorisidae (*Nycticebus coucang* et *Perodicticus potto*). Comparaison avec les lémuriens et les simiens. *Ann Genet* 1979; 22: 93–98.
64. Arnason U. Comparative chromosome studies in Cetacea. *Hereditas* 1974; 77: 1–36.

65. Arnason U, Bellamy H, Eyporsson P, Lutley R, Sigurjonsson J, Widegren B. Conventionally stained and C-banded karyotypes of a female blue whale. *Hereditas* 1985; 102: 251–253.
66. Camacho JPM, ed. B chromosomes in the eukaryote genome. Single topic volume. *Cytogenet Cell Genet* 2004; 106: 143–412.
67. Manolakou P, Lavranos G, Angelopoulou R. Molecular patterns of sex determination in the animal kingdom: a comparative study of the biology of reproduction. *Reprod Biol Endocrinol* 2006; 4: 59.
68. Reed KC, Marshall Graves JA, eds. *Sex Chromosomes and Sex-determining Genes*. Chur, Switzerland: Harwood Academic Publishers, 1993.
69. Wachtel SS, ed. *Molecular Genetics of Sex Determination*. San Diego: Academic Press, 1994.
70. Aasen E, Medrano JF. Amplification of the ZFY and ZFX genes for sex identification in humans, cattle, sheep and goats. *Biotech* 1990; 8: 1279–1281.
71. Griffiths R, Daan S, Dijkstra C. Sex identification in birds using two CHD genes. *Proc R Soc Lond* 1996; 263: 1251–1256.
72. Kahn NW, St. John J, Quinn TW. Chromosome-specific intron size differences in the avian CHD gene provide an efficient method of sex identification in birds. *Auk* 1998; 115: 1074–1078.
73. Neto, ED, Santos FR, Pena SDJ, Simpson AJG. Sex determination by low stringency PCR (LS-PCR). *Nucl Acids Res* 1993; 21: 763–764.
74. Wilson JF, Erlandsson R. Sexing of human and other primate DNA. *Biol Chem* 1998; 379: 1287–1288.
75. ISCN (1978): An International System for Human Cytogenetic Nomenclature. Bergsma D, ed. Birth Defects: Original Article Series 14(8) (ISCN). New York: The National Foundation. *Cytogenet Cell Genet* 1978; 21: 309–404.
76. ISCN (1981): An International System for Human Cytogenetic Nomenclature – High Resolution Banding (ISCN). Buckton K, Harnden DG, Klinger HP, Lindsten JE, eds. Birth Defects: Original Article Series 17(5). New York: March of Dimes Birth Defects Foundation. *Cytogenet Cell Genet* 1981; 31: 1–23.
77. ISCN (1985): An International System for Human Cytogenetic Nomenclature. Harnden DG, Klinger HP, eds. Birth Defects: Original Article Series 21(1) (ISCN). New York: March of Dimes Birth Defects Foundation, 1985.
78. ISCN (1991): Guidelines for cancer cytogenetics. Supplement to an International System for Human Cytogenetic Nomenclature (ISCN). Mitelman F, ed. Basel: S. Karger, 1991.
79. Paris Conference (1971): Standardization in Human Cytogenetics. Birth Defects: Original Article Series 8(7). New York: The National Foundation, 1971; *Cytogenet Cell Genet* 1972; 11: 313–362.
80. Paris Conference (1971), supplement (1975): Standardization in Human Cytogenetics. Birth Defects: Original Article Series 11(9). New York: The National Foundation, 1974. *Cytogenet Cell Genet* 1975; 15: 201–238.
81. ISCNDA (1989): International System for Chromosome Nomenclature of Domestic Animals. Di Berardino D, Hayes H, Fries R, Long S, eds. *Cytogenet Cell Genet* 1990; 53: 65–79.
82. ISSAK (1999): International system for standardized avian karyotypes: standardized banded karyotypes of the domestic fowl (*Gallus domesticus*). Ladjali-Mohammed K, Bitgood JJ, Tixier-Boichard M, Ponce de Leon FA. *Cytogenet Cell Genet* 1999; 86: 271–276.
83. ISCNH (1997): International system for cytogenetic nomenclature of the domestic horse (ISCNH). Bowling AT, Breen M, Chowdhary BP, Hirota K, Lear TL, Millon LV, Ponce de Leon FA, Raudsepp T, Stranzinger G (Committee). *Chromosome Res* 1997; 5: 433–453.
84. Richer CL, Power MM, Klunder LR, McFeely RA, Kent MG. Standard karyotype of the domestic horse (*Equus caballus*). *Hereditas* 1990; 112: 289–293.
85. Raudsepp T, Christensen K, Chowdhary BP. Cytogenetics of donkey chromosomes: nomenclature proposal based on GTG-banded chromosomes and depiction of NORs and telomeric sites. *Chromosome Res* 2000; 8: 659–670.
86. Ansari HA, Bosma AA, Broad TE, Bunch TD, Long SE, Maher DW, Pearce PD, Popescu CP. Standard G-, Q-, and R-banded ideograms of the domestic sheep (*Ovis aries*): homology with cattle (*Bos taurus*). Report of the committee for the standardization of the sheep karyotype. *Cytogenet Cell Genet* 1999; 85: 317–324.
87. Long SE. Standard nomenclature for the G-band karyotype of the domestic sheep (*Ovis aries*). *Hereditas* 1985; 103: 165–170.

88. Gustavsson I. Standard karyotypes of the domestic pig: committee for the standardized karyotype of the domestic pig. *Hereditas* 1988; 109: 151–157.
89. Cho K-W, Youn H-Y, Watari T, Tsujimoto H, Hasegawa A, Satoh H. A proposed nomenclature of the domestic cat karyotype. *Cytogenet Cell Genet* 1997; 79: 71–78.
90. Switonski M, Reimann N, Bosma AA, Long S, Bartnitzke S, Pienkowska A, Moreno-Milan AA, Fischer P. Report on the progress of standardization of the G-banded canine (*Canis familiaris*) karyotype. *Chromosome Res* 1996; 4: 306–309.
91. Committee for standardized karyotype of *Oryctolagus cuniculus*. Standard karyotype of the laboratory rabbit, *Oryctolagus cuniculus*. *Cytogenet Cell Genet* 1981; 31: 240–248.
92. Hayes H, Rogel-Gaillard C, Zijlstra C, de Haan NA, Urien C, Bourgeaux N, Bertaud M, Bosma AA. Establishment of an R-banded rabbit karyotype nomenclature by FISH localization of 23 chromosome-specific genes on both G- and R-banded chromosomes. *Cytogenet Genome Res* 2002; 98: 199–205.
93. Greenbaum IF, Gunn SJ, Smith SA, McAllister BF, Hale DW, Baker RJ, Engstrom MD, Hamilton MJ, Modi WS, Robbins LW, Rogers DS, Ward OG, Dawson WD, Elder FFB, Lee MR, Pathak S, Stangl FB Jr. Cytogenetic nomenclature of deer mice, *Peromyscus* (Rodentia): revision and review of the standardized karyotype. *Cytogenet Cell Genet* 1994; 66: 181–195.
94. Sawyer JR, Hozier JC. High resolution banding of mouse chromosomes: banding conservation between man and mouse. *Science* 1986; 232: 1632–1635.
95. Committee for a standardized karyotype of *Rattus norvegicus*. Standard karyotype of the Norway rat, *Rattus norvegicus*. *Cytogenet Cell Genet* 1973; 12: 199–205.
96. Soares MBM, Armada JLA, da Silva VF, Seuanez HN. Standardization of the karyotype of the Rhesus monkey, *Macaca mulatta*, and interspecific homologies with human chromosomes. *J Hum Evol* 1982; 11: 291–296.
97. Iannuzzi L. Standard karyotype of the river buffalo (*Bubalus bubalis* L.): committee for the standardization of banded karyotypes of the river buffalo. *Cytogenet Cell Genet* 1994; 67: 102–113.
98. Makinen A, Gustavsson I, Switonski M, Takagi N. The standard karyotype of the blue fox (*Alopex lagopus* L.). *Hereditas* 1985; 103: 33–38.
99. Makinen A, Isakova GK, Kuokkanen MT, Gustavsson I, Takagi N. The standard karyotype of the silver fox (*Vulpes fulvus* Desm.). *Hereditas* 1985; 103: 171–176.
100. Scherthan H, Cremer T, Arnason U, Weier H-U, Lima-de-Faria A, Frönicke L. Comparative chromosome painting discloses homologous segments in distantly related mammals. *Nat Genet* 1994; 6: 342–347.
101. Yang F, O'Brien PC, Wienberg J, Ferguson-Smith MA. A reappraisal of the tandem fusion theory of karyotype evolution in Indian muntjac using chromosome painting. *Chromosome Res* 1997; 5: 109–117.
102. Grutzner F, Rens W, Tsendl-Avush E, El-Mogharbel N, O'Brien PC, Jones RC, Ferguson-Smith MA, Marshall-Graves JA. In the platypus a meiotic chain of ten sex chromosomes shares genes with the bird Z and mammal X chromosomes. *Nature* 2004; 432: 913–917.
103. Chowdhary BP, Raudsepp T, Frönicke L, Scherthan H. Emerging patterns of comparative genome organization in some mammalian species as revealed by Zoo-FISH. *Genome Res* 1998; 8: 577–589.
104. Trifonov V, Yang F, Ferguson-Smith MA, Robinson TJ. Cross-species chromosome painting in the Perissodactyla: delimitation of homologous regions in Burchell's zebra (*Equus burchelli*) and the white (*Ceratotherium simum*) and black rhinoceros (*Diceros bicornis*). *Cytogenet Genome Res* 2003; 103: 104–110.
105. Yang F, Alkalaeva EZ, Perelman PL, Pardini AT, Harrison WR, O'Brien PCM, Fu B, Graphodatsky AS, Ferguson-Smith MA, Robinson TJ. Reciprocal chromosome painting among human, aardvark, and elephant (superorder Afrotheria) reveals the likely eutherian ancestral karyotype. *Proc Natl Acad Sci USA* 2003; 100:1062–1066.
106. Chowdhary BP, Raudsepp T. Chromosome painting in farm, pet and wild animal species. *Methods Cell Sci* 2001; 23: 37–55.
107. Carbone L, Nergadze SG, Magnani E, Misceo D, Cardone MF, Roberto R, Bertoni L, Attolini C, Piras MF, de Jong P, Raudsepp T, Chowdhary BP, Guerin G, Archidiacono N, Rocchi M, Guilotto E. Evolutionary movement of centromeres in horse, donkey, and zebra. *Genomics* 2006; 87: 777–782.

108. Chowdhary BP, Raudsepp T. Mapping genomes at the chromosome level. In: Ruvinsky R, Marshall Graves J, eds. *Mammalian Genomics*. Oxford, UK: CABI Publishing 2005, 23–66.
109. Kohn M, Högel J, Vogel W, Minich P, Hildegard K, Graves JAM, Hameister H. Reconstruction of a 450-My-old ancestral vertebrate protokaryotype. *Trends Genet* 2006; 22: 203–210.
110. Murphy WJ, Stanyon R, O'Brien SJ. Evolution of mammalian genome organization inferred from comparative gene mapping. *Genome Biol* 2001;2:REVIEWS0005 (<http://genomebiology.com/2001/2/6/reviews/0005.1>).
111. Solinas-Toldo S, Lengauer C, Fries R. Comparative genome map of human and cattle. *Genomics* 1995; 27: 489–496.
112. Ventura M, Archidiacono N, Rocchi M. Centromere emergence in evolution. *Genome Res* 2001; 11: 595–599.
113. Breen M, Langford CF, Carter NP, Fischer PE, Marti E, Gerstenberg C, Allen WR, Lear TL, Binns MM. Detection of equine X chromosome abnormalities in equids using a horse X whole chromosome paint probe (WCPP). *Vet J* 1997; 153: 235–238.
114. Ianuzzi L, Molteni L, DiMeo GP, DeGiovanni A, Perucatti A, Succi G, Incarnato D, Eggen A, Cribiu EP. A case of azoospermia in a bull carrying a Y-autosome reciprocal translocation. *Cytogenet Cell Genet* 2001; 95: 225–227.
115. Mastromonaco GR, Coppola G, Crawshaw G, DiBerardino D, King WA. Identification of the homologue of the bovine Rob(1;29) in a captive gaur (*Bos gaurus*). *Chromosome Res* 2004; 12: 725–731.
116. Molteni L, Perucatti A, Iannuzzi A, DiMeo GP, DeLorenzi L, DeGiovanni A, Incarnato D, Succi G, Cribiu E, Eggen A, Iannuzzi L. A new case of reciprocal translocation in a young bull: rcp(11;21)(q28;p12). *Cytogenet Genome Res* 2007; 116: 80–84.
117. Ramos PS, Bitgood JJ, Ponce de Leon FA. Novel chromosomal insertional translocation in chicken uncovered by double color FISH. *Anim Biotechnol* 1999; 10: 119–122.
118. Switonski M, Szczerał I, Skorczyk A, Yang F, Antosik P. Robertsonian translocation (8;14) in an infertile bitch (*Canis familiaris*). *J Appl Genet* 2003; 44: 525–527.
119. Zimin AV, Cornish AS, Maudhoo MD, Gibbs RM, Zhang X, Pandey S, Meehan DT, Wipfler K, Bosinger SE, Johnson ZP, Tharp GK, Marcais G, Roberts M, Ferguson B, Fox HS, Treangen T, Salzberg SL, Yorke JA, Norgren RB Jr. A new rhesus macaque assembly and annotation for next-generation sequencing analysis. *Biol Direct* 2014; 9(1): 20.

Contributed protocol section

IMPORTANT: No protocol included in this manual should be used for clinical testing unless the laboratory performing the procedure has properly validated that the test performs as expected and provides accurate and adequate results. Each laboratory should also consult the manufacturer's SDS for handling instructions, safety warnings, disposal, and labeling requirements for all chemicals used in the laboratory.

Common abbreviations in protocol description

The following abbreviations appear in the protocol section of this chapter.

Cfg: centrifuge

ddH₂O: double-distilled water

f.c.: final concentration

MetHb: (methemoglobin): A brownish-red crystalline organic compound formed in the blood when hemoglobin is oxidized either by decomposition of the blood or by the action of various oxidizing drugs or toxic agents. It contains iron in the ferric state and cannot function as an oxygen carrier.¹

q.s.: Latin quantum satis (as much as is enough).²

1. The *American Heritage® Medical Dictionary* Copyright © 2007, 2004 by Houghton Mifflin Company. Published by Houghton Mifflin Company. All rights reserved.
2. q.s. (n.d.). *The American Heritage® Abbreviations Dictionary*, 3rd ed. Retrieved July 16, 2011, from Dictionary.com website: <http://dictionary.reference.com/browse/qs>

Lymphocyte collection and processing

Protocol 24.1 Blood feather collection

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Materials

One Ziploc bag

II. Method

1. Locate blood feathers (feathers that are just beginning to emerge--see Figure 24.13) in the wing or tail by gently extending the wing so that each feather is visible or gently fanning the tail feathers. The older feathers will conceal the blood feathers, so it is necessary to look carefully at the base of the feathers and underneath the older feathers until a blood feather is located. Most adult birds usually have at least one blood feather at all times, but it can take several minutes of careful searching to locate them.
2. Once the feather is located, grasp the shaft at the base, as near to the follicle as possible and gently pull the feather out, being careful not to squeeze out the pulp contained within the feather shaft. Collect as many blood feathers as possible and place them into a CLEAN Ziploc bag (or similar bag). Label the bag with the ID of the bird and immediately place the bag in a small box or similar container that will prevent the feather from being crushed. It is very important that the pulp remain within the shaft and is not squeezed out. Protect the feather from extreme heat or cold. DO NOT FREEZE. See Protocol 24.12 for method to establish primary cultures from feather pulp.

Protocol 24.2 Avian lymphocyte culture (for large birds)

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Materials and reagents

For supplier's catalog number and contact information, refer to Tables 24.3 and 24.4, respectively.

- Iscove's Modified Dulbecco's Media (IMDM) supplemented with 1% L-Glutamine–Penicillin–Streptomycin
- Chicken serum



Figure 24.13 Blood feathers. In this photo only the top two feathers are blood feathers. The largest feather is nearly fully grown and does not contain sufficient pulp. See insert for color representation of this figure.

- 1-mL syringes
- 3-mL syringes
- 5-mL syringes
- 21G 1½ inches
- 15-mL centrifuge tubes
- Corning T25 flasks (nonfilter cap)
- Na heparin
- Heparinized vacutainer, 4 mL
- Phytohemagglutinin mitogen (PHA)
- Phorbol 12-myristate 13-acetate-4-0-methyl ether (working solution 200 µg/mL)
- Colcemid (working solution 10 µg/mL)
- Hypotonic: 0.067 M KCl
- Fixative “Fix”: 3 : 1 methanol–glacial acetic acid
- Ficoll Paque
- HBSS

Blood collection

1. Birds must be off feed for 3 hours prior to bleeding; otherwise you will get a heavy lipid contamination of the lymphocytes.
2. Using sterile technique draw approximately 0.1 mL of sodium heparin into a 5-mL syringe. Coat barrel of syringe.
3. Draw 2 mL of whole blood from brachial vein.
4. Invert syringe several times to mix blood and heparin.
5. If blood is to be transported a long distance, it should be transferred to a sterile heparinized vacutainer, 4 mL size. DO NOT remove vacutainer top to inject blood. Inject using sterile technique *through* the green top. Blood should be transported at ambient temperature and should be received at laboratory facilities within 24 hrs.

II. Method (use sterile technique throughout)

This technique requires the use of autologous plasma/buffy coat in the culture media. The plasma therefore must be allowed to settle out from the cell fraction.

1. Change the needle on the syringe to a new 21G 1½ inch needle. If the blood has been received in a vacutainer, transfer blood to a 5-mL syringe, then change the needle.
2. Tape the syringe against a wall (where it will not be disturbed) in a vertical position – needle up. If the ambient temperature is very warm, tape the syringes to an inside wall of a refrigerator.
3. Let blood settle out 2–3 hours, until there is clear separation of the plasma and red blood cells.

Table 24.3 Supply list. Purchasing information (item, part or catalog #, and supplier) for materials described in the protocol section of this chapter are listed in this table for quick reference. To locate a supplier's contact information, refer to Table 24.4. This list is not considered an endorsement for any product or supplier

Item	Part or catalog no.	Supplier
1-mL syringe	309602	Fisher Scientific
3-mL syringe	309585	Fisher Scientific
10-mL syringe	309604	Fisher Scientific
20G 1½ inch needles	NN*2038R	Fisher Scientific
21G 1 inch needles	305165	Fisher Scientific
22G 1 inch needles	5155	Fisher Scientific
22G × 3½ inch Sensi-Touch Spinal needles		
23G 1¼ inch needles	305120	Fisher Scientific
26G ½ inch needles	305111	Fisher Scientific
2-propanol	A416-1	Fisher Scientific
3×3 gauze sponges	22257155	Fisher Scientific
15-mL centrifuge tubes	05-539-1	Fisher Scientific
50-mL centrifuge tubes	05-539-6	Fisher Scientific
12.5 cm ² flask, Falcon	08-772-1E	Fisher Scientific
25 cm ² flask, Corning	430168	Fisher Scientific
25 cm ² Easy Flask filter cap, Nunc	156367	Fisher Scientific
75 cm ² flask, Corning	10-126-41	Fisher Scientific
8-mL Glass Culture Vial, Wheaton	225144	Fisher Scientific
Acetic acid, glacial	A38-500	Fisher Scientific
Actinomycin D (AMD)	3298	Merck & Co., Inc.
AmnioMAX™II complete medium (Gibco®)	11269-016	Life Technologies
Antibiotic-antimycotic (Gibco®)	15240-062	Life Technologies
Barium hydroxide (Ba(OH) ₂)	B46-250	Fisher Scientific
Cellgro Minimum Essential Medium (MEM) Alpha 1×	15-012-CY	Fisher Scientific
Chicken serum (Gibco®)	16110-082	Life Technologies
Clonetices FGM-2 SingleQuots	CC-4126	Lonza, Inc.
Clonetetics Fibroblast Basal medium	CC-3131	Lonza, Inc.
Colcemid™, KaryoMax® (Gibco®)	15212-012	Life Technologies
Cryogenic vials, internal threaded 2 mL	431386	Corning Inc.
Cryogenic vial cap inserts	430499	Corning, Inc.
Derma Punch Biopsy punch 2 mm	na	Huot Instruments
Derma Punch Biopsy punch 6 mm	na	Huot Instruments
Dimethyl sulfoxide (DMSO) Hybri-Max	D2650	Sigma-Aldrich, Inc.
Disposable pipette 10mL	13-678-11E	Fisher Scientific
Disposable pipette 5mL	13-678-11D	Fisher Scientific
Ethanol (ETOH)	36280-8	Sigma-Aldrich, Inc.
Ethidium bromide (EtBr)	E-1510	Sigma-Aldrich, Inc.
Ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA)	E5134	Sigma-Aldrich, Inc.
Fetal bovine serum (FBS)	16000	Life Technologies
Ficoll-Paque, Pharmacia		

Table 24.3 (Continued)

Forceps; curved, nonserrated	72910-7	EMS
Forceps; tissue, 1 × 2 teeth	72916	EMS
Fungin™	ANT-FN-1	InvivoGen
Gentamicin Solution	G1397	Sigma-Aldrich, Inc.
Gurr® buffer tablets	10582-013	Life Technologies
Giemsa Stain Improved R66 Solution	35086HE	CTL Scientific Supply Corp
Hanks' balanced salt solution (HBSS) (Gibco®)	14170	Life Technologies
Harleco Wright-Giemsa stain, EM Science	742	VWR
HyClone L-15 Leibovitz medium	SH30525.01	Fisher Scientific
Iscove's Modified Dulbecco's media (1×), (Gibco®)	12440-053	Life Technologies
Lectin	L-8777	Sigma-Aldrich, Inc.
L-Glutamine–penicillin–streptomycin solution stabilized	G6784	Sigma-Aldrich, Inc.
Liberase™	5401127001	Roche Diagnostics Corporation
Methyl alcohol, anhydrous	ME 151	Gallade Chemical, Inc.
Normocin™	ant-nr-1	InvivoGen
Pasteur pipette 5 3/4 inch	14673-010	VWR
Pasteur pipette 9 inch	13-678-20D	Fisher Scientific
Petri dish, glass	89000-300	VWR
Phorbol 12-myristate 13-acetate 4-O-methyl ether	P-3766	Sigma-Aldrich, Inc.
Phytohemagglutinin HA15 (PHA)	30852701	Fisher Scientific
Plate, ground glass	NA	NA
Potassium chloride (KCl)	P217-500	Fisher Scientific
Prep Blades, Weck	NC0006843	Fisher Scientific
Scalpel, No. 10 Disposable	089275A	Fisher Scientific
Sodium (Na) heparin salt solution	78083491	Patterson Veterinary
Sodium chloride (NaCl)	S271-3	Fisher Scientific
Sodium citrate, citric acid	S279-500	Fisher Scientific
Sodium phosphate, dibasic	S-0876	Sigma-Aldrich, Inc.
Sodium phosphate, monobasic	S-9638	Sigma-Aldrich, Inc.
Square media bottle, 125 mL	03-312-1	Fisher Scientific
Square media bottle, 250 mL	03-312-3	Fisher Scientific
SS Surgical blades No. 11	BD371211	VWR
SS Surgical Blades No. 10	82029-834	VWR
Stericap and Steritop Filtration System 150 mL	SCGVU01RE	Fisher Scientific
Trypsin 1:250	T-4799	Sigma-Aldrich, Inc.
Trypsin 250, Difco	215230	BD Bioscience, Inc
Trypsin-EDTA, (Gibco®)	25300-062	Life Technologies
Vacutainer 3.0 mL, ACD	O268877	Fisher Scientific
Vacutainer 8.5 mL, ACD	364606	Fisher Scientific
Vacutainer 10.0 mL, sodium heparin	367874	Fisher Scientific
Vinblastine sulfate USP (Velban)		
Whirl Pack bags, 18 oz.	B00736	VWR

Table 24.4 Supplier contact information. Contact information for the suppliers of the material listed in Table 24.3, and within the protocol section of this chapter, is provided in this table for a quick reference. This list is not considered an endorsement for any supplier

1. BD Bioscience, Inc. Sparks, MD 21152 800-675-0908 www.bdbiosciences.com	9. InvivoGen 3950 Sorrento Valley Blvd. Suite 100 San Diego, CA 92121 888-457-5873 www.invivogen.com
2. Corning Incorporated Corning, NY 14831 607-974-9000 www.corning.com	10. Lonza, Inc. 8830 Biggs Ford Rd. Walkersville, MD 21793-0127 301-898-7025 www.lonza.com
3. CTL Scientific Supply Corp. 1016 Grand Blvd # 3 DeerPark, NY11729 631-242-4249 http://www.ctlscientific.com	11. Merck & Co., Inc. 770 Sumneytown Pike P.O. Box 4 West Point, PA 19486 www.merck.com
4. Electron Microscopy Sciences (EMS) 800-523-5874 1560 Industry Rd. Hatfield, PA 19440 www.emsdiasum.com	12. Patterson Veterinary 137 Barnum Road Devens, MA 01434 800-225-7911 www.pattersonvet.com
5. Fisher Scientific 6722 Bickmore Ave. Chino, CA 91710 800-766-7000 www.fishersci.com	13. Roche Diagnostics Corporation 9115 Hague Ave. Indianapolis, IN 46250-0414 800-428-5433 www.roche.com
6. Gallade Chemical, Inc. 425 North Andreasen Dr. Escondido, CA 92029 760-489-0798 http://www.galladechem.com/	14. Sigma-Aldrich, Inc. 3050 Spruce St. St. Louis, MO 63103 314-771-5765 www.sigmaaldrich.com
7. Huot Instrument, LLC N50 13704 Overview Drive, Suite A Menomonee Falls, WI 53051 866-212-8466 www.startrol.com	15. VWR 1310 Goshen Parkway West Chester, PA 19380 800-932-5000 https://us.vwr.com
8. Life Technologies™ 9800 Medical Center Dr. P.O. Box 6482 Rockville, MD 20849 800-338-5772 www.lifetechnologies.com	

Preparation of culture flasks

4. Into one T25 culture flask place:
 - a. 19 mL of media (Iscove's)
 - b. 1 mL of chicken serum
 - c. 200 µL of heparin
5. Carefully remove syringe from wall and *bend needle until it is pointing downward*. Keeping syringe upright, gently express and discard the first few drops of plasma until there are no red blood cells discernible in the drops.

6. Add to the media in the flask as much plasma/buffy coat as possible one drop at a time, being careful not to add any red blood cells (or as few as possible).

Note: Avian red blood cells are nucleated and will compete with the lymphocytes, however will not provide useful metaphase spreads.

7. Ficoll-Paque separation of lymphocytes: (*Perform this step only if you have a small volume of blood or poor separation in step 3, otherwise proceed to step 8*).
 - a. Add approximately 10 mL of Ficoll–Paque to a sterile 15-mL centrifuge tube.
 - b. Put a new 21G needle on the syringe containing the residual blood.
 - c. Draw up an equal volume of HBSS as residual blood into the syringe. Gently invert to mix.
 - d. Layer this mixture on top of Ficoll gradient *very gently*.
 - e. Spin at 2000 RPM for 20–25 minutes.
 - f. Using sterile Pasteur pipette, draw off supernatant and discard.
 - g. Using sterile technique, pipette off white blood cell layer into a new 15-mL centrifuge tube containing 10 mL of HBSS.
 - h. Spin at 1000 RPM for 10 minutes. Using sterile technique aspirate off all but 0.25 mL supernatant.
 - i. Re-suspend purified lymphocytes in the remaining supernatant and add to the prepared culture flask. (If you have a very large amount of purified lymphocytes, add to additional flasks.)
8. Split the media/lymphocyte suspension between two T25 flasks (approximately 10.5 mL).
9. Add mitogens to each flask (record time):
 - a. 250 µL of lectin
 - b. 250 µL of PHA
 - c. 250 µL of phorbol
10. Incubate for 68–72 hours at 40°C.

Harvesting

For quick analysis add colcemid at 29 hours and harvest at 31 hours. Otherwise a normal harvest is done between 68 and 72 hours with a 1½ hour colcemid incubation.

1. On day 3, after approximately 68 hours add 50 µL of Colcemid to each culture and re-incubate for 1½ hours. (While you wait, place hypotonic solution in the water bath.)
2. Transfer contents of each culture flask to a 15-mL centrifuge tube. Centrifuge tubes at 1500 RPM for 15 minutes. Aspirate supernatant, leaving 0.5 mL, being careful not to disturb the pellet. Re-suspend pellet gently with a Pasteur pipette.
3. Add 5 mL of 0.067 M KCl (37°C). Re-suspend gently and place in water bath (37°C) for 10–20 minutes. Meanwhile, prepare fresh fix.
4. Add 3 drops of fix to the hypotonic suspension and mix gently with a Pasteur pipette.
5. Centrifuge at 1000 RPM for 10 minutes. Aspirate all but 0.5 mL of the supernatant, being careful not to disturb the pellet.
6. Re-suspend pellet and remaining supernatant well. Take up all of this material into your Pasteur pipette. Add 5 mL of fix to the tube. Place the pipette with your cells into the fix and carefully release the suspension in a steady stream, thoroughly mixing with a Pasteur pipette. Centrifuge at 1000 RPM for 10 minutes.
7. Rinse at least two more times with 5 mL of fix as in step 6, except reduce centrifugation to 1000 RPM for 10 minutes, re-suspending pellet well between each rinse. After the third rinse and spin, leave enough supernatant to make a cloudy cell suspension. This, of course, is dependent on the size of the pellet, but usually leaving approximately 0.5 mL of supernatant is an adequate amount. You are now ready to make slides. If slides are not to be made immediately, do not remove supernatant after last rinse; refrigerate (if to be used within 1–2 days) or freeze in liquid nitrogen until ready to use.

Note: The preceding protocol for avian lymphocyte culturing is optimized for condor/vulture bloods. We have had varying degrees of success using this exact protocol with other avian species.

III. Additional readings and references

Biederman BM, Lin CC. A leukocyte culture and chromosome preparation technique for avian species. *In Vitro* 1982; 18(4); 415–418.

Studies in avian karyology. In van Brink JM, Kiauta B, eds. *Genetica* 1984; 65; 117.

Wiley JE, Meisner LF. Synergistic effect of TPA and T-cell mitogens in nonmammalian vertebrates. *In Vitro* 1984; 20(12);

932–936.

Macgregor H, Varley J, eds. *Working with Animal Chromosomes*. New York: Wiley and Sons, 1983.

Protocol 24.3 Lymphocyte culture using whole blood

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Materials

For supplier's catalog number and contact information, refer to Tables 24.3 and 24.4, respectively.

- 1-mL syringes
- 3-mL syringes
- 21G needles
- 15-mL centrifuge tubes
- Corning T25 flasks (unfiltered caps)
- Completed Alpha MEM
- Lectin
- Na heparin
- Phytohemagglutinin mitogen (PHA) (9 mg/mL)
- Phorbol 12-myristate 13-acetate-4-O-methyl ether (working solution 200 µg/mL)
- Actinomycin D (working solution 100 µg/mL) (AMD)
- Colcemid (working solution 5 µg/mL)
- Ethidium bromide (stock solution 10 mg/mL) (EtBr)
- Hypotonic solution: 0.05 M KCl (37°C)
- Water bath (37°C)
- Fixative "Fix": 3:1 methanol–glacial acetic acid (make fresh daily)

II. Method (use sterile technique throughout)

Keep track of timing so you can start the harvest in 4 days; 92–96 hours after the addition of the mitogens. Harvest will begin on day 4, approximately 4 hours earlier than the addition of the mitogens (i.e., if you added mitogens at 1400, then start harvest at 1000.)

1. Using sterile technique, draw approximately 0.1 mL of heparin into a syringe; draw 5–10 mL of blood and immediately invert syringe several times to mix blood and heparin, or use a sterile heparinized vacutainer for collection. The heparin is an anticoagulant and prevents the blood from clotting. Keep the blood at ambient temperature. If atmospheric temperature is excessive, blood should be kept cool (with a cold pack in proximity, but not touching). DO NOT FREEZE!
2. Into each of the three T25 culture flasks place:
 - a. 9.5 mL of completed Alpha MEM
 - b. 0.1 mL of Na heparin
 - c. 0.5 mL of heparinized blood

Flask A	Flask B	Flask C
250 µL lectin	250 µL lectin	250 µL PHA
250 µL phorbol	250 µL PHA	250 µL phorbol
		250 µL phorbol

- d. Make sure blood drops into the media and not down the sides of the tube. Use slow, steady pressure on the syringe; otherwise the blood will spurt out.
- e. Lectin and PHA are two of a number of mitogens which stimulate lymphocytes to undergo cell division. For each new species a variety of flasks is set up with different mitogens. In general, lectin works well for most hoof stock. The primates work well with PHA.

- f. Phorbol is used as a comitogen. When used in conjunction with other mitogens, enhanced mitogenic response has been observed. This chemical is highly toxic and care should be taken since it is used in conjunction with DMSO. **ALWAYS WEAR GLOVES!** For more information about the use and toxicity of this compound refer to the SDS.
3. Gently mix contents of each flask, loosen caps and place flasks upright in an incubator (37°C) with 5–6% CO₂. If a CO₂ incubator is unavailable, use a closed system by capping flasks tightly and use a HEPES buffered media to maintain pH.
4. Gently mix cultures daily.
5. Late on day 3 change the media in the cultures. Transfer contents of each culture flask to sterile 15 mL centrifuge tube and centrifuge for 15 min at 1500 RPM. Aspirate supernatant leaving 0.5 mL above the pellet; re-feed with fresh, warmed media and place back in the original flask. (It is not necessary to add new mitogens).
6. **Harvesting:** On day 4, after approximately 92 hours add 50 µL of Colcemid and re-incubate for 30–60 minutes. (While you wait, place hypotonic solution in the water bath).
 - OR:** After 92–96 hours add 300 µL of 100 µg/mL stock solution of AMD, after 50 minutes add 50 µL of Colcemid and incubate for an additional 10 minutes at 37°C.
 - OR:** After 92–96 hours add 50 µL of Colcemid + 10 µL EtBr for 30–45 minutes.

Note: Treatment of cell cultures with Colcemid and its analogs arrests dividing cells in metaphase by preventing spindle formation, thereby assuring an adequate number of cells at a stage of division satisfactory for studying chromosomes. EtBr and the antibiotic AMD have been shown to induce chromosome elongation or stretching, which facilitates higher resolution G-banded chromosomes. However, AMD and EtBr will decrease the mitotic index of your harvest.

1. Transfer contents of each culture flask to a 15-mL centrifuge tube. Centrifuge tubes at 1500 RPM for 15 minutes. Aspirate supernatant, leaving 0.5 mL, being careful not to disturb the pellet. Re-suspend pellet gently with a Pasteur pipette.
2. Add 5 mL of 0.05 M KCl (37°C). Re-suspend gently and place in water bath (37°C) for 30–45 minutes. Longer hypotonic incubation times are necessary when using AMD or EtBr to ensure good spreading of the high-resolution chromosomes. Meanwhile, prepare fresh fix.
3. Add 3 drops of fix to the hypotonic suspension and mix gently with a Pasteur pipette.
4. Centrifuge at 1500 RPM for 15 minutes. Aspirate all but 0.5 mL of the supernatant, being careful not to disturb the pellet.
5. Re-suspend pellet and remaining supernatant well. Take up all of this material into your Pasteur pipette. Add 5 mL of fix to the tube. Place the pipette with your cells into the fix and carefully release the suspension in a steady stream, thoroughly mixing with a Pasteur pipette. Centrifuge at 1500 RPM for 15 minutes. (The fix treatment will lyse the remaining red blood cells, turning the hemoglobin brown and fix the lymphocytes.)
6. Rinse at least two more times with 5 mL of fix as in step 11, except reduce centrifugation to 1000 RPM for 10 minutes, re-suspending pellet well between each rinse. After the third rinse and spin, leave enough supernatant to make a cloudy cell suspension. This, of course, is dependent on the size of the pellet, but usually leaving approximately 0.5 mL of supernatant is an adequate amount. You are now ready to make slides. If slides are not to be made immediately, do not remove supernatant after last rinse; refrigerate (if to be used within 1–2 days) or freeze in liquid nitrogen until ready to use.

III. Additional readings and references

- Barch MJ, Knutsen T, Spurbeck JL, eds. *The AGT Cytogenetics Laboratory Manual*, 3rd ed. New York: Lippincott-Raven, 1997.
- Rybak J, Tharapel A, Robinett S, Garcia M, Mankinen C, Freeman M. A simple reproducible method for prometaphase chromosome analysis. *Human Genetics* 1982; 60: 328–333.
- Wiley JE, Meisner LF. Synergistic effect of TPA and T-cell mitogens in nonmammalian vertebrates. *In Vitro* 1984; 20(12): 932–936.

Protocol 24.4 Lymphocyte culture using autologous plasma/buffy coat (AP/BC)

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Principle

This technique utilizes autologous plasma and buffy coat lymphocytes (ap/bc) in place of whole blood.

II. Materials

For supplier's catalog number and contact information, refer to Tables 24.3 and 24.4, respectively.

- 1-mL syringes
- 3-mL syringes
- 21G needles
- 15-mL centrifuge tubes
- Corning T25 flasks (nonfilter cap)
- Completed Alpha MEM
- Na heparin
- Lectin
- Phytohemagglutinin mitogen (PHA) (9 mg/mL)
- Phorbol 12-myristate 13-acetate-4-O-methyl ether (working solution 200 µg/mL)
- Actinomycin D (working solution 100 µg/mL) (AMD)
- Colcemid (working solution 5 µg/mL)
- Ethidium bromide (working solution 10 mg/mL) (EtBr)
- Hypotonic: 0.05 M KCl
- Fixative "Fix": 3 : 1 methanol–glacial acetic acid (make fresh daily)
- Spinal needles 3½ inch, 18 or 20G
- Water bath (37°C)

III. Method (use sterile technique throughout)

1. Using sterile technique, collect 5 mL of whole blood and transfer to a heparinized vacutainer. Spin blood at 1500 RPM for 15 minutes.
2. Gently remove the top from the spun tube. Using a 3-mL syringe with an 18 or 20 gauge spinal needle, carefully remove the buffy coat and plasma layer (ap/bc) without disturbing the red blood cells. Pick up 0.5 mL of plasma per culture. Invert syringe several times to mix contents.

Note: Lymphocytes recovered from 5 mL of blood should be sufficient for 2–3 culture flasks. However, the lymphocyte count on some animals or species may require a higher or lower concentration of cells per culture. You must work it out for your particular species, but in general we use lymphocytes recovered from 5 mL of whole blood for two 10-mL cultures. You must also test for the optimal mitogen to use for a particular species. If dealing with a species not encountered before, we set up a series of three flasks as set out below. From this test, you should be able to determine which mitogen works best for subsequent cultures for that species.

Set up two or three T25 flasks (depending on how much ap/bc recovered) as follows:

- 9.5 mL completed Alpha MEM
- 100 µL sodium heparin
- 0.5 mL ap/bc
- 250 µL phorbol

Flask A	Flask B	Flask C
250 µL lectin	250 µL PHA	250 µL lectin
		250 µL PHA

- Make sure blood drops into the media and not down the sides of the tube. Use slow, steady pressure on the syringe; otherwise the blood will spurt out.
- Lectin and PHA are two of a number of mitogens which stimulate lymphocytes to undergo cell division. For each new species a variety of flasks is set up with different mitogens. In general, lectin works well for most hoof stock. The primates work well with PHA.
- Phorbol is used as a comitogen. When used in conjunction with other mitogens, enhanced mitogenic response has been observed. This chemical is highly toxic and care should be taken since it is used in conjunction with DMSO. ALWAYS WEAR GLOVES! For more information about the use and toxicity of this compound refer to the SDS.

3. Gently mix contents of each flask, loosen caps and place flasks upright in an incubator (37°C) with 5–6% CO_2 . If a CO_2 incubator is unavailable, use a closed system by capping flasks tightly and use a HEPES buffered media to maintain pH.
4. Gently mix cultures daily.
5. Late on day 3, change the media in the cultures. Transfer contents of each culture flask to sterile 15-mL centrifuge tube and centrifuge for 15 minutes at 1500 RPM. Aspirate supernatant leaving 0.5 mL above the pellet; re-feed with fresh, warmed media and place back in the original flask. (It is not necessary to add new mitogens).
6. **Harvesting:** On day 4, after approximately 92 hours add 50 μL of Colcemid and re-incubate for 30–60 minutes. (While you wait, place hypotonic solution in the water bath).

OR: After 92–96 hours add 300 μL of 100 $\mu\text{g}/\text{mL}$ stock solution of AMD; after 50 minutes add 50 μL Colcemid and incubate for an additional 10 minutes at 37°C

OR: After 92–96 hours add 50 μL of Colcemid + 10 μL of EtBr for 30–45 minutes.

Note: Treatment of cell cultures with Colcemid and its analogs arrests dividing cells in metaphase by preventing spindle formation, thereby assuring an adequate number of cells at a stage of division satisfactory for studying chromosomes. EtBr and the antibiotic AMD have been shown to induce chromosome elongation or stretching, which facilitates higher resolution G-banded chromosomes. However, AMD and EtBr will decrease the mitotic index of your harvest.
7. Transfer contents of each culture flask to a 15-mL centrifuge tube. Centrifuge tubes at 1500 RPM for 15 minutes. Aspirate supernatant, leaving 0.5 mL, being careful not to disturb the pellet. Re-suspend pellet gently with a Pasteur pipette.
8. Add 5 mL of 0.05 M KCl (37°C). Re-suspend gently and place in a water bath (37°C) for 30–45 minutes. Longer hypotonic incubation times are necessary when using AMD or EtBr to ensure good spreading of the high-resolution chromosomes. Meanwhile, prepare fresh fix.
9. Add 3 drops of fix to the hypotonic suspension and mix gently with a Pasteur pipette.
10. Centrifuge at 1500 RPM for 15 minutes. Aspirate all but 0.5 mL of supernatant, being careful not to disturb the pellet.
11. Re-suspend pellet and remaining supernatant well. Take up all of this material into your Pasteur pipette. Add 5 mL of fix to the tube. Place the pipette with your cells into the fix and carefully release the suspension in a steady stream, thoroughly mixing with a Pasteur pipette. Centrifuge at 1500 RPM for 15 minutes then aspirate the supernatant. (The fix treatment will lyse the remaining red blood cells, turning the hemoglobin brown and fix the lymphocytes.)
12. Rinse at least two more times with 5 mL of fix as in step 11, except reduce centrifugation to 1000 RPM for 10 minutes, re-suspending pellet well between each rinse. After the third rinse and spin, leave enough supernatant to make a cloudy cell suspension. This, of course, is dependent on the size of the pellet, but usually leaving 0.5 mL of supernatant is an adequate amount. You are now ready to make slides. If slides are not to be made immediately, do not remove supernatant after last rinse; refrigerate (if to be used within 1 - 2 days) or freeze in liquid nitrogen until ready to use.

IV. Additional readings and references

- Barch MJ, Knutsen T, Spurbeck JL, eds. *The AGT Cytogenetics Laboratory Manual*, 3rd ed. New York: Lippincott-Raven, 1997.
- Rybak J, Tharapel A, Robinett S, Garcia M, Mankinen C, Freeman M. A simple reproducible method for prometaphase chromosome analysis. *Human Genetics* 1982; 60: 328–333.
- Wiley JE, Meisner LF. Synergistic effect of TPA and T-cell mitogens in nonmammalian vertebrates. *In Vitro* 1984; 20(12); 932–936.

Protocol 24.5 Horse lymphocyte culture method

Contributed by Molecular Cytogenetics Laboratory, M.H. Gluck Equine Research Center, University of Kentucky, Lexington, KY

I. Materials and reagents

For supplier's catalog number and contact information, refer to Tables 24.3 and 24.4, respectively.

- **RPMI-1640 with Glu + Hepes**, store in dark at 4–8 °C.
- **L-Glutamine**, liquid, aliquot and store at –20 °C.
- **Antibiotic-antimycotic**, liquid, store at –20 °C.
- **FBS - Fetal Bovine Serum**, heat inactivated, mycoplasma tested, aliquot in 20 mL portions and store at –20 °C.
- Na heparin, 10 mL = 10,000 units, use 3 drops from 25G needle per 9.5 mL of medium (100 $\mu\text{L}/10\text{ mL}$ medium). Store at room temp.

- **Phorbol**, working solution 200 µg/mL; 5 mg of phorbol is dissolved in 10 drops DMSO (break off glass top and add DMSO to vial), aliquot 25 mL of complete medium into a sterile 50-mL cfg tube. Add 1 mL medium to glass vial and mix with phorbol/DMSO. Transfer to cfg and rinse vial. Mix gently. Filter through 0.2 µm **nylon** filter. Aliquot and store at -20 °C until ready to use.
- **Colcemid**, Zoo Stock: KaryoMax stock solution 10 µg/mL; working solution 5 µg/mL made by diluting stock 1 : 1 with PBS. Store at 4 °C.
- **Lectin** from pokeweed. Sterile. Resuspend in 5 mL of sterile PBS. Aliquot and store at -20 °C.
- **Pokeweed Mitogen**, 1 mg powder in vial, reconstitute with 1 mL sterile PBS or medium. Filter sterilize through 0.2-µm syringe filter into sterile tubes (conc 1 µg/µL). Add 250 µL to each 10 mL culture. Recommended amount is 25 µL but doesn't work. Store stock at -20 °C for at least 1 year.
- **KCl hypotonic solution** –0.067 M KCl, 2.49 g potassium chloride q.s. to 500 mL with sterile mpH₂O; or 0.075 M KCl, 2.79 g q.s. to 500 mL. Place at 37 °C prior to use. Store at 4 °C.
- **Fixative** – 3 : 1 (75 mL methanol; 25 mL acetic acid). Put in bottle and place at -20 °C. Must make fresh each day.
- **Slides** – To clean slides place Fisher Premium slides in acetone up to a level just below the specimen label, use glass beaker. Cover with foil and let sit overnight under fume hood. Next day pour off acetone, back into bottle and mark "used." Rinse slides under tap water, soak in alconox solution for several hours, rinse well in tap water then distilled water. Fill beaker 70% ethanol, cover will foil and mark with contents and store at 4 °C until needed. Take number of slides needed out of beaker and wipe dry with KimWipe. Air dry. Rinse in distilled water. Fill small beaker with distilled water and slides, chill at 4 °C and slides are ready for slide making.
- Medium

RPMI-1640 with Glu + HEPES
 1% L-glutamine
 1% Antibiotic–antimycotic
 10% FBS
 Aliquot 9.5 mL into T25 flasks

II. Method

1. Centrifuge whole blood for 20 minutes at 1000 RPM to separate buffy coat. One buffy coat from 6–10 mL of blood will inoculate two T25 flasks containing 9.5 mL of medium.
2. Add 3 drops (100 µL) sodium heparin. Determine which combination of phorbol, lectin, PWM and/or PHA to add to each flask. This depends on number of blood tubes sent by vet.
 Always set up a lectin and a lectin/phorbol flask as it may work best:

F1	phorbol + lectin
F2	phorbol + lectin
F3	lectin + PWM
F4	lectin + PWM
Use:	250 µL lectin
	250 µL PWM
	250 µL phorbol

3. Pull buffy coat with autologous plasma to give 1 mL. Add 0.5 mL to each flask of 5 mL medium. Total: 9.5 mL complete medium
 3 drops Na heparin
 + desired phorbol/lectin/PWM combination
 add 0.5 mL buffy coat + autologous serum
4. Culture 68–70 hours then add 50 µL Colcemid (Zoo Colcemid Concentration: stock = 10 µg/mL; working dilution = 5 µg/mL; final conc = 0.2 µg/mL). Incubate for 30 minutes (15–30 minutes may work too). Pour flask contents into 15 mL conical bottom centrifuge tube.
5. Centrifuge at 1000 RPM in our centrifuge to pellet. Remove all but 0.5 mL supernatant and resuspend pellet by flicking tube with finger or by gentle pipetting with Pasteur pipette. *It is very important that the cells are resuspended well and not clumped.* Add 6 mL 0.067 M KCl prewarmed to 37 °C. Invert tube to mix. Incubate 30 minutes at 37 °C.

6. Add 3 drops of 3 : 1 fix and invert tube to mix. Cfg at 1000 RPM in our centrifuge. Remove all but 0.5 mL of supernatant.
7. Pull all the suspension into the pipette. Add 6 mL of fix to tube and slowly drop cells from pipette into the fix. Add more fix to a total volume of 8–10 mL. Resuspend cells by inversion. Cfg and remove supernatant as before. Repeat this fixative change at least two more times using 10 mL of fix. The pellet should be almost white.
8. Dilute pellet with fix until mixture is slightly cloudy. Cell density determination takes some experience. Drop 5–6 drops onto clean, wet slides. Allow to air dry at temp about **70 °F (21.1 °C)** at **55–60% humidity**. Check spread quality by phase contrast microscopy or by Giemsa staining prior to making a lot of slides. Use only cells from best preparation for FISH or G-banding. Be sure to record results in Clinical Case logbook.

If spreading is not good, try changing fix to 2 : 1 methanol–acetic acid.

Protocol 24.6 Rhino blood culture

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Materials

For supplier's catalog number and contact information, refer to Tables 24.3 and 24.4, respectively.

- 1-mL syringes
- 3-mL syringes
- 21G needles
- Corning T25 flasks (nonfilter cap)
- 15-mL centrifuge tubes
- Completed Alpha MEM (**NOTE:** RPMI media works equally well.)
- Na heparin
- Lectin
- Phytohemagglutinin mitogen (PHA) (9 mg/mL)
- Phorbol 12-myristate 13-acetate-4-O-methyl ether (working solution 200 µg/mL)
- Velban (0.5 µg/mL)
- Actinomycin D (working solution 100 µg/mL) (AMD)
- Colcemid; working solution 5 µg/mL
- Hypotonic: 0.075 M KCl
- Fixative "Fix": 3 : 1 methanol–glacial acetic acid (make fresh each day)
- Spinal needles 3½ inch, 18 or 20G
- Water bath (37 °C)

II. Method (use sterile technique throughout)

1. At least 10 mL of blood is needed. Blood should be drawn into a sterile heparinized vacutainer tube filled only half full. If full, extra heparin (0.1–0.2 mL) should be added. Mix well.
2. The blood sample will be setup using two different conditions:
 - a. Flask A is set up using whole blood.
 - b. Flasks B and C use autologous plasma and buffy coat (ap/bc) recovered after the blood has been spun.
Remove 0.5 mL of whole blood for Flask A and proceed to step 3 for ap/bc recovery for Flasks B and C.
3. Centrifuge blood at 1500 RPM for 15 minutes. Carefully remove the ap/bc using a spinal needle and syringe and mix well.
(Avoid disturbing the red blood cells). Split this ap/bc between flasks B and C.
4. Set up three T25 flasks (A, B, C) as follows:

A	B and C
9.5 mL completed Alpha MEM	9.5 mL completed Alpha MEM
0.5 mL whole blood	0.5 mL ap/bc
6 drops heparin	6 drops heparin
250 µL lectin	250 µL lectin
250 µL phorbol	250 µL phorbol

Note: The duplicate flasks B and C allow you to test different harvest timings or other harvest procedures, such as PHA instead of lectin as the mitogen. See Protocol 24.3 Lymphocyte Culture Using Whole Blood for additional information.

5. Gently mix contents of each flask, loosen caps and place flasks upright in an incubator (37°C) with 5–6% CO₂. If a CO₂ incubator is unavailable, use a closed system by capping flasks tightly and use a HEPES buffered media to maintain pH.
6. Late on day 3 change the media in the cultures. Transfer contents of each culture flask to sterile 15 mL centrifuge tube and centrifuge for 15 minutes at 1500 RPM. Aspirate supernatant leaving 0.5 mL above the pellet; re-feed with fresh, warmed media and place back in the original flask. (It is not necessary to add new mitogens.)
7. **Harvesting:** On day 5 at 114 hours add 0.2 mL of AMD and 0.2 mL of Velban. Re-incubate for 1 hour. (While you wait, place hypotonic solution in the water bath).
8. Transfer contents of each culture flask to 15-mL centrifuge tubes. Centrifuge tubes at 1500 RPM for 15 minutes. Aspirate supernatant leaving 0.5 mL above the pellet. Re-suspend the pellet gently using a Pasteur pipette.
9. Add 5 mL of 0.075 M KCl (37°C). Re-suspend gently and place in water bath (37°C) for 30 minutes. Meanwhile, prepare fresh fix.
10. Add 3 drops of fix to the hypotonic suspension and mix gently with a Pasteur pipette.
11. Centrifuge at 1500 RPM for 15 minutes. Remove all but 0.5 mL of supernatant above the pellet, being careful not to disturb the pellet.
12. Re-suspend pellet and remaining supernatant well. Take up all of this material into your Pasteur pipette. Add 5 mL of fix to the tube. Place the pipette with your cells into the fix and carefully release the suspension in a steady stream, thoroughly mixing with a Pasteur pipette. Centrifuge at 1500 RPM for 15 minutes. (The fix treatment will lyse the remaining red blood cells, turning the hemoglobin brown and fix the lymphocytes.)
13. Rinse at least two more times with 5 mL of fix as in step 12, except reduce centrifugation to 1000 RPM for 10 minutes, re-suspending pellet well between each rinse. After the third rinse and spin, leave enough supernatant to make a cloudy cell suspension. This, of course, is dependent on the size of the pellet but usually leaving 0.5 mL of supernatant is an adequate amount. You are now ready to make slides. If slides are not to be made immediately, do not remove supernatant after last rinse; refrigerate (if to be used within 1–2 days) or freeze in liquid nitrogen until ready to use.

Solid tissue collection and long-term culturing/processing

Protocol 24.7 Organ tissue collection protocol from carcass

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Method

1. Wet the entire carcass in 70% ETOH and blot with sterile absorbent gauze.
2. Use sterile lens scissors to remove the skin and feathers.
3. With fresh sterile scissors, cut through the body wall and aseptically retrieve the desired tissues using sterile tweezers and sterile scissors.
4. Immediately place tissue in biopsy vials with transport medium (provided) to keep tissue moist. Seal well with Parafilm and ship as soon as possible using an insulated container to protect sample from temperature extremes.

Protocol 24.8 Skin biopsy procedure

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

IMPORTANT: Tissue cultures must be sterile; bacteria or fungi kill the cultures. The fresher the sample is, the better it will grow. No antiseptic (such as iodine-based antiseptic scrub) should be used before biopsy.

I. Materials

(For supplier's catalog number and contact information, refer to Tables 24.3 and 24.4, respectively.)

1. Electric clippers
2. Straight-edge razors
3. 70% alcohol

4. Sterile gauze
5. Sterile forceps and scalpel
6. Biopsy vial with Growth medium: Cellgro Minimum Essential Medium Alpha 1×, (Fisher Scientific) with 10% FBS, supplemented with 1% L-glutamine penicillin–streptomycin solution stabilized (Sigma-Aldrich) and 1% Gibco antibiotic–antimycotic.

II. Method

1. Using electric clippers, clip the hair in the area to be sampled, if excessive.
2. Moisten with alcohol.
3. Use single-edged razor blade and shave closely so that all hair is removed from the area.
4. Use a 70% alcohol-drenched sponge to clean the shaved spot *very, very* well.
5. Get the recipient bottle ready, which contains tissue culture medium. Using sterile forceps and scalpel, grasp a piece of skin from the center of the shaved area and cut off a full thickness of dermis about the size of a small bean or pea. Place immediately into recipient bottle and close cap tightly.
6. Using a permanent marker, label the biopsy vial with the species name, gender, date, animal identification number (ISIS # and/or house ID) and biopsy location.
7. If the biopsy cannot be set up the same day as it was collected, keep the vial refrigerated until the sample can be processed. Biopsies that were collected completely aseptically can be held in the refrigerator for up to 3 weeks before processing, as long as no contamination develops; however, it is best to set up the biopsy as soon after collection as possible.
8. Discard scalpel in biohazard sharps container. Should you contaminate the scalpel accidentally before biopsy by touching hair, etc., then use a new instrument.

Protocol 24.9 Placenta biopsy procedure

Contributed by Dr. Kurt Benirschke, University of California, San Diego

I. Principle

1. The placenta has the same genotype as the fetus;
2. At times it is the only tissue available;
3. It often grows better than fetal tissues and, most importantly, even in stillborns, (and perhaps at times in cases of macerated fetuses), the placental biopsy will still be available. Naturally, it should be sampled as soon as possible after birth.

Type of tissue

The best tissue to grow in the placenta (see Figure 24.14) is from the fetal surface and to take tissues from the surface chorion with some smaller fetal blood vessels included.

II. Method

Collection

Pour a little alcohol/methanol over the fetal surface of the placenta to ‘sterilize’ it somewhat. Then, with sterile tweezers, peel off the amnion that is never totally attached (just pushed against) the underlying chorion.

Harvest

Next, sterilize (or have available) a small pair of scissors and take a small piece of surface placental tissue chorion that has a small vessel with it, with tweezers and scissors, and place it into the tissue culture fluid (see Protocol 24.8, Skin biopsy procedure).

Culture

Culture follows the same way as fibrous tissue culture. See Protocol 24.14 Preparation of primary cultures using enzyme digestion.

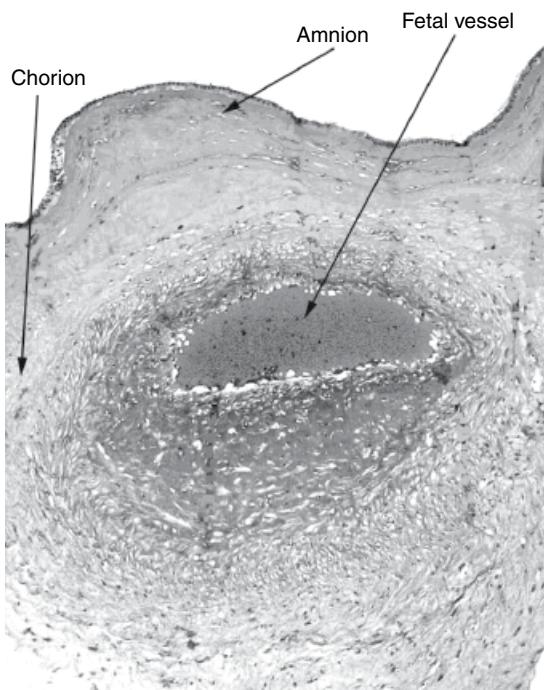


Figure 24.14 Placental parts.

Protocol 24.10 Freezing of fibroblast cell cultures

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Materials

Refer to Tables 24.3 and 24.4 for supplier's catalog and contact information, respectively.

1. CoolCell® (alcohol free) or Mr. Frosty® Freezing Container (needs isopropyl alcohol)
2. -80°C freezer
3. Liquid nitrogen freezer
4. 15-mL centrifuge tubes
5. Completed Alpha MEM
6. Dimethyl sulfoxide (DMSO)
7. HBSS
8. 0.05% Trypsin-EDTA
9. 2 mL of cryogenic freezing vials (cryo vials)
10. Ice bath

II. Method

Use sterile technique throughout.

1. Enter all data into freeze book or appropriate database and note freeze number in regular logbook.
To determine the number of vials to freeze: one T75 flask yields approximately 2 vials (depending upon confluency):
 - a. Multiply the # of T75 flasks by 2.
 - b. Add 1 additional vial for the viability.
 - c. This is the total number of vials to be frozen (e.g., four T75 flasks would yield 9 vials).
(The "+1" is the viability vial and should not be allotted a space in the official records since it is to be thawed immediately for testing).

2. Label a 2-mL cryogenic freezing vial with appropriate identifiers, including as much information as possible. An example of our labeling system follows:

Example:	
Lab # - Freeze #	3324-550
Sex	Male
Type of Animal	Black rhino
Date	12 Dec 81
Passage #:	P5

3. Prepare freeze media using completed Alpha MEM; add DMSO to a final concentration of 10% and mix in a centrifuge tube (make fresh each day). Immediately place on ice. Make enough to allow for 1 mL of freeze media per vial to be frozen.
4. Remove cells from flasks as follows:
 - a. Aspirate the media from flask without disturbing the cells.
 - b. Add 2 mL of HBSS to the flask to rinse all media out. Gently rock the flask to coat the cells in HBSS and then aspirate.
 - c. Add 2 mL of trypsin-EDTA to the flask and gently rock the flask to make sure all cells are coated. Place on warming tray (37°C) until cells are detached (~1–2 minutes). Rap sides of flask gently to detach cells. If cells are slow to detach, it may be necessary to remove the initial trypsin-EDTA and add fresh trypsin-EDTA. Check under microscope to make sure all cells are removed.
 - d. Add 8 mL of HBSS and rinse all cells out with a pipette, transferring rinse into a labeled centrifuge tube. (Adjust the amount of HBSS used to rinse the flasks depending upon the number of flasks you are freezing; i.e., for 4 flasks, use 2 mL of HBSS for the first rinse and pool all the flasks together for a total of 10 mL.) Do a second rinse with 5 mL of HBSS and also place into the 15-mL tube.
5. Centrifuge for 10 minutes at 1000 RPM. Remove supernatant, leaving approximately 0.5 mL of HBSS to avoid disturbing the cell pellet.
6. Add appropriate amount of freeze media (i.e., 1 mL/vial) to the cell pellet and re-suspend by gently pipette-mixing. Make sure to break up any clumps of cells. Dispense 1 mL of cell suspension per vial.
7. Place samples in freezing container and put in –80°C freezer for a minimum of 4 hours.
8. Remove vials from container and place them in appropriate assigned spaces in liquid nitrogen tank. Remove viability test vial (see later).

Thawing and checking a viability

1. Immediately thaw the frozen vial in a water bath (37°C). Do not immerse. This should take less than 5 minutes to thaw.
2. Wipe the vial with 70% ethanol and allow it to dry.
3. Carefully pour contents of vial into T25 flask containing 4 mL of appropriate media.
4. Incubate in a 5–6% CO₂ incubator at appropriate temperature.
5. Check after 24 hours for the percentage of cells that have reattached and started dividing. Rank this percentage from poor to excellent for future reference.
6. Record viability in freeze log or appropriate database. The scoring of the viability is purely subjective and varies from person to person and with each cell line. Fill in the table below with your personal notes on viabilities. Base your score on:
 - a. Confluence
 - b. Cell morphology
 - c. Amount of floating cells
7. When the viability becomes confluent, split the flask into two T25 flasks. The second T25 will provide you with a back up flask for harvesting.

Poor	Fair	Good	Very Good	Excellent
<25% confluent				Almost confluent in 24 hours
Mostly dead, unattached floating cells				Very few/no dead unattached cells

Protocol 24.11 Freezing tissue biopsy samples for later initiation of cell culture (tissue piecing)

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Materials

Refer to Tables 24.3 and 24.4 for supplier's catalog and contact information, respectively.

1. Ground glass plate and/or Petri dish
2. Biopsy vial with 5 mL of biopsy medium
3. Completed Alpha MEM
4. Dimethyl sulfoxide (DMSO)
5. 15- or 50-mL centrifuge tubes
6. HBSS supplemented with 1% P/S/F
7. Pipettes (5 and 10 mL)
8. Forceps and scalpel in 70% ethanol
9. 2 mL of cryogenic freezing vials (cryo vials)
10. Liquid nitrogen
11. Ice bath

II. Method

Use sterile technique throughout.

1. Take biopsy sample as described in Protocol 24.8 Skin biopsy procedure. A 6 mm² biopsy will provide enough material for one frozen vial.
2. Prepare freeze medium using completed Alpha MEM; add DMSO to a final concentration of 10%, and mix in a centrifuge tube (make fresh each day). Immediately place on ice. Make enough to allow for 1 mL of freeze media per vial of tissue to be frozen.
3. On a ground glass plate (or Petri dish) mince tissue biopsy into tiny fragments (0.5–1 mm³) with sterile forceps and scalpel. Use clean cuts in a rocking motion; do not shred or tear the tissue. Do not let fragments dry out; keep moistened with media or HBSS. The larger the pieces, the greater chance that the DMSO will not penetrate the tissue, resulting in cell death.
Note: If biopsy sample was not adequately prepared at initial sampling, it may be necessary to rinse the tissue in HBSS or medium and remove any excessive amount of hair, fur, etc. This usually needs to be done if working from ear notch samples.
4. Gather tissue pieces with forceps and carefully place into sterile cryo vial.
5. Add 1 mL of cold freeze media to each vial. Cap carefully but securely, making sure that silicone gasket does not bulge out. If capped too tightly, liquid nitrogen will seep inside and cause contamination and/or explosion upon thaw. Gently agitate cryo vial to expose all tissue pieces to the freeze medium.
6. Label vials with appropriate information, using a water and alcohol resistant marker.
7. Place directly into liquid nitrogen for storage.

Note: Tissue that has been frozen in this manner tends to take longer to initiate growth and may also be a slow growing culture in general.

To thaw a tissue piece

Use sterile technique throughout.

1. Quick-thaw sample in a water bath (37°C). Wipe the outside of the vial with 70% ethanol-drenched gauze. Transfer contents of vial to a 15 mL centrifuge tube containing 10 mL of HBSS.
2. Centrifuge at 1000 RPM for 10 minutes.
3. Remove as much supernatant as possible. If the pieces look adequately diced, proceed with step 3 in Protocol 24.18 Preparation of primary cultures using enzyme digestion. If the tissue pieces were frozen in larger fragments than looks reasonable for adequate digestion, remove the washed tissue to a sterile Petri dish and dice the pieces smaller. Proceed with step 3 in Protocol 24.18.

Alternate method

For use when a centrifuge isn't available.

1. Using a sterile Petri dish, pour HBSS + P/S/F into the dish, enough to cover the tissue pieces.
2. Wipe the outside of the vial with 70% ethanol and allow it to dry. Pour the contents of the vial into the Petri dish.
3. Carefully rinse the tissue pieces in the fresh HBSS with the forceps. If the pieces look adequately diced, proceed with Protocol 24.18 Preparation of primary cultures using enzyme digestion.

Protocol 24.12 Preparation of primary cultures from feather pulp

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Materials

Refer to Tables 24.3 and 24.4 for supplier's catalog and contact information, respectively.

1. Whirl-Pak® or other clean plastic bags
2. 0.125 mg/mL Liberase™ (i.e., collagenase)
3. Ground glass plate and/or Petri dish
4. 70% ethanol
5. Gauze sponge
6. Scalpel and forceps in 70% ethanol
7. 15-mL centrifuge tubes
8. HBSS + 1% Pen-Strep Fungizone®
9. FGM supplemented with 20% FBS and 1% Pen-Strep-L-Glutamine
10. T12.5 or T25 culture flasks

II. Method

Sample collection

Large Birds (storks, cranes, eagles, etc.)

1. Pull a total of three feathers (primary, secondary and tail feathers work best).
2. At three weeks (21 days) for storks and cranes or approx. 5 weeks for eagles (check at week 3, 4, and 5), the regenerating feathers will be optimal for obtaining feather pulp. It is important to collect the regenerating feather just as the feather begins to emerge from the shaft. Carefully pull the regenerated feather by grasping it as close to the base as possible, being certain not to squeeze the pulp out in the process.
3. One optimal regenerated feather will yield approximately 0.2 mL of pulp, which is sufficient material to set up four T25 flasks in dilutions of 40%, 30%, 20%, and 10% (see step #7 below in Culture Protocol). If a larger volume of pulp is recovered, adjust flask dilutions to smaller percentages or use only half of the pulp.

Small Birds (tanagers, doves, etc.)

1. Pull at least three feathers (primary, secondary and tail feathers are the only ones large enough to work with).
2. At 1 or 2 weeks the regenerating feathers will be optimal for obtaining feather pulp. It is important to collect the regenerating feather just as the feather begins to emerge from the shaft. Carefully pull the regenerated feather by grasping it as close to the base as possible, being certain not to squeeze the pulp out in the process.
3. Three optimal regenerated feathers will yield very little pulp. Place **all** of this material into one T12.5 flask (see Culture protocol next).

Culture protocol

Use sterile technique throughout.

1. Remove the feather from plastic bag, wipe the shaft with a gauze sponge soaked in 70% ethanol, and place onto the glass plate (or Petri dish).

2. Using scalpel and forceps, cut the shaft of the feather lengthwise to expose the feather pulp. Grasp the pulp with the forceps and peel it out of the shaft. It is usually easiest to grasp the distal end of the pulp. Place it in a small amount of HBSS +1% P/S/F (approximately 0.3 mL), and mince it with clear cuts of the scalpel. Do not shred or tear the pulp. Rinse carefully in HBSS to remove blood cells or feather debris from the tissue.
3. Gather the minced pulp into a clump. Using the forceps, push the pieces to the bottom of a 15 mL conical tube.
4. Add ~0.3–0.5 mL Liberase™ to the pulp so it is completely covered. Gently agitate to expose all surfaces of the pulp to the enzyme. Be careful not to let any of the pulp stick to the side of the tube above the liquid.
5. Loosen the cap and place the tube into incubator (37°C).
6. Incubate for approximately 1–6 hours until the pulp begins to look slightly digested. During the incubation the tube should be gently agitated approximately once each half hour to check on the progress of the digestion.
7. When the pulp looks sufficiently digested, add 4–5 mL of medium (or 1.5 mL of medium for a T12.5) to the tube and pipette-mix the digested tissue mass to dislodge any cells that are loosely attached to the pulp. Place entire contents of the tube into appropriate size culture flask. Depending on the amount of pulp recovered, it may be necessary to make serial dilutions of the cell material in order to obtain proper cell confluence for harvesting.
8. Incubate the flask(s) at 40°C with 5–6% CO₂.
9. The next day examine the flask(s) under the microscope for fibroblast cells. If there is good cell growth, replace half of the media to remove floating feather debris.

Protocol 24.13 Preparation of primary cultures from solid tissue (explants)

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Materials

Refer to Tables 24.3 and 24.4 for supplier's catalog and contact information, respectively.

- Warming tray
- Ground glass plate and/or Petri dish
- Forceps and scalpel in 70% ethanol
- 70% ethanol
- T25 culture flasks
- T75 culture flasks
- Pipettes (5 and 10 mL)
- Completed Alpha MEM (or species appropriate media)
- HBSS +1% P/S/F
- HBSS
- 0.05% trypsin-EDTA

II. Method

Use sterile technique throughout.

1. Place biopsy on a sterile glass plate (or Petri dish) with a few drops of HBSS +1% P/S/F. Clean away any additional hair/debris that was not removed during the sampling process. Mince the tissue biopsy into tiny fragments (approximately 0.5 mm³) with sterile forceps and scalpel. Use clean cuts in a rocking motion, do not shred or tear the tissue. **Do not let fragments dry out;** keep moistened with media or HBSS.
 2. Move a few pieces of tissue to a dry area of the plate/dish and dab to remove excess liquid. Carefully place these pieces of tissue onto the back/bottom of a T25 culture flask. Continue in this manner, a few pieces at a time, until all tissue has been placed in the flask.
- Note:** Space the fragments so that there is sufficient room for outgrowth, approximately 2–3 mm apart.
3. Briefly place on a warming tray (37°C), approximately 1–2 minute, until the fragments have adhered, but DO NOT let the pieces dry out!
 4. Carefully add 5 mL of complete media per flask without disturbing the pieces. Incubate at species appropriate temperature with 5–6% CO₂.
 5. After 24 hours check cultures. If there are many fragments floating in the media, replant these pieces into another flask.

6. Check and feed the cultures as needed (3 times a week). The feeding schedule may be varied based on cell growth rate. For slow growing cultures feeding 2 times a week may be sufficient.
 7. When outgrowth is sufficient to cover the bottom of the flask the cells must be “passed” to a larger flask (secondary culture). Remove the media. Rinse with 1 mL HBSS. Remove HBSS. Add 1 mL trypsin-EDTA solution and swirl around flask to coat. Place flask on warming tray set at 37°C until cells have detached.
 8. Add 9.5 mL of media to the flask and swirl around to re-suspend all the cells in the media. Transfer to a T75 culture flask. Re-feed the 1° culture with 4.5 mL media. Incubate both 1° and 2° cultures. Secondary cultures should be checked every other day and fed accordingly.
 9. Cultures may be frozen when sufficient numbers of 2° flasks have reached confluence or harvested when active growth is most evident.
- Note:** Cultures set up in this manner generally take longer to initially produce cells, however in some cases the cell morphology may be better than with enzyme digestion. This method is recommended for many amphibian cell cultures.

Protocol 24.14 Preparation of primary cultures using enzyme digestion

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Materials

Refer to Tables 24.3 and 24.4 for supplier’s catalog and contact information, respectively.

1. 0.125 mg/mL Liberase™ (Roche: i.e., collagenase)
2. Ground glass plate and/or Petri dish
3. Scalpel and forceps in 70% ethanol
4. 15-mL centrifuge tubes
5. HBSS with 1% P/S/F
6. 5-mL pipettes
7. Pasteur pipettes
8. Completed Alpha MEM (or species appropriate medium)
9. T25 culture flask

II. Method (use sterile technique throughout)

1. Using forceps, place the biopsy on the glass plate with a few drops of HBSS + 1% P/S/F. DO NOT LET THE SAMPLE DRY OUT. Clean away any additional hair/debris that was not removed during the biopsy sampling process. (If the HBSS becomes dirty with debris, place a few drops of fresh HBSS on a clean area of the glass plate and move the tissue over before cutting.) Using scalpel and forceps, cut the tissue into small squares (approximately 0.5 mm³). Use clean cuts in a rocking motion; do not shred or tear the tissue. Smaller pieces will digest more rapidly than larger pieces.
2. Using the forceps, gather the pieces together in a clump and push them to the bottom of a 15 mL conical tube. It is easiest to do this with the pieces in a clump rather than individually.
3. Add sufficient Liberase™ (approximately 0.3–0.5 mL) to the pieces so they are completely covered and mix thoroughly, keeping the pieces in the bottom of the tube and covered with the enzyme.
4. Loosen cap and place the tube in a 37°C incubator. (This is the optimal temperature for the action of collagenase. The only exception is amphibians, which are incubated at room temperature.)
5. About once each hour, gently agitate the tissue pieces in the tube being careful not to let any pieces stick to the side of the tube above the collagenase.
6. After 3–6 hours the tissue pieces should look “chewed” (crisp edges become fuzzy looking) and the collagenase becomes cloudy with cells and tissue debris.
7. When the tissue looks sufficiently digested, add 4–5 mL of media to the tube and pipet the digested tissue pieces and cell pellet in and out of a 5-mL pipette to dislodge any cells that are loosely attached to the tissue. Place entire contents of the tube into a T25 flask (primary culture; 1°). If the pieces are still too large to pipet, place the medium in the conical tube, agitate to re-suspend the pieces, and carefully pour the contents into your primary flask.
8. Distribute the media and cells evenly on the bottom of the flask with a gentle rocking/swirling motion and incubate at 37°C (or species appropriate temperature), with 5–6% CO₂, in a humidified incubator.

9. Check the next day for contamination and examine under the microscope for small patches of cells (there may be only a few patches of 3–10 cells). If a few or no cell patches are seen, replace approximately 2.5 mL of medium and observe again in 2 days. If many patches are seen, replace approximately 4.5 mL of medium. Be careful not to remove any pieces of tissue while feeding as these may still attach to the flask and produce cells.
10. When outgrowth is sufficient (i.e., cells almost completely cover the flask) the cells must be “passed” to a larger flask (secondary culture; 2°).

Protocol 24.15 Harvesting of fibroblast cell cultures

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Materials

Refer to Tables 24.3 and 24.4 for supplier’s catalog and contact information, respectively.

- Colcemid: working solution 5.0 µg/mL
- 15-mL centrifuge tubes
- HBSS
- 0.05% trypsin-EDTA
- 0.05 M KCl (37°C)
- Fixative “Fix”: 3 parts absolute methanol to 1 part glacial acetic acid (make fresh each day)
- Pasteur pipettes (5¾ inch) and bulbs
- Water bath (37°C)
- Pipettes (5 and 10 mL)

II. Method

1. Check cultures for optimal growth of cells. This usually occurs approximately 24 hours after a 1 : 2 passage. Evidence of mitotic doublets and rounded-up cells should be evident and *abundant*.
2. To each T25 flask add 50 µL Colcemid for 15 minutes to 1 hour prior to the start of harvest. (The timing varies for each species and must be found through trial and error. For example, most birds require approximately 4 hours in Colcemid.) **Note:** The longer exposure of cells to Colcemid will increase the mitotic index, however, the length of the chromosomes will also decrease. Shorter chromosomes provide less information during analysis.
3. Transfer culture fluid to a 15-mL centrifuge tube. Add 1 mL of HBSS to the flask to rinse all media out. Pour this rinse into your centrifuge tube. Add 1 mL trypsin-EDTA to the flask and make sure all the cells are coated. Wait 1–2 minutes. Rap sides of flask gently to detach cells. Check under microscope to make sure all cells are removed. Add 3 mL of HBSS and rinse the flask, placing the cell/HBSS mixture in the 15-mL tube. If necessary perform an additional rinse with 5 mL of HBSS for a total of 15 mL in the centrifuge tube.
4. Centrifuge for 10 minutes at 1000 RPM. Remove supernatant, leaving approximately 0.5 mL of HBSS. Re-suspend pellet gently with Pasteur pipette.
5. Add 5 mL of 0.05 M KCl (37°C). Re-suspend gently. Incubate in a water bath (37°C) for 10–60 minutes. Incubation time varies for different cultures; you will have to find the best times by trial and error. Meanwhile, prepare fix.
6. Add 3 drops of fix to the cells and hypotonic solution while gently agitating the tube.
7. Centrifuge at 1000 RPM for 10 minutes.
8. Remove all but 0.5–1 mL of supernatant. Re-suspend *well by pipette mixing with the Pasteur pipette*. Carefully pull up the suspension into the Pasteur pipette. Add approximately 5 mL of fix to the tube. Hold the pipette approximately 2 inches above the fix and release the cell suspension slowly into it. (Alternate method: place the pipette with your cells into the fix and carefully release the suspension in a slow steady stream.) Re-suspend thoroughly with pipette. Spin down at 1000 RPM for 10 minutes.
9. Wash with fix (step 8) at least two more times.
10. After the last rinse, depending on size of pellet, leave approximately 0.5 mL of fix to make a cloudy suspension. You are now ready to make slides.

III. Additional readings and references

Schubert BA, Meilinger CA, Anderson MA, Meyer KJ, Spurbeck JL, Stupca PJ, Jalal SM (2007). Chromosome spreading is a function of time and temperature of hypotonic solution. *The Journal of the Association of Genetic Technologists* 2007; 33(1); 178–182.

FISH analysis preparation

Protocol 24.16 Preparation of competitor DNA for FISH hybridization

Contributed by Molecular Cytogenetics Laboratory, M.H. Gluck Equine Research

I. Materials and reagents

Refer to Tables 24.3 and 24.4 for supplier's catalog and contact information, respectively.

- 10× Nick Translation (NT) buffer
- 0.5 M EDTA
- 20% SDS
- M Na acetate
- 70% and 95% ETOH
- DNase Working Solution
- TE

II. Method

1. Combine DNA samples to give a total of 1 mg DNA.

2. Prepare **10× nick translation buffer**

0.5 M Tris-Cl pH 7.8–8.0

50 mM MgCl₂

0.5 mg/mL BSA

Stock

1 M MgCl	20.33 g/100 mL ddH ₂ O, sterilize
----------	--

1 M Tris-Cl	12.1 g free base/100 mL ddH ₂ O, pH to 8.0 with conc HCl
-------------	---

10 mg/mL BSA	1.0 g BSA Fraction V/100 mL sterile ddH ₂ O; filter- sterilize and store at 4 °C.
--------------	--

Working dilution

50 mL 1 M Tris-CL

5 mL 1 M MgCl₂

5 mL 10 mg/mL BSA

q.s. to 100 with sterile ddH₂O

3. Add 500 µL of 10x NT Buffer to DNA and bring up volume to 5 mL with sterile DNase free water.

4. Shear DNA-buffer solution through 10 cc syringe with 18G needle 2x, then 2x thru 22G and 30G needles. Vortex to remove air bubbles and place in 15 °C water bath.

5. On ice, add 1 µL of DNase I stock solution (1 mg/mL) to 1000 µL H₂O (cold, iced) to give DNase working solution.

6. Add 10 µL of DNase working solution to the genomic DNA-NT buffer mix. Incubate at 15 °C in water bath for 2–12 hours.

7. After 2 hours stop reaction by placing DNA at -20 °C and test 7 µL of DNA solution on 2% gel with 1 KB ladder. Denature DNA by heating at 95 °C for 5 minutes, ice 2 minutes prior to loading on gel.

8. If DNA not at 300–500 bp fragment size, add 10 µL of fresh DNase working solution until desired fragment size is reached. Test every 2 hours on gel and add fresh DNase solution each time.

9. Terminate the reaction by adding 125 µL of 0.5 M EDTA (f.c. 10–15 mM) and 25 µL 20% SDS (f.c. 0.1%) to each 5 mL of DNA solution and heat at 65 °C for 20 minutes to inactivate DNase.
10. Split samples into two oakridge tubes and precipitate with 1/10 vol. 2.5 M Na acetate and 2.5× vol. 95% ETOH. Cfg to pellet.
11. Wash pellet in 70% ETOH, cfg, dry in savant and resuspend in 200–400 µL TE. Quantitate on spectrophotometer.
DNase I stock: 1 mg/mL in 50% glycerol/0.15 M NaCl=>1 µg/mL stock. Store at -20 °C.

Protocol 24.17 In situ hybridization of BAC clones labeled with spectrum fluorochromes: probe and slide preparation

Contributed by Molecular Cytogenetics Laboratory, M.H. Gluck Equine Research Center, University of Kentucky, Lexington, KY

I. Materials and reagents

Refer to Tables 24.3 and 24.4 for supplier's catalog and contact information, respectively.

- **40% Dextran Sulfate Stock Solution** in H₂O, autoclave, store at room temp
- **20× SSC Stock Solution**, pH 5.3, store at 4 °C (higher salt = lower stringency, lower salt = higher stringency)
- **20× SSC Stock Solution**, pH 7.0, store at 4 °C; also filter sterilize small aliquots for use with dextran sulfate and store at room temp
- **2× SSC Working solution**, pH 7.0, store at 4 °C
- 20% Dextran Sulfate/2× SSC Working Solution for Hyb: (see preparation below), make fresh each time
- **ETOH Series (2) I** 70%, 90%, 100% at room temp for dehydration of slides from freezer **II** 2–70%, 1–90%, 1–100% at -20 °C and on ice for dehydration of slides after denaturation
- **100% Formamide**, aliquoted and stored at -70 °C
- Competitor DNA (see separate protocol on preparation) (generally at 1.0–1.5 µg/µL concentration)
- **ssDNA Carrier** (see separate protocol on preparation) (stock at 40 mg/mL or 40 µg/µL); working dilution generally about 1–1.5 µg/µL. Current stock at 40 µg/µL, add 3.4 µg stock to 96.6 µL TE → 1.35 µg/µL working dilution
- **Probe** (see Vysis and Lear protocol on preparation) (generally at 15–30 ng/µL)
- **1× PBD**, pH 8.0 at room temperature. Phosphate buffered detergent, see reagent prep cards. Purchased from Oncor. Store at 4 °C
- **DAPI II or III counterstain working solution.** Generally, DAPI concentrations of less than 50 ng/µL works best to produce banding patterns and for the best images
- **Denaturation Solution**
28 mL formamide
4 mL 20× SSC, pH 5.3
8 mL ddH₂O
40 mL final concentration is 70% formamide/2× SSC
Make fresh and pre-warm to 70 °C in a water bath. pH should be around 7.3, but definitely between 7.0 and 8.0.
- **Wash solution**
20 mL formamide
4 mL 20× SSC, pH 5.3
16 mL ddH₂O
40 mL final concentration is 50% formamide/2× SSC.
Make fresh and pre-warm to 37 or 42 °C in a water bath. pH should be around 6.9–7.0.

II. Method

Probe and slide preparation for hybridization

1. Dehydrate slides from -70 °C freezer in **ETOH series I**. Check slides for good metaphase spreads using phase contrast microscopy. Premark best viewing areas with diamond tip pencil. Then age slides that are less than one month old in drying oven at 55–65 °C for 45 minutes.

2. Prewarm the coverslips (usually the 22 mm × 22 mm) you will need on a slide warmer set at 42 °C. Label the slide with the probe name using special color pencils and mark the areas for the different probes with diamond tip pencil.
3. In a 0.5 mL snapcap microcentrifuge tube prepare the Probe Mix:
for one **22 × 22 mm** area:

DNA (conc varies)	X µL	total 100 ng
Competitor DNA	X µL	total 4.0 µg*
ssDNA Carrier	X µL	total 6.0 µg*

For **24 × 50 mm** area: double the reagents.

* The ratios of competitor DNA to ssDNA Carrier should equal 10 µg total for each reaction. Probes with high amount of repeat sequence like BACs may require more competitor DNA to block background hybridization signal, longer preannealing time, or higher wash temperatures. This may need to be determined empirically.

If performing Multicolor FISH use 50–100 ng of each labeled BAC and place them in the same tube. You do not need to add additional competitor or ssDNAs.

Mix the components of the Probe Mix well, cfg briefly, put on vortex shaker for at least 30 minutes. This can be prepared the day before the hybridization and precipitated overnight at 4 °C as in step 5.

4. In a 0.5 mL snapcap microcentrifuge tube, prepare dextran sulfate/2× SSC solution from previously autoclaved or filter sterilized stocks (to reduce background). This can be mixing while you are precipitating your probe mix.

Prewarm stock Dex sulfate to 42–50 °C

For 100 µL:

50 µL 40% Dextran Sulfate Stock*

10 µL 20× SSC

40 µL sterile filtered water

Place in 42 °C water bath to go into suspension, then put on shaker and back to 42 °C water bath. *Be sure to measure the dextran sulfate carefully because it is difficult to pipette. Using graduated pipette tips helps to reduce error.

5. After the Probe Mix is thoroughly mixed, precipitate the solution with **1/10 Volume 3 M Na acetate and 2× Volume Cold 100% ETOH**. Place at –70 °C for 30 minutes to 1 hour or at –20 °C overnight.

Centrifuge for 30 minutes at 12,000 g @ 4 °C (10,000 RPM on our cfg), remove supernatant **carefully** so you don't dislodge the DNA pellet. Gently rinse the pellet in 50 µL of cold 70% ETOH, cfg for 10 minutes at 12,000 g, remove supernatant carefully and dry probe mix in savant vacuum dryer (20–30 minutes).

6. To prepare the Probe/Hyb Mix:

Place the formamide (small aliquot in brown microcentrifuge tube) on shaker to mix briefly. For most hybridizations you will use a **22 × 22 mm** area of the slide. This, add **5.5 µL of formamide** to probe mix, cfg briefly to bring mix off sides of tube, and allow it to mix on the vortex shaker for about 30 minutes. Then add **5.5 µL of dextran sulfate/2× SSC mixture**. Cfg quickly to bring mix off sides of tube. Shake again for 20 minutes.

If the amount of probe has been doubled for a 24 × 50 mm area or for two 22 × 22 mm areas, add 11 µL of formamide to lyophilized Probe Mix, mix on vortex shaker for 30 minutes, then add 11 µL of the 20% dextran sulfate/2× SSC solution; place the mix on the shaker for 20 minutes.

(Final conc. = 50% formamide, 10% dextran sulfate, 1× SSC).

7. Place the Probe/Hyb Mix in a water bath @ 75 °C for 10 minutes to denature the probe. Cfg quickly to bring mix off sides of tube and immediately place the Probe/Hyb Mix in a water bath @ 37 °C for 30 minutes to 1 hour to allow the competitor DNA to preanneal to the repetitive DNAs.

8. While the probe is preannealing, denature the slides for 2 minutes @ 70 °C. Denature no more than 3 slides at a time. Allow for a 1° drop in temperature of denaturation solution for each slide placed together in a Coplin jar. Therefore, if denaturing 3 slides at once, make sure the solution is at 73 °C. *Use specified FISH calibrated thermometers inside the Coplin jar to test temperature of denaturation solution.*

Dehydrate the slides immediately after denaturation by passing them through **cold ETOH series II** stored at –20 °C for 2 minutes each. Place slides on slide warmer. (If processing more than 3 slides put ethanol series in ice bath to keep it cold.)

9. Remove the tubes containing the Probe/Hyb Mix from the 37 °C water bath. Cfg briefly to bring mix off sides of tube. Place tube on slide warmer just prior to putting probe on slide. Place 11 µL (or 22 µL) of the Probe/Hyb mix on the specific slide area and coverslip being careful not to get air bubbles under the coverslip. Seal with rubber cement and place in a humidified chamber overnight @ 37 °C. *Heterologous probes can hybridize up to 5 days, but generally 3 days is sufficient.*

Post hybridization slide washes

Do not allow slides to dry out during the following steps AND keep the lighting level very low after hybridization with probes labeled with Spectrum fluors.

1. Carefully remove the rubber cement with forceps, then gently lift up or slide off the coverslip so you don't scratch the slide. Immerse the slide immediately in the pre-warmed wash solution of **50% formamide/2× SSC** (pH should be around 7.0) – 1× for 5 minutes, 2× for 3 minutes @ 40–42 °C. Be sure to agitate slides every 15 seconds to help remove nonspecific probe hybridization.
Perform all washes at 37 °C for heterologous probes. Be sure the temperature of the solution inside Coplin jar is accurate by measuring with FISH calibrated thermometer.
2. Next, wash slides (BAC clones) 3× at 42 °C in **2× SSC, pH 7.0**, 5 minutes for wash 1, then 3 minutes for washes 2 and 3. Be sure to agitate slides.
For heterologous probes wash 3× in 2× SSC, pH 7.0 at 37 °C 3–5 minutes each (total of 9–15 minutes).
3. Place slides in 1× PBD at room temp and apply 20–30 µL of DAPI II or DAPI III to each slide. Place a glass coverslip (22 mm × 50 mm) on the slide. Place the slide between two sheets of bibulous paper sitting on a hard surface such as the bare bench top. Gently press out air bubbles by sliding your finger along the top sheet of paper directly over the slide.
4. Place the slide in a slide holder, label the holder with the experiment and date. Close the folder with a spring clip. Slides with DAPI should sit for about an hour at room temp so the DAPI is absorbed by the DNA. Store slides at –20 °C when not viewing. After placing oil on the slide put a KimWipe over the slide so the coverslip does not stick to the top of the slide folder.

Protocol 24.18 Labeling DNA with spectrum fluorochromes

Contributed by the San Diego Zoo Institute for Conservation Research, Genetics Division

I. Standard reaction setup (keep on ice and in dark)

Refer to Tables 24.3 and 24.4 for supplier's catalog and contact information, respectively.

1. Turn water bath on to 15 °C. Put clone DNA on shaker. Fill ice bucket, turn down lights and put the following components of Spectrum Kit on the ice. Then, in a 1.5-mL screw cap microfuge tube (with colored caps) mix together the following components in the order listed:

XXX µL of sterile ddH₂O (varies depending on DNA concentration)

XXX µL of clone DNA up to 1 µg ** (DNA plus H₂O cannot exceed 17.5 µL)

2.5 µL of Spectrum Red, Green or Orange

5.0 µL of dTTP

10.0 µL of dNTP

5.0 µL of Buffer

10.0 µL of Enzyme

50.0 µL Total

**(If the DNA you want to label is at 100 ng/µL, you would need 10 µL to give 1 µg or 1000 ng of DNA). The total reaction volume should not exceed 50 µL. So, if your DNA is too dilute it will make the total volume over 50 µL. The DNA must be precipitated, dried and resuspended in less liquid in order to reach the appropriate concentration.

2. Briefly mix and centrifuge components in the tube and immediately place in water bath for 12–24 hours. After 16 hours put tubes on ice and test fragment size on a 2% agarose gel. Do this by taking 3 µL from each tube (plus 1 µL loading buffer) and load on gel with Biomarker Low Ladder (5 µL ladder + 1 µL loading buffer). Run gel at 60–70 volts for about 45 minutes.
3. Check gel. Fragment sizes (smear) should run between 50–300 bp. Spectrum Red may run higher, around 500 bp. Bright glob may show up on the gel; this may be excess fluorochrome.
4. If optimal sizes are achieved stop the reaction by heating at 70 °C for 10 min in water bath or heat block, then place on ice. Add 1/10 volume of 3 M Na acetate + 2.5× volume of cold 100% ethanol place in freezer to precipitate.
5. Centrifuge as usual. Rinse with 70% ethanol. Dry in savant about 20 minutes. Resuspend dried samples in 40 µL TE on shaker 30 minutes. Quantitate on gel plate with standards.
6. If optimal sizes are not reached, add 10 µL of enzyme to each sample and incubate in water bath an additional 2–4 hours depending on the size of the fragments tested previously.

CHAPTER 25

Online genetic resources and references

Wahab A. Khan

Icahn School of Medicine at Mount Sinai, New York, NY, USA

25.1 Introduction

The following is a collection of scientific journals, textbooks, support groups, vendors, exam references and organizations primarily related to the subspecialties of cytogenomics and genetics. This chapter highlights prominent resources available to professionals in the field, as well as the lay public. It is, however, not exhaustive of all possible reference materials. The selected references were compiled from various cytogenetics and molecular genetics laboratories as well as online sources. References on exam study material for licensure in cytogenetics and molecular genetics, support groups, and relevant textbooks are also provided below. Updated editions to textbooks can be found on the publisher's website. All web addresses were retrievable when compiling the information contained in these sections. If you have ebook access to this chapter, and in the event a weblink does not work, please copy and paste the link directly into your web browser and/or search for the reference by its provided name (in bold). AGT is not responsible for the content of external internet sites nor is inclusion (or exclusion) within this list an indication of endorsement for the company or their products.

25.2 Resource information

25.2.1 Databases, laboratory tools and educational tutorials

1. **Atlas of Genetics and Cytogenetics in Oncology and Hematology** provides a referencing system for acquired neoplastic rearrangements. Searches are accessed by chromosome, rearrangement, gene or disease. Discussion includes etiology, epidemiology, clinical manifestations, treatment and prognosis. Laboratory findings seen via cytology, pathology, cytogenetics, molecular and morphological genetics are also described when appropriate, along with gene and protein involvement as a result of a rearrangement. Diagrams and sample photos further aid the user in understanding and recognizing these formations. ISCN idiograms can also be saved from this website. <http://atlasgeneticsoncology.org> or <http://atlasgeneticsoncology.org/dblinks.html#GC>
2. **BAC Resource Consortium** provides a genome-wide list of large-insert clones that have been FISH-mapped and sequence-tagged along with a list of clone distributors to integrate cytogenetic, radiation-hybrid, linkage, and sequence maps of the human genome. <http://www.ncbi.nlm.nih.gov/genome/cyto/hbrc.shtml>
3. **Cancer Chromosome Aberration Project (CCAP)** is an initiative to develop a set of tools to define and characterize chromosomal alterations associated with malignant transformation. This project is supported by the National Cancer Institute (NCI) and the National Center for Biotechnology Information (NCBI). <http://cgap.nci.nih.gov/Chromosomes/CCAP>
4. **Chromosome Abnormalities in Cancer at University of Wisconsin-Madison** provides training tools on understanding basic cytogenetics, learning through case conferences, and links to specific genetic disorders. It also shows thumbnail images of karyotypic changes, where possible, in solid tumors and leukemia. <http://www.slh.wisc.edu/clinical/cytogenetics/cancer/>

5. **College of American Pathologists** provides accreditation and proficiency testing for Cytogenetics and Molecular Genetics laboratories, as well as guidelines for definitive cancer reporting, among other educational and advocacy services. <http://www.cap.org>
6. **dbVAR** of the National Center for Biotechnology Information (NCBI) is a structural variation database designed to store data on variant DNA that is ≥ 1 base pair in size. <http://www.ncbi.nlm.nih.gov/dbvar/>
7. **Developmental Genome Anatomy Project (DGAP)** identifies apparently balanced chromosomal rearrangements in patients with multiple congenital anomalies and uses this information to identify the responsible genes disrupted in critical stages of human development. <http://www.bwhpathology.org/DGAP/>
8. **Drawing Derivative chromosomes** extracts chromosomal gains and losses as well as breakpoints from ISCN cytogenetic nomenclature and converts them into a chromosome ideogram. The ideogram is automatically drawn containing the derivative chromosome, which links to NCBI or Ensembl map viewer. <http://www.cydas.org/OnlineAnalysis/WebExample2.aspx>
9. **Electronic Scholarly Publishing (ESP)** offers classical papers in genetics which can be browsed by author, title or date of publication. <http://www.esp.org/>
10. **GeneTests** is a publicly funded medical genetics information resource that was developed for physicians and researchers. Search options include disease by name, which links to participating clinical sites performing the tests and also includes clinical genetics directories, categorized by service type. A wide range of educational materials, including an illustrated glossary of medical genetic terms, and links to Internet resources that provide information for health professionals and consumers is provided. <http://www.genetests.org>
11. **Gestational age calculator** provides tools for estimating gestational age, best times for prenatal genetic screening, and estimated due date. The Estimated Date of Delivery (EDD) calculator can be accessed through the “professional toolbox” resource. <http://www.perinatalservicesbc.ca/health-professionals/professional-resources>
12. **GraphPad Software** provides an online scientific calculator. Working solution volumes are also featured. Other calculations include statistical distributions and *P* value interpretation, observed vs. expected frequencies (chi square), predictive values from sensitivity, specificity, prevalence, and confidence interval calculations. <http://www.graphpad.com/quickcalcs/Molarityform.cfm>
13. **London Laboratory Services Group**, a joint venture of London Health Sciences Centre in London, Ontario, provides incidence rates of major chromosome abnormalities at birth. <http://www.lhsc.on.ca/lab/cytogen/stats.htm>
14. **mFISH**, created by Thomas Lier, PhD, at the Institute of Human Genetics in Jena, Germany, lists publications on mFISH (multicolor fluorescent *in situ* hybridization), marker chromosomes, and uniparental disomy. <http://www.fish.uniklinikum-jena.de>
15. **Mercer University School of Medicine's** Internet Pathology Laboratory for Medical Education provides an image index of gross pediatric pathology, including malformations and deformations. <http://library.med.utah.edu/WebPath/PEDHTML/PEDIDX.html>
16. **Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer** relates chromosomal aberrations to tumor characteristics, based on individual cases or associations. <http://cgap.nci.nih.gov/Chromosomes/Mitelman>
17. **National Cancer Institute (NCI)** at the National Institutes of Health (NIH) provides online information on cancer diagnosis, treatments, and clinical trials. www.cancer.gov
18. **National Center for Biotechnology Information (NCBI)** provides access to biomedical and genomic information. <http://www.ncbi.nlm.nih.gov>
19. **National Cancer Institute (NCI)** at the National Institutes of Health provides, in layman's terms, a discussion of common cancer types, coping with cancer, and how to find treatments. <http://www.cancer.gov/cancertopics>
20. **National Human Genome Research Institute (NHGRI) Newsroom** provides news updates on the genome, media resources, and educational tutorials. <http://www.genome.gov/ResearchFunding/>
21. **National Society of Genetic Counselors** and the **Canadian Association of Genetic Counselors** provide information on both health care and genetic counseling. <http://www.nsgc.org> and <http://cagc-acgc.ca>
22. **Online Mendelian Inheritance in Man® (OMIM)**, provides full-text overviews of known Mendelian disorders, focusing on the relationship between phenotype and genotype. It is updated daily, and provides links to many other genetics resources. <http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>
23. **Open Directory Project (ODP)** is a directory of resources hosted by Netscape Communication Corporation, including genetic journals, support groups, and informational websites by disease. Because it is primarily dependent on volunteers, not all links may be current. <http://dmoz.org/Science/Biology/Genetics/>
24. **OpenHelix** provides a search portal for genomics resources and training in using these resources. Several narrated tutorials of web-based bioinformatic tools are provided. Unless otherwise specified, a registration fee (personal or institutional) is required for tutorial access. <http://www.openhelix.com/>

25. **PROGENETIX** database for interpreted (“called”) whole genome CGH profiles provides an overview of copy number abnormalities in human cancer from Comparative Genomic Hybridization (CGH) experiments. The database collects genomic gain/loss information of individual cancer and leukemia cases, published in peer-reviewed journals. <http://www.progenetix.org/>
26. **PubMed**, the National Library of Medicine (NLM®) journal literature search system, provides access to information for research, health care, and education. Since 1996, it has provided free access to MEDLINE, the online database that contains journal citations and abstracts for biomedical literature from around the world. <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed>
27. **PubMed Central** provides free access to most life sciences journals. <http://www.ncbi.nlm.nih.gov/pmc/>
28. **PubMed Commons** permits authors to share opinions on scientific findings by commenting on peer-reviewed publications indexed in PubMed. <http://www.ncbi.nlm.nih.gov/pubmedcommons/>
29. **Sanger Institute** is a genomic research center where a series of projects are being carried out to understand the role of genetics in health and disease. <http://www.sanger.ac.uk/>
30. **SKY/M-FISH and CGH Database** allows investigators to share and compare their molecular cytogenetic data. It is open access and users can view an individual investigator’s public data or compare public cases from different investigators. <http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi>
31. **The Cancer Genome Atlas (TCGA)** is part of the National Institutes of Health initiative to identify cancer genes from over 500 samples (deposited as of February 2014) and different cancer subtypes. <http://cancergenome.nih.gov/>
32. **The Center for Applied Genomics (TCAG)** hosts a database of genomic variants that catalogs structural variation in the human genome. One of the aims of the project is to correlate large-scale genomic variation with phenotypic data. TCAG homepage can be found at <http://www.tcag.ca/> and database of genomic variant browser at <http://dgv.tcag.ca/dgv/app/home>
33. **Clinical Genome Resource** contains a growing number of patient cases related to clinical genetic testing using new molecular cytogenetic technologies. It provides clinical decision-making tools to enhance patient care for individuals with phenotypes including intellectual disability, autism, and developmental delay. The consortium consists of a group of clinical cytogenetics and molecular genetics laboratories that are seeking to build a genomic knowledge base to improve patient care through data sharing and collaboration. <http://clinicalgenome.org>
34. **University of Kansas Medical** Genetic Education Center provides clinical, research, and educational resources that are used by educators, students, genetic counselors, families, geneticists, and other health care professionals. The website also provides links for teaching resources in cytogenetics/genomics, e.g., chromosome composition, abnormalities, online karyotyping activities along with references for genetic conditions and numerous clinical resources (i.e., OMIM, GeneReviews). <http://www.kumc.edu/gec/geneinfo.html>

25.2.2 Bioinformatic resources

1. **ARRAYEXPRESS**, accessed via European Bioinformatics Institute (EBI), stores functional genomics experiments, including gene expression, where one can query and download data collected to MIAME (minimum information about a microarray experiment) and MINSEQE (minimum information about a high-throughput sequencing experiment) standards. Gene Expression Atlas contains a subset of archived data which can be queried for individual gene expression under different biological conditions across experiments. <http://www.ebi.ac.uk/services>
2. **BioCyc** is a collection of Pathway/Genome databases. Each database in the BioCyc collection describes the genome and metabolic pathways of a single organism. Pathway analysis of transcription and metabolism can be accessed at <http://biocyc.org>, which also provides webinars to help navigate through the information.
3. **HumanCyc** is a bioinformatics database that describes human metabolic pathways as an organizing framework for the human genome. <http://humancyc.org>
4. **DECIPHER** (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) is a consortium which aids in the interpretation of submicroscopic chromosomal imbalance, for example microdeletions/duplications/insertions, translocations and inversions. It displays this information on an interactive human genome browser. <http://decipher.sanger.ac.uk/>
5. **ENCODE** (encyclopedia of DNA elements) consortium is an international collaboration of research groups with the goal to build a comprehensive list of functional elements in the human genome. These elements act at the protein and RNA levels, and also contain regulatory sequences that control cells in which a gene is active. <https://www.encodeproject.org/>
6. **ENSEMBL Genome Browser** is a joint project between EMBL – EBI (European Bioinformatics Institute) and the Wellcome Trust Sanger Institute to maintain annotations on selected eukaryotic genomes. <http://www.ensembl.org/index.html>

7. **GALAXY** is developed by the Nekrutenko lab in the Center for Comparative Genomics and Bioinformatics at Penn State and the Taylor lab at Emory University. It acts as a genomic database and a multi-threaded job manager for genomic queries. It is designed for those who do not have a computer programming background but would like to perform analysis on specific chromosomal targets or whole genome analysis of their data. It is also for computational biologists who have the computer background but need to interface with genome browsers to extract meaningful information. <http://main.g2.bx.psu.edu>
8. **GBG** (Genomics and Bioinformatics Group) provides a series of software packages to manage molecular interaction data from multiple platforms to increase understanding of these interactions on the chemo-sensitivity of cancer. A suite of tools to help evaluate the impact of gene splicing variation is also available. <http://discover.nci.nih.gov/>
9. **GENE** is a database maintained by NCBI which keeps records of gene nomenclature, Reference Sequences (RefSeq), maps, pathways, phenotypes, and links to genome, phenotype, and locus-specific resources worldwide. <http://www.ncbi.nlm.nih.gov/gene>
10. **GENE ONTOLOGY PROJECT** is an initiative to standardize representation of gene and gene products across species and databases. Vocabulary standards and gene product annotation data offers consistent descriptions of gene products in different databases. <http://www.geneontology.org/>
11. **Genomic Clone Database**, initiated by The Centre for Applied Genomics and the Hospital for Sick Children in Toronto, Canada, provides a repository of human genomic clones. <http://projects.tcg.ca/gcd>
12. **GEO** (Gene Expression Omnibus) is a functional genomics data repository supporting “Minimum Information about a Microarray Experiment” data submissions. Resources for transcriptomics data can also be accessed from GEO. <http://www.ncbi.nlm.nih.gov/geo/>
13. **HUGO** of Gene Nomenclature Committee (HGNC) provides a repository of approved gene names and symbols along with gene families, proteomic and phenotypic information. www.genenames.org
14. **IGSR** or The International Genome Sample Resource (IGSR) provides usability of data generated by the 1000 Genomes Project. <http://www.1000genomes.org/>
15. **PROTEIN DATA BANK** (PDB) archive is a repository of information about the 3D structures of large biological molecules, including proteins and nucleic acids. <http://www.rcsb.org/pdb/home/home.do>
16. **SAGE** (Serial Analysis of Gene Expression) is a tool, which provides visual representation of gene expression in a given cell population. The project originally assembled over 5 million transcript tags from more than 100 human cell types on a genome wide scale. Tools on the SAGE Genie website include: Anatomic Viewer, Digital Gene Expression Displayer, SAGE Experimental Viewer, SAGE Absolute Level Lister, SAGE Library Finder, SAGE Genomics Finder, and Digital Karyotyping data among others. <http://cgap.ncbi.nih.gov/SAGE>
17. **SeattleSNPs** Programs for Genomic Applications (PGA) focus on identifying, associations between single nucleotide polymorphisms (SNPs) in candidate genes and pathways underling inflammatory responses in humans. <http://pga.gs.washington.edu/>
18. **SIFT** (Sorting Intolerant from Tolerant) Human Genome database uses an algorithm to separate a single amino acid substitution in a protein sequence (non-synonymous single nucleotide polymorphism or nsSNP). It then determines whether the amino acid change is tolerant or damaging to protein function. <http://sift.jcvi.org>
19. **USCS Genome Browser**, developed and maintained by the Genome Bioinformatics Group at the University of California Santa Cruz (UCSC), contains the Reference sequence and working draft assemblies for a large collection of genomes. [http://genome.ucsc.edu/](http://genome.ucsc.edu)
20. **VEGA** (Vertebrate Genome Annotation) database is a repository for gene models comprising of manual annotation of vertebrate finished genome sequence. <http://vega.sanger.ac.uk/index.html>
21. **VISTA**, a genome browser hosted at Genomics Division of Lawrence Berkeley National Laboratory and US Department of Energy Joint Genome Institute, provides programs and databases of orthologous and paralogous gene relationships for comparative analysis of genomic sequences. <http://genome.lbl.gov/vista/index.shtml>

25.2.3 Links to cytogenetics and genomics support groups

1. **Support Groups for Chromosomal Conditions** of the University of Kansas Medical Center’s Genetic Education Center provides a comprehensive list of cytogenetics support groups, catalogued by chromosome number, for most well-known disorders. <http://www.kumc.edu/gec/support/chromoso.html>
2. **Genetic Alliance** includes more than 1000 disease-specific advocacy organizations, as well as thousands of universities, private companies, government agencies, and public policy organizations. Their website allows search by organization, disease, or resource repository. <http://www.geneticalliance.org>

3. **National Organization for Rare Diseases** (NORD) is a US-based federation with partners in Europe and Japan helping individuals with rare diseases through programs of education, advocacy, research, and patient services. Their database provides information and links for many different rare diseases. Rare Connect is a social network of Rare Disease Communities led by NORD and EURORDIS in partnership with leading disease-specific patient groups. <http://www.rarediseases.org/>
4. The **European Organization for Rare Diseases** (EURORDIS) is an alliance of organizations and individuals, representing rare diseases and patient organizations in over 45 countries. <http://www.eurordis.org/>
5. **Orphanet**, led by a European consortium, is the reference portal for information on rare diseases and orphan drugs. Its aim is to help improve the diagnosis, care, and treatment of patients with rare diseases and its database provides information on over 6000 diseases. www.orpha.net
6. **UNIQUE** is a “rare chromosome disorder” support group based out of the United Kingdom. Their database provides a leaflet for many reported genetic disorders, which can be downloaded or accessed through their site. A karyotype search by chromosome or disorder can also be performed. <http://www.rarechromo.org/html/home.asp>
7. The **Canadian Association of Genetic Counselors** (CAGC) has compiled the Canadian Directory of Support Services as a resource for families and professionals seeking information on genetic support groups in Canada. Canadian Directory of Support Services is cataloged by support group and disorder and is maintained by the London Health Sciences Center. http://www.lhsc.on.ca/Patients_Families_Visitors/Genetic_Support_Directory

25.2.4 Prominent peer-reviewed journals pertaining to genetics

1. *American Journal of Human Genetics* published by the University of Chicago Press can be accessed at <http://www.ajhg.org>
2. *American Journal of Medical Genetics* can be accessed through <http://www3.interscience.wiley.com/cgi-bin/jhome/33129>
3. *Archives of Pathology and Laboratory Medicine*, an official publication of the College of American Pathologists (CAP), is a monthly journal which provides open access to many articles. Data obtained from CAP surveys are summarized in this journal. <http://www.archivesofpathology.org/>
4. *Cancer Genetics and Cytogenetics* can be accessed through <http://www.sciencedirect.com/science/journal/01654608>
5. *Chromosoma*, published by SpringerLink, can be accessed from <http://link.springer.com/journal/412>
6. *Cytogenetic and Genome Research* by Karger publishers, formerly known as *Cytogenetics and Cell Genetics*, can be accessed from <http://www.karger.com/Journal/Home/224037>
7. *European Journal of Medical Genetics* can be accessed at <http://www.sciencedirect.com/science/journal/17697212>
8. *Genes, Chromosomes and Cancer* can be accessed from <http://onlinelibrary.wiley.com/browse/publications>
9. *Genetics in Medicine*, the official journal of the American College of Medical Genetics, can be accessed from <http://www.geneticsinmedicine.org>
10. *Human Genetics* can be accessed from <http://link.springer.com/journal/439>
11. *Journal of the Association of Genetic Technologists*, published by the Association of Genetic Technologists (AGT) can be accessed online at <http://www.agt-info.org/Pages/default.aspx>. A reading list of prominent articles related to cytogenetics and molecular genetics are also published in the journal for CEU credits.
12. *Journal of Clinical Oncology* can be accessed from <http://www.jco.org>
13. *Journal of Medical Genetics* can be accessed from <http://jmg.bmj.com/>
14. *Journal of Pediatrics*, provides search functions for cytogenetic topics in the periodical itself or on MedLine®. <http://www.jpeds.com/>
15. *Leukemia* can be accessed from <http://www.nature.com/leu/index.html>
16. *Prenatal Diagnosis* can be accessed at <http://obgyn.onlinelibrary.wiley.com/>
17. *Molecular Cytogenetics* can be accessed at <http://www.molecularcytogenetics.org/>
18. *Nature* can be accessed from <http://www.nature.com/nature/index.html>
19. *Nature Genetics* can be accessed from <http://www.nature.com/ng/index.html>
20. *Nature Review Genetics* can be accessed from <http://www.nature.com/nrg/index.html>
21. *Obstetrics and Gynecology* can be accessed at <http://www.greenjournal.org>

25.2.5 Cytogenetics and medical genetics textbooks

(Note: Textbook editions may have been revised since compiling this list. Please check for updated versions.)

1. *AGT Cytogenetics Laboratory Manual*, 4th ed., edited by Marilyn S. Arsham, Margaret J. Barch and Helen J. Lawce (Wiley Blackwell, 2016).

2. *Cancer Cytogenetics: Chromosomal and Molecular Genetic Aberrations of Tumor Cells*, 3rd ed., edited by Sverre Heim and Felix Mitelman (John Wiley & Sons, Inc., New York, 2009).
3. *Cancer Cytogenetics: Methods and Protocols*, edited by John Swansbury (The Royal Marsden NHS Trust, Sutton, UK). (Humana Press Inc., Totowa, NJ, 2003).
4. *Catalogue of Unbalanced Chromosome Aberrations in Man*, 2nd ed., edited by Prof. A. Schinzel (Walterde Gruyter, Berlin and New York 2001).
5. *Chromosome Abnormalities and Genetic Counseling*, 4th ed., edited by R. J. McKinlay Gardner, Grant. R. Sutherland, and Lisa G. Shaffer (Oxford University Press, New York 2012).
6. *Culture of Animal Cells: A Manual of Basic Techniques and Specialized Applications*, 6th ed., by R. Ian Freshney (John Wiley & Sons Inc., New Jersey, 2010).
7. *Cytogenetics, FISH and Molecular Testing in Hematologic Malignancies* by Wojciech Gorczyca (Informa UK, London, 2008).
8. *Fluorescence in situ Hybridization (FISH): Protocols and Applications*, Methods in Molecular Biology series, volume No. 659, edited by Joanna M. Bridger, Emanuela V. Volpi (Humana Press, New York, 2010).
9. *Human Cytogenetics: A Practical Approach: Constitutional Analysis*, 3rd ed., volume 1, edited by D.E. Rooney and B. Czepulkowski (Oxford University Press, New York, 2001).
10. *Human Cytogenetics: A Practical Approach: Malignancy and Acquired Abnormalities*, 3rd ed., volume 2, edited by D.E. Rooney and B. Czepulkowski (Oxford University Press, New York, 2001).
11. *Lab Math: A Handbook of Measurements, Calculations, and Other Quantitative Skills for Use at the Bench*, by Dany Spencer Adams (Cold Spring Harbor Laboratory Press, New York, 2003).
12. *Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench* by Jane Roskams (Cold Spring Harbor Laboratory Press, New York, 2002).
13. *Management of Genetic Syndromes*, 3rd ed., by Suzanne B. Cassidy and Judith E. Allanson (John Wiley & Sons Inc., Hoboken, New Jersey, 2010).
14. *Molecular Diagnostics: For the Clinical Laboratorian*, 2nd ed., edited by William B. Coleman and Gregory J. Tsongalis (Humana Press, New York, 2006).
15. *Practical Genetic Counseling*, 7th ed., by Peter S. Harper (Hodder Arnold Publication, London, 2010).
16. *Smith's Recognizable Patterns of Human Malformation*. 6th ed., by Kenneth Lyon Jones (Elsevier Inc., Philadelphia, 2006).
17. *Statistical Methods for Microarray Data Analysis* (Methods in Molecular Biology), edited by Andrei Y. Yakovlev, Lev Klebanov, and Daniel Gaile (Humana Press, New York, 2012).
18. *Thompson & Thompson Genetics in Medicine*, 7th ed., by Robert L. Nussbaum, Roderick R. McInnes and Huntington F. Willard (Elsevier Inc., Philadelphia, 2007).
19. *The Cytogenetic Symposia*, 2nd ed., edited by Betty Dunn, Patricia Mouchrani, and Martha Keagle (Association of Genetic Technologists, Lenexa, Kansas, 2006).
20. *The Principles of Clinical Cytogenetics*, 3rd ed., edited by Steven L. Gersen and Martha B. Keagle (Springer Publications, New York, 2012). This book is written on an array of topics related to the practice of Medical Cytogenetics. It would serve as excellent resource for students in cytogenetics programs as well as those preparing for the American Board of Medical Genetics examination.
21. *WHO Classification of Tumors of Haematopoietic and Lymphoid Tissue* (IARC WHO Classification of Tumors) of the International Agency for Research on Cancer, 4th ed., edited by S. Swerdlow, E. Campo, N. Lee Harris, E.S. Jaffe, S.A. Pileri, H. Stein, J. Thiele, J.W. Vardiman (World Health Organization, Lyon, 2008).
22. *Human Genetics: Concepts and Applications*, by Ricki Lewis, is a non-science major's human genetics text that explains what genes are, how they function, how they interact with the environment, and how our understanding of genetics has changed since completion of the human genome project. The content is hosted on an online learning center and contains flashcards, multiple choice quizzes, and mini chapter outlines on human genetics concepts and applications.

25.2.6 Vendor products/equipment and lab support

1. **Abbot Molecular** provides FISH, aCGH, and oligonucleotide probe reagents. Apart from providing instruments and reagents for DNA and RNA analysis, it also gives physicians information from early detection of pathogens and subtle changes in genes and chromosomes. www.abbtmolecular.com
2. **Affymetrix** provides microarray diagnostic development tools and molecular biology kits for genome-wide analysis studies and a range of products for cellular and protein analysis. <http://www.affymetrix.com>
3. **Applied Biosystems** offers assays and array products as well as informatics software. <http://www.appliedbiosystems.com>

4. **Applied Spectral Imaging** (ASI) provides cytogenetics, pathology imaging, and data management applications that offer quality image capture (acquisition) capabilities in fluorescent, transmission (Brightfield) and spectral imaging, such as the Cytovision Karyotyper and metaphase and interphase FISH scoring scanners, as well as integration into the Laboratory Information System (LIS). Company offices are in the USA, Canada, and Europe. <http://www.spectral-imaging.com>
5. **Bioview** offers automated scanning applications and systems for cytogenetics with interphase FISH analysis capability. Their scanning software can be integrated for both clinical and research purposes. <http://www.bioview.com>
6. **Carl Zeiss Microscopy** provides optical instruments (e.g., brightfield, confocal, electron, and super-resolution microscopy systems), fluorescent dyes, and filters, as well as medical and surgical tools. Brochures and order information can be obtained from their website. <http://www.zeiss.com>
7. **Cytocell** offers a comprehensive range of classical FISH probes for subtelomeric and pericentromeric regions, cryptic chromosome deletions, whole chromosome painting and prenatal analysis, as well as chromophore multiprobe systems for cancer diagnosis and prognosis. <http://www.cytocell.com>
8. **Cytognomix Inc.** provides proprietary genome-coordinate defined Single Copy (SC) FISH probe technology for various congenital disorders, leukemia and other cancer abnormalities. SC technology can also be used in genomic microarray designs. It also offers software for high-throughput interpretation of genomic variation. <http://www.cytognomix.com/>
9. **Dako** diagnostic reagents, kits and instruments specialize in serving cancer-related testing in immunohistochemistry, molecular pathology and flow cytometry. <http://www.dako.com>
10. **Molecular Devices** provides instruments, software, reagents and supplies for research, biotechnology, and pharmaceutical customers. Systems include microarray scanners, microscope slide loaders, and Cytovision capturing and analysis program. <http://www.moleculardevices.com/>
11. **Genial Genetics** is a UK-based company providing cytogenetic reagents, harvest automation, including MultiPrep Robotic Harvester for cytogenetic samples, MultiPrep CellSprint™ Automated Cytogenetic Suspension Culture Harvester automation, and SHIRE genetic database (LIS) software. <http://www.genialgenetics.com>
12. **Genisphere** offers DNA labeling kits for use with Affymetrix®, GeneChip®, and microRNA microarrays. It also provides fluorescently labeled signal amplifiers, constructed as “DNA dendrimers,” which can be used in FISH-based applications. Additional product information and international distributors can be found on their website. <http://www.genisphere.com/>
13. **Invitrogen Life Technologies (Thermo Fisher Scientific)**, formed by the merger between Invitrogen and Applied Biosystems in 2008, provides Gibco® cell culture products, such as trypsin, collagenase, growth factors, PHA and other reagents specific to cytogenetic laboratory use; Dynabeads® (DYNAL® Magnetic Beads) for cell separation; DNA reagents and consumables; Molecular Probes® for detection and labeling; TaqMan® for real-time PCR (qPCR); and Applied Biosystems® for an integrated system for sequencing, flow cytometry, and real-time and end point PCR – from sample prep to data analysis. <http://www.thermofisher.com/us/en/home.html>
14. **Iris Sample Processing**, previously known as StatSpin®, provides diagnostic products, such as small, quiet benchtop to high speed centrifuges, a molecular workstation for processing FISH slides, hybridization equipment for FISH and various other sample collection and processing devices. <http://www.statspin.com>/
15. **Irvine Scientific** provides a comprehensive range of prenatal, diagnostic, and cytogenetics products, including AmnioStat-FLM®-PG, Chang Medium®, Mycotrim®. Chang Medium In Situ, Chang Medium C, and Chang Medium D are specially optimized for culturing human amniotic fluid cells, chorionic villi samples (CVS) during prenatal care. Chang Medium BMC and MF are specially optimized for culturing bone marrow and peripheral blood, respectively during postnatal care. Mycotrim® is a unique triphasic culturing system for identification, isolation, and transport for rapid mycoplasma screening. *U. urealyticum* and *M. hominis* control organisms are also available. <http://www.irvinesci.com>
16. **LeanAdvisors** offer approaches to eliminate waste from a series of processes in a healthcare setting. It has helped to cut the “fat” from diagnostic labs so that tasks are completed efficiently. <http://www.leanadvisors.com>
17. **Leica Microsystems** provide quality viewing, capturing, annotating, and archiving of images. Their introduction of High Definition Imaging Systems combines high resolution and image speed for both clinical laboratory and educational environments. <http://www.leica-microsystems.com/home/>
18. **Metasystems** is an employee-owned German company specializing in automated scanning and image analysis for genetic applications. It provides an interactive karyotyping system known as IKAROS and an automated system for high-throughput FISH signal detection and capture from the Metafer hardware. In 2009, the automated virtual slide scanning (Vslide) system was added and its probe kit portfolio, which already supported telomere analysis, comparative genomic hybridization (CGH) analysis, filter-based 24-color FISH (mFISH) and high resolution multicolor banding (mBAND), was expanded to include locus-specific probe kits (XL) for hematology, whole chromosome probe kits (XCP) and arm-specific mFISH kits (XaCyte). <https://metasystems-international.com/>

19. **Nikon** offers a variety of microscopes including stereomicroscopes and super-resolution instruments, which exceed the traditional diffraction limits of optical microscopy. It also supplies objective lenses and microscope imaging software for advance or basic research purposes. <http://www.nikoninstruments.com/>
20. **Olympus Microscopes** supplies a complete line of microscopes, including inverted, upright, photo, digital, fluorescent, scanner, optics and accessories. Annual Olympus BioScapes Imaging Competition winners can be viewed at <http://www.olympusamerica.com/>. For an excellent primer on the anatomy of a microscope, go to the Olympus Microscopy Resource Center at <http://www.olympusmicro.com/>
21. **PerkinElmer** supplies a complete molecular karyotyping solution: microarrays, aCGH reagents, ScanRI™ microarray scanner with image processing software and aCGH data analysis software. Genomic technologies such as its Constitutional Chip™ focuses on well characterized chromosomal regions and provides high resolution coverage for detection of copy number imbalances throughout the genome. BAC DNA is available for result verification. Their aCGH data analysis solutions range from simple, easy-to-use software (SpectralWare®) to advanced, state-of-the art solutions (One Click and Fusion, PerkinElmer Editions). <http://www.perkinelmer.com/>
22. **Rainbow Scientific Inc.** offers a wide range of cytogenetics, molecular, hematology and clinical chemistry products, such as diagnostic test kits, bio-reagents, scientific instruments, and database software. <http://www.rainbowscientific.com>
23. **Sigma-Aldrich**® provides a complete line of cell culture products including specialty media, Fetal Bovine Serum, supplements, reagents, and labware. It also offers a variety of cell culture supplies that specifically support the needs of researchers working with cell culture applications such as 3D cell proliferation, cell/tissue culture, cell preservation and storage, cell visualization, scale-up cell culture and stem cell/tissue engineering. <http://www.sigmaldrich.com/>
24. **Softgene** is a fully integrated Laboratory Information System (LIS) providing solutions to automate the workflow in the cytogenetics laboratory. <http://www.softcomputer.com/>

25.2.7 Credentialing and guidelines

NOTE: Please check with appropriate laboratory policy or laboratory director for variations on published guidelines, as it may differ on a case by case basis. These guidelines are also subject to change with technology advancement.

1. **American College of Medical Genetics** (ACMG) provides standards and guidelines for clinical genetics laboratories which can be found at http://www.acmg.net/StaticContent/SGs/Section_E.html. Please refer to the main ACMG website page at www.acmg.net for educational resources and updates to guidelines. The standards and guidelines section on the website also includes criteria for microarray analysis for constitutional cytogenetic abnormalities.
2. **American Society for Cell Biology** (ASCB) is a nonprofit membership organization. Membership in the ASCB is open to all research scientists, students, educators (high school, undergraduate, and graduate level), and technicians who have expertise in cell biology or an allied field. <http://www.ascb.org>
3. **American Society for Clinical Pathology** (ASCP) combined the National Credentialing Agency for Laboratory Personnel, Inc. (NCA) and the Board of Registry (BOR) of the ASCP to form a new credentialing agency. It provides credentials through board certified examinations and certification maintenance programs for cytogenetic and molecular genetic technologists, among others laboratory medicine subspecialties. <https://www.ascp.org/>
4. The **Association of Clinical Cytogeneticists** (ACC) provides information on the practice of cytogenetics in the UK. It also contains links for interested individuals wishing to pursue certification in cytogenetics in the UK. In order for international technologists to practice in the UK, it is recommended that they contact the laboratories, NHS hospital trusts or the cytogenetics laboratory directors directly for additional information pertaining to their experience eligibility. Updates are posted on the ACC website when policies change. <http://www.cytogenetics.org.uk>
5. **Canadian College of Medical Geneticists** (CCMG) provides professional guidelines, membership, training, and assessment of credentials for Clinical Cytogeneticists practicing in Canada. <https://www.ccmg-ccgm.org/>
6. The **Canadian Society for Medical Laboratory Science** (CSMLS) is the national certifying body and professional association for medical laboratory technologists and medical laboratory assistants in cytogenetics and other MLT-related fields who wish to practice in Canada. The site provides details on prior learning assessment documents that need to be completed in order to sit for the exam or do additional training hours. <http://www.csmls.org/>
7. **College of American Pathologists** (CAP) provides an extensive checklist for cytogenetic analysis, and is part of their biennial inspection for laboratory accreditation. <http://www.cap.org>
8. **EuroGentest** is an EU-funded Network of Excellence looking at aspects of genetic testing - quality management, information databases, public health, new technologies and education. <http://www.eurogentest.org/web/index.xhtml>

9. **European Cytogenetic Association** (ECA) provides guidelines and a common European framework for constitutional and acquired cytogenetic investigations. It also provides assessment, and quality control of cytogenetic services in Europe and the general representation of professionals in the field.

25.2.8 Genetics training programs and courses

1. The **Association of Genetic Technologists** (AGT), the organization for cytogenetic and molecular professionals, provides information and contact personnel for genetics technology educational programs in the U.S. and Canada with NAACLS accreditation and CSMLS approval, respectively. It also provides continuing educational opportunities through online education, an annual scientific conference, a journal club, and “Test Yourself” activities. <http://www.agt-info.org/Pages/default.aspx> or <http://www.agt-info.org>
2. **British Columbia Institute of Technology** (BCIT) post diploma training program is split between a didactic and a practicum component spent at one or more sites affiliated with BCIT. After successful completion of the program, graduates are eligible to write the Certification Examination of the Canadian Society for Medical Laboratory Science (CSMLS), which leads to the qualification of Registered Technologist (RT) in Clinical Genetics, the nationally recognized qualification for employment in the field in Canada. <http://www.bcit.ca>
3. **Kennesaw State University**, Department of Biological and Physical Sciences offers a Bachelor of Science (BS) degree in Biology or Biotechnology with a concentration in cytogenetics. Graduates are eligible to sit for the Technologist in Cytogenetics examination given by the Board of Certification of the American Society for Clinical Pathology (ASCP). The program is approved by NAACLS (National Accrediting Agency for Clinical Laboratory Sciences). <http://www.kennesaw.edu>
4. The **Michener Institute for Applied Health Sciences** provides a Genetics Technology program consisting of course work and clinical simulation at the Michener Institute preceding hospital-based hands on clinical education in Cytogenetics and Molecular Genetics. Graduates receive Michener's Graduate Diploma in Genetics Technology and are eligible to write the Clinical Genetics national certification examination conducted by the Canadian Society for Medical Laboratory Science (CSMLS). Please check directly with the institution for updated contact information, and program expectations as they may change. http://www.michener.ca/ce/postdiploma/genetics_technology.php
5. **Michigan State University**, Genetics PhD Program offers research in plant genetics and genomics, microbial and viral genetics, genetics of cancer, population genetics and evolution. Please refer to program's website for specific updates to their graduate curriculum. <http://www.genetics.msu.edu/>
6. **Texas Tech University Health Sciences Center** offers a Master of Science degree in Molecular Pathology. Besides graduate seminars and course-work, students also observe the roles of genetic counselors, fellows and consultants. The molecular laboratory facility is accredited by the College of American Pathology. <http://www.ttuhs.edu/sah/msmp/>
7. **University of Connecticut**, Department of Allied Health Sciences, offers a Bachelor's degree in Diagnostic Genetic Sciences with concentrations in Cytogenetics and Molecular Diagnostics. It also offers a certificate program with concentrations in Cytogenetics and Molecular Diagnostics, to individuals with a bachelor's degree in Biology, Medical Laboratory Science or a related subject area that includes coursework in biochemistry, microbiology and statistics, and who have completed the appropriate prerequisite courses. All of the program options include a 6-month clinical or practicum semester conducted at various clinical, research, and veterinary laboratories throughout the United States. The program is accredited by the National Accrediting Agency for Clinical Laboratory Sciences (NAACLS). <http://www.cag.uconn.edu/ahs/ahs/>
8. **University of Connecticut**, Department of Allied Health Sciences also offers a Masters degree in Health Care Genetics. The specific plan of study and admission requirements can be accessed through <http://www.alliedhealth.uconn.edu/graduate/>
9. **University of Kansas Medical Center**, School of Health Professions offers a Master of Science in Molecular Biotechnology with a core curriculum that includes biochemistry, molecular genetics, cell structure, cell signaling, research ethics, biotechnology issues, scientific writing, and radiation safety. Practical skills are built through lecture and laboratory coursework in the theory and application of a broad range of nucleic acid and protein-based molecular biotechnologies. The program is accredited by the National Accrediting Agency for Clinical Laboratory Sciences (NAACLS). <http://www.mb.kumc.edu/>
10. **University of Texas Health Science Center** at San Antonio, School of Health Professionals offers undergraduate post-Baccalaureate Certificate program in Cytogenetics. Graduates of the Bachelor's degree program are eligible to take the Technologist in Cytogenetics examination given by the Board of Certification of the American Society for Clinical Pathology (ASCP). This program is accredited by the National Accrediting Agency for Clinical Laboratory Sciences (NAACLS). <http://uthscsa.edu/shp>

11. **University of Texas M.D. Anderson Cancer Center**, School of Health Professions, offers training in cytogenetic technology, molecular genetic technology, cytology, diagnostic imaging, histotechnology, medical dosimetry, radiation therapy, and clinical laboratory science as part of their Bachelors of Science Degree Programs. All programs are accredited by the National Accrediting Agency for Clinical Laboratory Sciences (NAACLS). <http://www.mdanderson.org/>

25.2.9 Professional organizations

1. The **Association of Genetics Technologists** (AGT) supports cytogenetic and molecular genetics technologists internationally with a scientific journal and publications, journal club reading lists, annual meetings, job offerings, educational opportunities and peer networking. <http://www.agt-info.org/Pages/default.aspx>
2. **Children's Oncology Group** (COG) provides a spectrum of research studies, among which is the cytogenetic laboratory providing chromosomal analysis, as a standard of care, in particular with pediatric leukemias. The Group provides key information to help support children and their families from the time of diagnosis, through treatment and following cure. Institutional or international membership applications to become a COG-recognized laboratory can be found at <http://www.childrensoncologypgroup.org>
3. The **Foundation for Genetic Technology** (FGT) promotes education in genetics technology with awards, scholarships and grants for both undergraduate and graduate students. <http://www.agt-info.org/Pages/fgt.aspx>
4. **Genetic Professional Societies & Organizations** provides a list of clinical genetic organizations catalogued by country. <http://www.kumc.edu/gec/prof/soclist.html>
5. The **National Society of Genetic Counselors** advances the various roles of genetic counselors in health care by fostering education, research, and public policy to ensure the availability of quality genetic services. <http://www.nscc.org>
6. **Ontario Laboratory Accreditation** (OLA), a quality management program, provides information for cytogenetic or molecular diagnostic laboratories wishing to gain OLA accreditation in Ontario, Canada. <http://www.qmpls.org>

25.2.10 Job search

1. **Salary Survey** A salary survey for genetic technologists is also conducted biennially and is posted on the AGT website <http://www.agt-info.org/Pages/default.aspx> or <http://www.agt-info.org>. Another website provides a basic market pricing report of cytogenetic technologist salaries in the United States; however, value range may not be accurate for the inquired area. To access this data: copy and paste the following link into browser or search on 'Salary wizard' directly through Google. http://swz.salary.com/salarywizard/layouthtmls/swzl_comprest_national_HC07000132.html
2. **Job Postings** can be found at <http://www.agt-info.org/Pages/default.aspx> or www.AGT-info.org, as well as many professional organizations. Google "cytogenetic technologist jobs" (and add a desired location) for a host of websites where jobs are posted.

Index

Page numbers in *italics* refer to illustrations; those in **bold** refer to tables

abortus tissue 134
FISH studies 164, 803–806
cultured non-mitotic abortus tissue 804–806
direct preparations 164, 803–804
accreditation 1021
agencies 1002–1003, 1021
acetyl groups 494
achromatic lens 690
acridine orange 48, 238
R-banding 238–239
sister chromatid exchange testing protocols 289–294
acrocentric chromosomes 330, 356, 360
variations 331
acrocentric derivative 396–397
actinomycin D 48, 49
acute lymphoblastic leukemia (ALL) 517–520
CRLF2 abnormalities 520
del(9p) 519
dicentric chromosomes 520
high hyperdiploidy 517, 517
hypodiploidy 518, 518
iAMP21 amplification 520
KMT2A rearrangements 519
low hyperdiploidy 517
Philadelphia chromosome 519
pseudodiploidy 518
T-lineage ALL 520
t(1;19)(q23;p13.3) translocation 519
t(12;21)(p13;q22) translocation 519
see also leukemia
acute myeloid leukemia (AML) 492–493, 508–514
cytogenetic abnormalities **500–501**, 510–514, 511–512
11q23 abnormalities 513–514
inv(16)(p13.1q22)/t(16;16)(p13.1;q22) AML 510
Philadelphia chromosome positive AML 514
t(8;21)(q22;q22) AML 510
t(15;17)(q24;q21) APL 513
presentation 509
therapy-related myelodysplastic syndrome 514
see also leukemia
acute promyelocytic leukemia (APL) 510, 513
addition 939
additional material 399
adenoma 662
pleomorphic 604, 605
adenosine triphosphate (ATP) 4
adult T-cell leukemia/lymphoma 520
agammaglobulinemia 660, 662
agenesis 662

Alagille syndrome **438**
probe **731**
Albright hereditary osteodystrophy-like syndrome **435**
ALK1-positive lymphoma 525
alkylating agents 662
all-trans retinoic acid (ATRA) therapy 517
alleles 15–16
allelic recombination 455
alpha fetoprotein 662
alpha satellite DNA probes 724–726, 725, 777
alpha-thalassemia/mental retardation (ATR-16)
syndrome **437**
alphabetic symbols 367, **368–369**
Alu sequences 228
banding 740
alveolar rhabdomyosarcoma 596–597, 596, 611
alveolar soft part sarcoma (ASPS) 598
amethopterin 46–48, 49
amniocentesis 173
versus chorionic villus sampling 180–181
amniocyte fluid (AF) cell types 120, *120*, 121, 174
AmnioMAX complete medium 149
amniotic fluid 173–175
analysis 178–180
determining harvest times 178
number of slides per patient 179
staining 179
time to report cases 179
cell types 175
characteristics 173–174, *174*
collection container 175
culture 175–178
bloody specimens 177
in situ versus flask growth methods 176
maternal cell contamination 180, *760*
media and additives 176, 177
microbial contamination treatment 177
number of cultures per specimen 176
number of days to first culture check 178
open versus closed system 175
poor growth 178
precipitation treatment 177
protocols 188–191, 195–197
subculture 195–197
FISH studies 745, *746*
protocol 801–803
harvesting procedures 178, 191–197
mosicism 180
interpretation criteria 198–199

- amniotic fluid (*cont'd*)
 slide-making 178–179
 specimen size 175
 specimen transport 175
- amphotericin 132
- amyloidosis 522
- analploid chromosomes 464
- analytic performance monitoring 1029
- anaphase lag 63, 444, 445
- anaplastic large cell lymphoma (ALCL) 525
- androgen therapy 662
- anemia 555, 662
- aneuploidy 61, 62, 379, 433, **435–439**, 441
 clinical consequences 434
 interphase FISH studies 419–420, 420
 mapping **440**
 mechanisms 434–447
 preimplantation screening 771, 818–821
 ring chromosome 467
 sex chromosome 379–380, 434, **439**
 constitutional karyotype 379
 constitutional notation in a neoplastic karyotype 380
 neoplastic karyotype 379–380
see also specific chromosomes
- aneurysmal bone cysts (ABC) 599
- Angelman syndrome **437**, 488–490, 488
 clinical features **489**
 CMA case example 917
 FISH analysis 762–763
 probes **731**, 732, 733
 methylation assay 492
- angiomatoid fibrous histiocytoma 598
- anhidrosis 492, 494
- animal cytogenetics 1057
 captive management role 1059–1059
 chromosome abnormalities 1059–1058
 species integrity 1059
 studbooks 1058–1059
- cattle 1057
- chromosome analysis 1064–1070
 banding 1065–1066, **1067**, 1068
 conventional staining 1064
 digital imaging 1066
 karyotype standards and precedents 1068–1070
 karyotyping 1066–1068
- fibroblast cell culture 1062–1063
 bioresource banking 1062
 culture conditions 1063, **1065**, **1064**
 freezing 1092–1093
 harvesting 1098–1099
 sample source 1062
- FISH analysis protocols 1099–1102
 competitor DNA preparation 1099–1100
 labeling with spectrum fluorochromes 1102
 probe and slide preparation 1100–1102
- horses 1058, **1058**
- lymphocyte culture protocols 1078–1089
 avian lymphocytes 1078–1084
 horse lymphocytes 1087–1089
- supplies **1080–1082**
 using autologous plasma/buffy coat 1085–1087
 using whole blood 1084–1085
- molecular and comparative cytogenetics
 1070–1071
 BAC maps 1071, **1073**
 future directions 1071
 reciprocal chromosome painting 1070
 Zoo-FISH 1070
- peripheral blood culture 1063–1064
 rhino blood culture protocol 1089–1090
- primary culture preparation
 from feather pulp 1095–1096
 from solid tissue 1096–1097
 using enzyme digestion 1097–1098
- sample collection 1060–1062
 placenta 1091, **1092**
 record keeping 1062
 regulations 1060
 skin biopsy procedure 1090–1091
 tissue collection from carcass 1090
- tissue biopsy freezing for later culture 1094–1095
- wildlife conservation 1059
- animal models 840
- antelope
 sable 1059
Suni **1067–1068**
- anti-fatigue mat 999
- Anti-Kickback Statute (AKS) 1002
- antibiotics, culture media 94, 127
 treated media for contamination 158, 177
- antibody banding 252
- anticoagulants 92
- Aotus* monkeys 1059
- aplasia 662
- apochromatic lens 690
- apoptosis 555
- appraisals 1035
- approximation range 399–400
- apraxia 663
- archiving 71
- arithmetic mean 962–965
- arm FISH 835
- arteriosclerosis 663
- artifact 612
- ascertainment bias 612
- ascites 132
- Ashkenazi Jewish population 658, 663
- assessments 1018–1021
 external 1021
 audits/accreditation 1021
 proficiency testing 1021
- internal 1019–1020
 audits 1019
 competency program 1020
 quality indicators 1019–1020
- astrocytomas 602
- low-grade (LGA) 602
- ataxia 663

- ataxia-telangiectasia (A-T) 88, 653, **655**, 658–659
 diagnosis 658–659
- atresia 663
- audits
 external 1021
 internal 1019
- Auer rods 555
- aurora kinases 608
- autoclaving 123
 safety considerations 992
 sterilization protocol 1003
- automated hybridization system 997
- autopsy material 134
- average 963
- avidin 753–754
- B cells 90–91
 lymphoblastoid cell lines 132
see also lymphocytes
- B-prolymphocytic leukemia (B-PLL) 521
- bacterial artificial chromosomes (BACs) 737
 BAC maps 1071, *1073*
- bacteri-plasmid cloning 19
- balanced salt solutions (BSSs) 126–127
 Hanks' 138–139
- balanced translocation 61
see also translocation
- band levels 67–68, **67**
 determination of 364
see also chromosome bands
- banded karyogram 67
- banding resolution 356, 706
 G-bands 355
- banding techniques 27, 28, 43, 58–59, 213–215, 214
 amniotic fluid 179
 animal cytogenetics 1065–1066, *1067*, 1068
 antibody banding 252
 banding pattern definition 215
 bands per haploid set (BPHS) 45, 45
- C-banding 27, 28, 215, 230–233, 231
 protocols 268–274
- Cd banding 235–236
 protocol 285–286
- codes 216
- CT-banding 252
 protocol 295
- G-banding 27, 28, 43, 52, 58–59, 214, 215,
 222–230
 protocols 208–209, 278–285
 quality comparison 54
- N-banding 244
- Q-banding 27, 28, 58–59, 215, 220–222, 220
 protocol 266–268
- R-banding 27, 28, 58, 215, 228, 238–240
 protocols 277–278
- replication banding 14, 229, 248, 249, 250–251
 clinical significance 250–251
- restriction endonuclease banding 252
- Rx-FISH technique 769, 770
- sequential methods 233, 253
- simultaneous methods 233
- T-banding 228, 252
 protocol 294–295
- variation **217**
see also chromosome bands
- Barr body 12–13
- basophilia 555
- basophils 555
- BCR/ABL1 translocation 391, 415, 417
 chronic myelogenous leukemia 515
see also Philadelphia chromosome (Ph)
- Beckwith–Wiedemann syndrome **436**, 486, 486, 487
- bell-shaped curve 962, 963, 964, 965
- biohazard symbol 978
- bioinformatics 1052
 resources 1107–1108
- biological hazards 977
 spill management 982
see also safety considerations
- biological safety cabinets 995
- bioresource banking 1062
- bisphenol A (BPA) exposure 449
- bladder cancer 776, 777
- Blaschko's lines 447
- blepharophimosis 490, 494
- blood *see* peripheral blood
- blood feathers 1062, *1079*
 collection protocol 1078
- bloodborne pathogen precautions *see* safety considerations
- Bloom syndrome 88, **655**, 658
 diagnosis 654, 658
- body habitus 663
- bone core biopsy 560–561
- bone marrow
 protocols 557–571
 G-banding 278–280, 575–576
 transplant chimerism 422–423
- bonobo *1060*
- brainstorming 1024–1025
- BrdU 46–47, 49
 cell culture synchronization at S-phase 163–164
 chemical structure 246
 incorporation into DNA 247, 250
 lateral asymmetry 249
 post-FISH BrdU antibody detection 823
 replication banding 250–251
 sister chromatid exchange visualization 247, 248, 249,
 250, 251
 protocols 289–294
 staining technologies 246–251
- break-apart probes (BAP) 418–419, *419*, 729–730, 730
- breast tissue
 comparative genomic hybridization 852
 FISH analysis 824–831
 pretreatment time 745–747, 747
- brightfield microscopy 695–697
 coverglass 696
 eyepiece adjustment 697

- brightfield microscopy (*cont'd*)
 filters 695
 immersion oil 696
 Köhler illumination 695, 696, 706–707
 slides 696
see also microscope
- brightfield photography 701–702
 cameras 701–702
 filters 701
- 5-bromo-2'-deoxyuridine *see* BrdU
- budget *see* financial management
- buffers
 culture media 94
 FISH probes 740–741
 phosphate buffer solution 141
- bulbar conjunctiva 663
- Burkitt lymphoma/leukemia 523
- C-bands 27, 28, 215, 230–233, 231
 amniotic fluid 179
 animal cytogenetics 1066
 clinical significance 233, 233
 historical and theoretical perspectives 231–232
 protocols 268–274
 blood slides 272–274
 technical considerations 234
see also banding techniques
- café-au-lait spots 663
- cancer
 blood G-banding protocol 278–280
 cytogenetics protocol 557–561
 disease progression studies 911
see also solid tumors; *specific types of cancer*
- candidate gene search 909–910
- capital budget 1040
 capital expenditure 1041–1042
 depreciation 1042
 internal rate of return (IRR) 1042
 inventory 1042
 net present value (NPV) 1041
 payback period 1042
 weighted average cost of capital (WACC) 1041
see also financial management
- captive management *see* animal cytogenetics
- carcinoma 581
 ex pleomorphic adenoma (CexPA) 605
 hepatocellular 664
 in situ (CUS) 607
 lung 603
 non-small-cell lung carcinoma (NSCLC) 603–604
 small-cell lung carcinoma (SCLC) 603, 603
 renal 607
see also renal cell carcinoma (RCC)
 urothelial 610
 with t(15;19) translocation 604
- Carnoy's fixative 49
- case review 1027–1028
- cash budget 1040
see also financial management
- cat-eye syndrome 439
- CATCH22 syndrome 410
- cattle 1057
- cause and effect diagrams 1024, 1025
- CD antigen 555
- Cd staining 235–236
 clinical significance 236
 historical and theoretical perspectives 235–236
 protocol 285–286
 technical considerations 236
- CD138 antigen 816
 CD138-positive cell selection 817–818
- cell counting
 chamber method 161
 hemacytometer use 968–972, 969, 970
- cell cultures *see* cultures
- cell cycle 14–15, 15
 cell division 14–15
 interphase 14
 regulation 14
- cell harvesting 35
 amniotic fluid 178, 191–197
 automatic harvesting devices 49
 maintenance 997–998
 chorionic villus samples 184, 184, 191–193, 203, 205–207
 FISH specimens 747–748
 harvest medium 139
 in situ harvesting 41–42
 manual harvest for flasks 157–158
 peripheral blood cultures 95
 protocols 152, 154, 157–158, 191–197
 animal fibroblast cultures 1098–1099
 blood 100, 106, 112, 117, 563–565, 570
 hematologic disorders 563–565, 570–575
 plasma cells 571–575
 tumor cells 635–636, 641–642, 644–646, 649–650
 quality control 1026
 tumor cell cultures 585, 586, 635–636, 641–642,
 644–646, 649–650
- cell lines 119–120
 multiple 404
- cell membrane 1–2
 composition 1
 glycoprotein functionality 2
 selective permeability 2
- cells 1, 2
 cytoplasm 3–5
 generation time 119
 viability assessment 161, 972–973
- CENP staining 236–238, 237
 protocol 286–287
- centiMorgan 780
- centrifugation 35–36
 safety considerations 992
- centriole 4–5
- centromere 60, 235, 235, 356, 360–361, 360
 positions 360
 probes 726
 staining 235–238

- Cd staining 235–236
 CENP staining 236–238
 centrosome 4
 cerebellar dysfunction 663
 Chang medium 140
 Charcot–Marie–Tooth disease 21, **438**
 CHARGE disorder 908–909
 charge-coupled device (CCD) cameras 703
 Chediak–Higashi syndrome 492, 494
 chemical emergencies 987–988
 eye contact 987
 ingestion 988
 inhalation 987
 skin contact 987
 Chemical Hygiene Plan (CHP) 984
 chemical safety *see* safety considerations
 chiasmata 18
CHIC2 deletion 516
 chimerism 62
 bone marrow transplant 422–423
 chondrodystrophy 1072
 chondroid lipoma 594
 choreoathetosis 663
 chorionic villus sampling (CVS) 28, 180–184
 analysis 184–186
 confined placental mosaicism 185
 harvesting procedures 184, **184**, 191–193, 203,
 205–207
 maternal cell contamination 186, 207
 protocols 199–208
 sample weight determination 183
 specimen cleaning 182, 182, 183
 specimen transport 182
 structure and cell types 181–182, *181*
 versus amniocentesis 180–181
 villus culture 184, **184**
 chromatin 5, 224
 condensation 5, 225
 differential packaging 227
 euchromatin 5, 10, 227
 heterochromatin 5, 10–11, 227, 230–231, 494
 chromogenic *in situ* hybridization (CISH) 610
 chromomeres 18, 227
 chromomycin A3 240, 240
 staining protocol 277–278
 chromomycin/distamycin R-banding protocol 277–278
 Chromoprobe Multiprobe[®] panels 799–800
 chromosomal imbalance 474
 chromosomal microarray (CMA) 423–424, 611–612, 719,
 903, 913–915
 advantages 905–906, **908**
 algorithm 916
 applications 907–912
 acquired genetic change characterization 909
 cancer progression and clinical translation 911
 candidate gene search 908–909
 chromosome region-specific arrays 909–910
 chromosome-specific arrays 910
 constitutional abnormalities 907–909
 critical region determination for specific disease 908
 developmental delay 907–908
 evolutionary characterization 912
 genome-wide arrays 910
 genomic variation determination 911–912
 genotype phenotype correlations 909
 mental retardation 907–908
 mosaicism detection 447–448, 920
 multiple congenital anomalies 907–908
 prenatal diagnosis 911
 subtelomeric regions 907
 case examples 916–922
 confirmation of abnormal results 776, 915
 data analysis 914
 general considerations 913
 limitations 912–913
 methods 906–907
 microarray CGH 29
 platforms **905**
 principle 903–905, *904*
 reporting results 915
 specimens 913
 types of microarray 914
 validation 914–915
 see also comparative genomic hybridization (CGH)
 chromosome abnormalities 60–62
 animal cytogenetics 1057–1058
 breakage 65–66, 229, 367–369, 371
 see also chromosome instability syndromes
 mosaicism 62–65, *64*
 numerical abnormalities 433
 see also aneuploidy; euploidy
 solid tumors 587–592, **588–592**
 see also specific tumors
 structural rearrangements 453–458, *454*
 complex rearrangements 475
 mechanisms 455–458
 see also deletions; duplications; insertions; inversions;
 rings; translocation
 Chromosome Anomaly Collection 913
 chromosome bands 356
 band levels 67–68, **67**, 364
 dark band 356
 derivation 363
 landmarks *304*, *305*, 362, 363
 light band 357
 notation 363
 numbering system *304*, *305*
 see also banding techniques; *specific chromosomes*
 chromosome instability syndromes 88–89, 653–654, **655**
 ataxia–telangiectasia (A-T) 88, 653, 658–659
 Bloom syndrome 88, 654, 658
 cytogenetics versus molecular diagnosis 654
 immunodeficiency, centromeric instability, and facial
 anomalies (ICF) syndrome 660, 662
 Nijmegen breakage syndrome 659, 659
 proficiency testing 662
 Roberts syndrome 661
 Rothmund–Thomson syndrome 662

- chromosome instability syndromes (*cont'd*)
 Werner syndrome 661
see also Fanconi anemia
- chromosome paints 416, 722, 723, 735, 745, 779
 animal cytogenetics 1070
 multicolor FISH 842
 protocol 796–799
- chromosomes 5, 10–11, 25, 60
 A group 302
 anticontraction methods 43–46, 44, 48–49, **48**
 B group 302
 C group 303
 categorization 66–67
 chromosome 1 306
 banding variations 307
 terminal deletion **435**
 chromosome 2 308
 deletion **435**
 pericentric inversion 309, **435**
 chromosome 3 310
 duplication **435**, 460
 Q-banding variations 311
 chromosome 4 312
 deletion **435**
 FISH signal variations 313
 G-banding variations 313
 Q-banding variations 313
 reciprocal translocation **435**
 chromosome 5 314
 deletions **435**
 pericentric inversion 461
 Q-banding variations 315
 ring chromosome **467**
 chromosome 6 316, 317
 chromosome 7 318
 banding variations 319
 deletion **435**
 duplication **435**
 chromosome 8 320
 banding variations 321
 deletions **435**, **436**
 duplication **436**
 pericentric inversion **436**, 460–462
 trisomy **436**
 chromosome 9 322
 banding variations 323
 deletion **436**
 pericentric inversion **436**
 trisomy **436**
 chromosome 10 324
 banding variation 325
 deletion **436**
 chromosome 11 326
 banding variation 327
 deletions **436**, **437**
 duplication **436**
 paracentric inversion **437**
 reciprocal translocation **437**
 chromosome 12 328
 paracentric inversion **462**
- tetrasomy **437**
 variants 329
- chromosome 13 330, 332
 banding variations 333
 deletion 459
 trisomy **437**
- chromosome 14 334
 banding variations 335
- chromosome 15 336
 banding variations 337
 deletions **437**, 489, 490
 duplication **437**
- chromosome 16 338
 banding variations 339
 deletions **437**, **438**
- chromosome 17 340
 banding variations 341
 deletions **438**
 duplications **438**
- chromosome 18 342
 banding variations 343
 deletions **438**, 459
 FISH variation 343
 insertion 473
 trisomy **438**
- chromosome 19 344
 banding variations 345
- chromosome 20 346
 banding variations 347
 deletion **438**
- chromosome 21 348
 banding variations 349
 trisomy *see* Down syndrome
- chromosome 22 348
 banding variations 349
 deletions **438**, **439**
 duplication **439**
- chromosome count 365–367
- condensation 225, 228
- D group 303
- E group 303
- F group 303
- G group 303
- heteromorphisms 301–302, 356
- homologues 361–362
- identification 301
 shapes 302–303
- microscopy/analysis 59–60
- preparation methods 26
- random chromosome loss 62
- shapes 61
- staining *see* banding techniques; staining
- stalks 60, 302, 357
- structure 224
- translocation 61
- see also* chromosome abnormalities; chromosome bands; chromosome instability syndromes; karyogram; sex chromosomes
- chromothripsis 458
- chronic eosinophilic leukemia (CEL) 516

- chronic lymphocytic leukemia (CLL) 521
 protocol 566–571
- chronic myelogenous leukemia (CML) 515
- chronic myelomonocytic leukemia (CMML) 515–516
- chronic neutrophilic leukemia (CNL) 516
- cilia 5
- clastogen 656–657, 663, 671, 676
- clear cell renal cell carcinoma 606, 606
- clear cell sarcoma (CCS) 598
- clinodactyly 663
- clone 405
 mainline clone 405–406
 neoplastic clonal evolution 405, 406
 positional cloning 666
 sideline clone 406–407, 406
 stemline clone 406–407, 406
 multiple stemlines 408
- CM-FISH 835
- COBRA 719, 834–835
- Colcemid(R) 27, 28, 36–37
 concentration 39
 effects on chromosomes 36
 effects on melanoma cells 38
 mitotic arrest 37
 use on FISH specimens 747–748
- colchicine 5, 27, 37
- collagenase 138
- comparative genomic hybridization (CGH) 29, 833, 834, 838, 849–861
 advantages and limitations 851
 applications 851–853
 solid tumors 611–612, 853, 855
- DNA quantification and purity determination 973–974
 methods 841, 853–861
 genomic DNA 855
 hybridization 856, 900
 image acquisition 856–861, 857, 860
 metaphase preparation 853–855
 nick translation 855, 894–895, 898
 post-hybridization wash 856
 precipitation and denaturation 856, 895–896
- online databases 833–834
- protocols 891–901
 theory 851, 852
 troubleshooting 860, 861
see also chromosomal microarray (CMA)
- compensating eyepieces 693
- competency 1034–1035
- competency program 1020
- complete cytogenetic study 68
- compliance 1001–1002
 laboratory information system 1048
- composite karyotype 402
- compressed gas cylinder safety 992
- computer workstations 1000, 1000
- condor, California 1059, 1069
- confined placental mosaicism (CPM) 87–88, 185
- confocal microscopes 703
- congenital mesoblastic nephroma (CMN) 607
- consensus sequence 13
- conservation 1059
- constitutive heterochromatin 11, 227, 230–231, 494
see also C-bands; heterochromatin
- contamination
 amniotic fluid culture 177
 maternal cell contamination 180
 antibiotic addition to culture media 94, 127
 treated media for contamination 158
 chorionic villus sample 186
 monitoring 127
Mycoplasma testing 159–160
 continual improvement 1024–1025
- continuous cell line 119
- copy number probes (CNPs) 914
- copy number variations (CNVs) 21, 920
 identification of 911–912
- cordocentesis 186
- core binding factors (CBF) 555
- core needle biopsy 612
- Cornelia de Lange syndrome 435
- cost per test 1038
- Cot curve 8
- Cot-1 DNA 739–740, 842–843
- coverslips 215–216
 FISH specimens 752
 solid tissue culture protocols 145–150
see also slide-making
- CpG islands 228, 494
 imprinted genes 484
- credentialing 1002–1003, 1021, 1112–1113
- CREST syndrome 236, 237, 286
- cretinism 664
- Cri-du-Chat syndrome 435
 probe 731
- cristae 4
- critical limits 1028–1029
- CRLF2* abnormalities 520
- crocodile
 Nile 1059
 slender-snouted 1070
- crossing over 18
- cryopreservation 131–132
 programmed freezing 131
 protocols 165–166, 171–172
 animal fibroblast culture 1092–1093
 lymphoblastoid cell lines 171
 Nalgene cryogenic container 166–167
 tissue biopsy freezing for later culture 1094–1095
- storage 131
 records 132
 thawing 132, 172
- cryptic species 1059
- cryptorchidism 664, 1072
- CT-banding 252
 protocol 295
- culture media 34, 93–94, 125–126
 amniotic fluid culture 176
 medium addition or changes 177
 treated media for contamination 158
- tumor cell culture 583

- cultures 33–35, 34, 57, 119, 121–127
 absolute plating efficiency 120
 ambient conditions required 124–125
 amniotic fluid 175–178
 protocols 188–191, 195–197
 antimicrobial agents 94, 127
 balanced salt solutions (BSSs) 126–127
 blood cultures 92–93
 equipment and supplies 93–94
 protocols 98–112, 117
 troubleshooting 97
 buffers 94
 contamination management *see* contamination
 cryopreservation protocols 165–166, 171–172
 Nalgene cryogenic container 166–167
 culture containers 122–123
 culture protocols 141–154
 chorionic villus samples 199–206
 culture from feather pulp 1095–1096
 culture from solid tissue 1096–1097
 culture maintenance 154–155, 163
 culture using enzyme digestion 1097–1098
 hematologic disorders 562–575
 reagents 138–140
 subculture 155–156
 tissue collection and transport 134–138
 dispersion of monolayer cells for subculture 127
 fibroblasts *see* fibroblast cultures
 growth characteristics 120
 growth rate estimation 162–163
 growth factors 94
 media *see* culture media
 monolayer 120
 morphology 120, 120
 quality control 1026
 S-phase synchronization 163–164
 sterile technique 121, 122, 123–124
 suspension 120
 time factors 119–120
 tissue source 121
 tissue transport 128, 132, 134–135
 transport media 135–138
 tumor cells 581, 584, 584
 growth patterns 586
 protocols 631–644, 646–651
 villus 184
 water for 124
see also cryopreservation; culture media
 cutaneous T-cell lymphoma (CTCL) 525
 cyclin-dependent kinases (CDKs) 14
 inhibitors (CKIs) 14
 cyclins 14
 cystic hygroma 132, 433
 fluid for prenatal diagnosis 209–210
 Cytocell Chromoprobe Multiprobe® panels 799–800
 CytoGen system 1048–1051
 case log-in and specimen processing 1049, 1050
 report writing 1049, 1050
- cytogenetics 25
 comparative 840
 complete cytogenetic study 68
 historical background 25–28, 30–31
 laboratory procedure manual 69–70
 methods 29–49
 centrifugation 35–36
 chromosome anticontraction methods 43–46, 44, 48–49, 48
 culture methods 33–35, 34
 fixation 40–41, 49
 harvesting 35, 41–42, 49
 hypotonic treatment 37–40, 39
 removal of attached cells 35
 specimen logging 32–33
 specimen procurement 29–32
 synchrony chemicals 43, 46–48, 46, 49
see also karyotyping
 cytogenetics support groups 1109
 cytopenia 555
 cytoplasm 3–5
 cytosol 3
- dactomycin 48
 daily productivity 1039
 DAPI 241, 242, 242, 754, 763–764, 763, 778
 DAPI/distamycin A staining 241–243, 241, 778
 clinical significance 243
 FISH DAPI bands 252
 protocol 275–276
 dark band 356
 databases 912, 1052, 1103–1105
 online 833–834
 DECIPHER database 912
 dedifferentiation 612
 delays in test results 1028
 deletions 380–381, 445, 449–450
 gorilla 1061
 interstitial 381, 449–450, 450
 microdeletion 28, 381, 382, 449–450
 terminal 373, 380–381, 449–450, 450
see also specific chromosomes
 denaturation 8, 717, 777
 comparative genomic hybridization 856, 895–896
 FISH 751–752, 792–793, 847
 multicolor FISH 847, 886, 889–890
 protocol 792–793
 deoxyribonucleic acid *see* DNA
 depreciation 1042
 derivative chromosomes 394
 homologue derivative 394, 395
 isoderivative 395
 multiple events derivative 395–396
 neocentromere derivative 396
 one centric derivative 394
 ring derivative involving more than one chromosome 396
 whole arm derivatives 396–397, 398
 desmoplastic small round cell tumor (DSRCT) 598, 730

- destaining slides 252
 protocol 298–299
- developmental delay 907–908, 916, 918, 921
- diakinesis 18
- dicentric chromosomes 381–383, 383, 455–456
 acute lymphoblastic leukemia 520
 pseudodicentric 383
see also isodicentric chromosome
- diepoxybutane (DEB) 664
- diffuse large B-cell lymphoma (DLBCL) 522
- DiGeorge critical region (DGCR) 410, **468**
- DiGeorge syndrome (DGS) 21, 382, **470, 471**
 probes **731**
- digital imaging 702–703, 705
 advanced contrast 710–712, **711**
 brighten/darken slider 711
 cutoff % slider 711–712
 pink/blue sliders 711
 animal cytogenetics 1066
 archiving 715
 enhancement 710
 contrast 710
 sharpening 710
 FISH imaging 703, 713–714
 microscope setup 713
 probe enhancement 715
 thresholding 714, **716**
- image capture 707–710
 comparative genomic hybridization 856–861, **857, 860**
 computer gray levels 707, 707
 FISH signals 764
 gray scale 708–, 708, 710
 indicator chromosomes 710
 multicolor FISH 848–849
- image quality improvement 705
 metaphase preparation 706
 macro programming 712, **712, 713**
 printing 715
 quality control 715, 1027
- digoxigenin (DIG) 719, 778
- dik-dik, Kirk's 1057
- dilution *see* solutions
- dishwashing procedure 1003–1004
- disinfectants 979
- disjunction
 meiotic 61–62
 mitotic 63
- distal direction 361, **361**
- distamycin A 241
 chromomycin/distamycin R-banding protocol 277–278
 DAPI/distamycin A staining 241–243, **241, 242**
 protocol 275–276
- division 939–940
- DNA 5, 6–9, **718**
 condensation 225, 228
 consensus sequence 13
- denaturation 8, 717, 778
- comparative genomic hybridization 856, 895–896
- FISH 751–752, 792–793, **847**
 multicolor fish 847, 886, 889–890
 protocol 792–793
- fingerprinting 21
- genomic variation 21–22
- G–C content 8–9
- lesions 653
- methylation 12, 484, 494, 495, 665
 assay 492
 hypomethylation in ICF syndrome 660
- mitochondrial (mtDNA) 4
- permanganate effect 224
- purity determination 973–974
- quantification 973–974
- renaturation 717
 FISH specimens 753
 repair 653, 654
 replication 7, 7, 14
 replication banding 14, 229, 248, 249, 250–251
see also late replication analysis
- satellite DNA 13, **13, 301, 724**
- sequencing 19
 structure 6–7, 6, **718**
 transcription 6, 9, 9
- DNase I sensitivity 228
- DNMT3B 664
- documents 1015–1018
 creation and control 1016–1018
 elements of **1016**
- dosage compensation 12, 15
- double minute 60, 402, 578
- double ureters 664
- double-hit lymphoma 524, **524**
- Down syndrome critical region (DSCR) 409
- Down syndrome (trisomy 21) **59, 470**
 clinical consequences 430, 433
 isochromosome and **387**
 karyogram **432**
 mosaicism **920**
 Robertsonian rearrangements 62
- dry heat sterilization 123
- drying 58
see also slide-making
- dual-fusion FISH
 interphase **414, 415**
 metaphase **413, 415**
- duplications 383, 445, 450–451, **451**
 direct 450
 inverted 451
see also specific chromosomes
- dynamic mosaicism 458
- ectoderm 87
- Edwards syndrome **433, 470**
- karyogram **432**
- EGR1* deletion probe **734, 734**

- electrical safety 987–988
 electrophoresis 19
 Emanuel syndrome 471
embryo diagnosis *see* preimplantation genetic FISH
 embryonal rhabdomyosarcoma 596
 encephalocele 664
 ENCODE project 22
 endoderm 87
 endodermal sinus tumors 493, 494
 endoplasmic reticulum (ER) 3
 endoreduplication 378, 378, 664
 environmental control chamber 992–993
 epicanthal folds 664
epidermolysis bullosa 492, 494
 epigenetic mechanism 494
 epithelial (E-type) cells 120, 120, 121, 174
 Eppendorf pipette calibration 1004–1006
Epstein–Barr virus (EBV) 167–168
 equipment operation, maintenance and safety 988–990, **989**
 autoclaves 990
 automated hybridization system 995
 biological safety cabinets 993
 centrifuges 990
 compressed gas cylinders 990
 environmental control chamber 992–993
 fume hoods 995
 incubators 994–995
 microscopes 992
 ovens 992
 pH meter 994
 pipettes 993–994
 refrigerators/freezers 991–992
 robotic harvester 995–996
 scales 994
 thermometers 991
 timers 994
 water baths 992
ERBB2 gene amplification 418–420, 419
 ergonomics 996–997, **996**, 997, 998
 error, sources of 69
 erythema 664
essential thrombocythemia (ET) 516
 ethanol
 dilutions 950
 solution preparation 947
 euchromatin 5, 10, 227
 euploidy 429, 441–442
 event symbols 367, **368–369**, **370–371**
 evolutionary comparisons 912
Ewing sarcoma 599, 601, 610, 730
 exons 8
 exonuclease 664
 expense projection 1040
 exponent 937
extrakeletal myxoid chondrosarcoma (EMC) 598
 eyepieces 693

 facultative heterochromatin 11, 227, 494
 see also heterochromatin
 False Claims Act 999–1000

 Fanconi anemia 88–89, 653, **655**, 656–658
 analytic performance monitoring 1029
 cancer susceptibility 656
 clinical features 656
 diagnosis 654, 656–657, 657
 protocols 671–674, 676–685
 somatic mosaicism 658
 Federal Anti-Kickback Statute (AKS) 1000
 fetal blood sampling 186
FGFR1 rearrangement 516
 fiber FISH technique 770–771
 fibroblast 664
 fibroblast cultures 120, 120, 121, 128–132
 animal cytogenetics 1062–1063, **1063**, **1064**, 1092–1093
 cryopreservation 131–132, 1092–1093
 Nalgene Cryogenic container 166–167
 programmed freezing 131
 protocol 165–166
 records 132
 storage 131
 thawing 132
 growth rate estimation 162–163
 lymphoblastoid cell lines 132
 preparation for analysis 130–131
 biochemical protocols 130
 chromosome analysis 130
 molecular genetics protocols 130
 synchronization and phasing 130, 163–164
 protocols 141–142, 145–147, 150–156
 culture maintenance 154–155, 163
 subculture 155–156
 routine handling and maintenance 129
 S-phase synchronization 163–164
 sister chromatid exchange testing protocol 291–294
 specimen setup 128–129
 subculture 129, 155–156
 tissue sampling 128
 mosaicism exclusion 128
 tissue transport 128, 132
 tracking culture age 131
 Ficoll separation of whole blood 675–676
 filter sterilization 124–125
 final report 68–69
 financial management 1037–1038
 budget development and monitoring 1039–1043
 capital expenditure 1041–1042
 forecast 1039–1040
 trends and measures 1042–1043
 cost per test 1038
 fine needle aspiration (FNA) 612
 fingerprinting 21
 fire safety 986, 988
 drills 986
 fire extinguishers 986
 fire prevention unit 986
 fishbone diagram 1022
 fixation 40–41, 49
 blood cells 106–107
 fixative ratio 56
 G-banding 224

- tumor cells 586
see also slide-making
- fixative 140
 quality and freshness 56
 solution preparation 950
- flagella 5
- flow chart 1017, 1022
 laboratory information system interfaces 1046
- fluorescence 741, 778
- fluorescence *in situ* hybridization (FISH) 28–29, 28, 214, 408–420, 718, 719, 834
- abortus tissue 164, 803–806
 cultured non-mitotic abortus tissue 804–806
 direct preparations 164, 803–804
- amniotic fluid 745, 746, 801–803
- analysis 757–765
 image capture 764
 microscopy 757–757, 758, 795
 performance monitoring 131
 scoring 758–764, 759, 760, 762, 763, 765
- animal cytogenetics 1099–1102
- array CGH confirmation FISH 776, 915
- automated process 811–814
- background signal interference 743–744, 744, 745
- bone marrow transplant chimerism 420
- breast tissue analysis 824–831
- chromosomal variations
 chromosome 4 313
 chromosome 15 337
 chromosome 18 343
- digital imaging 703, 713–714
 microscope setup 713
 probe enhancement 714
 thresholding 714, 714
- fiber FISH 770–771
- FISH DAPI bands 252
- fusion probes 410, 415, 726–729, 727–728, 729
 dual-fusion FISH 413, 414, 726
 extra signal 415, 726, 729
 interphase fusion karyotype 414–415, 415
 metaphase fusion strategy 412–413, 415
 single-fusion FISH 412–413, 414, 726
- hematologic malignant conditions 532
- historical development 717–719
- interphase FISH karyotype 413–414
 string of interphase FISH results 416–418, 417
 variations 761
- metaphase FISH karyotype 410–412
 abnormal results 412, 412
 normal results 411–412
 variations 761
- methods 744–757
 counterstains 754
 coverslips and sealants 752
 denaturation 751–752, 792–793
 detection 754–755
 harvest/cell preparation 747–748
 post-hybridization wash 753
 pretreatments 749–751, 800–801
 probe preparation for use 756
- renaturation 753
- slide preparation 58, 748, 754, 755, 792–794
- slide storage 748–749, 754
- specimen 745–747
- molecular mechanisms 717
- mosaicism detection 438, 765
- multicolor FISH *see* multicolor (multiplex) FISH (M-FISH)
- multiprobe® FISH panels 799–800
- oncological specimens 734
 bladder cancer 776, 777
 paraffin-embedded malignant tissue 418–420, 419, 608, 810–811
 plasma cell targeting 814–815
 separation and harvest procedures 567–571, 815–818
- preimplantation genetic FISH 771–775, 818–823
 methods 774–775
 obtaining samples 772–773
 preimplantation abnormalities 773–774
 probe design 773
 scoring 775
 technical issues 773, 774
- pretreatments 749–751
 2x SSC treatment 749
 aging and baking slides 749
 formalin-fixed paraffin-embedded tissues 750–751
 protease pretreatment 750, 800–801
 RNase treatments 750
 salvaging overbaked slides 749
 probe adjuvants 739–741
 buffers 740–741
 Cot-1® blocking DNA 739–740
- probe designs 720–721, 722–730, 727–728
 analyte specific reagents 722
 break-apart probes (BAP) 410, 415–416, 416, 729–730, 730
- cancer-related probes 734–736
- centromere-specific probes 726, 735
- chromosome paints 416, 722, 723, 735, 745, 779, 796–799
- deletion probes 730–732, 731, 732, 733, 734–735, 737, 736
- duplication probes 730–732
- fusion probes 140, 412–414, 415, 726–729, 727–728, 729
- in vitro diagnostics (IVD) 722
- interphase probes 734
- locus-specific probes 722, 723, 735, 778, 779, 796–799
- NOR probes 726
- off-label use 739
- preimplantation genetic FISH 773
- satellite DNA probes 724–726, 725, 777
- subtelomere-specific probes 732–733, 734, 777, 780
- telomere probes 733, 780
- probe mixtures 738, 739
- probe name 410
- probe sources and characteristics 736–738
- probe concentration 738
- probe size 737
- probe storage 738
- probe terminology/nomenclature 738

- fluorescence in situ hybridization (FISH) (*cont'd*)
 probe validation 408–409, 765–768
 controls 768
 cutoff establishment 766–767, 956–961
 efficiency 766
 multiplication rule 959–961
 sensitivity 765–766
 special considerations 767
 specificity 766
 protocols 790–831
 animal cytogenetics 1099–1102
 quality control 1027
 reporting results 765
 Rx-FISH 769, 770, 834
 safety precautions 757, 790
 signal patterns 409–410
 smears 806–808
 solid tumor applications 608–610, **609**
 special investigation 768–771
 sequential staining methods 768–769
 sperm FISH 775, 776
 stringency 741–743, **743**
 troubleshooting **784–789**
 variants in signal pattern 777
 alpha satellite DNA probes 777
 locus-specific probes 777
 subtelomere-specific probes 777
 very small samples 808–810
 Zoo-FISH 1070
 fluorescence microscopy 697–699, 698, 757–758, 757
 filters 699
 light sources 697–699
 see also microscope
 fluorescent dyes 215, **218**
 fluorescent R-banding 238–241, 241
 acridine orange 238–239
 chromomycin A3/methyl green 240, 240
 protocol 277–278
 fluorite objective lens 690
 fluorochromes 697
 fluors 741, **742**, 778
 multicolor FISH **843**
 focal nodular hyperplasia 664
 follicular lymphoma (FL) 523
 forecast 1039–1040
 Fork Stalling and Template Switching model (FoSTeS) 448–449
 formamide 742–743
 formula weight (FW) 943
 fragile sites 66, 89, 404, 653
 common 404
 rare 404
 fragile X syndrome 66, 89, 404
 freezers 991–992
 freezing *see* cryopreservation
 full-time equivalents (FTEs) 1040
 fume hoods 984–985, 995
 fungizone–mycostatin solution 158
 fusion 778
 fusion probes *see* fluorescence in situ hybridization (FISH)
 G-11 staining 233–235, **234**
 clinical significance 234
 historical and theoretical perspectives 233–234
 protocol 274–275
 technical considerations 235
 G-banding 27, 28, 43, 52, 58–59, 214, 215, 222–230, **834**
 amniotic fluid 179
 animal cytogenetics 1065, *1065*, *1066*
 base composition and isochores 227–228
 blood lymphocyte slides 284–285
 bone marrow 278–279, 571–575
 cancer blood cells 278–279
 dye structure and binding 225, 226–227
 GTG technique 223
 historical and theoretical perspectives 222–224
 Leishman's stain protocol 208–209
 protein role 224–226, 229
 protocols 278–285, 571–575
 quality comparison **54**
 quality control 1026
 replication timing 228
 late replication analysis 297–298
 resolution determination 355
 Johnson and Stallard method 355, **356**
 quality control 355
 Vancouver method 355, **355**
 Welborn method 355
 structural and functional relationships of bands 227
 technical aspects 229–230, 229, 230
 tumor cells 586–587
 variations
 chromosome 4 **313**
 chromosome 9 **323**
 chromosome 13 **333**
 chromosome 22 **349**
 see also banding techniques; Giemsa stain
 gametic complementation 494
 gazelle, Soemmerring's 1057, *1058*
 gene dosage effect 494
 genes
 alleles 15–16
 replication 6, 7, 7
 transcription 6, 9, 9
 genetic code 8, **8**
 genome 21–22
 genomic DNA variation 21–22
 genomic imprinting *see* imprinting
 germ cell tumors (GCTs) 493, 578, 607
 giant cell tumor of bone (GCTOB) 599
 Giemsa stain 219–220
 protocol 266
 restaining permanently mounted slides 300
 see also G-11 staining; G-banding; R-bands; staining
 glioblastoma 602
 glioblastoma multiforme (GMB) 610
 gliomas 602
 gloves 977, 978
 glycolipids 3
 glycoproteins, cell membrane 2

- goal setting 1032
 Golgi complex 3
 gonadal ridge 494
 gorilla 1061
 grammatical symbols 367, **370–371**
 granulocytic sarcoma 534
 gray scale 708–710, 708
 gross revenue budget 1040
see also financial management
 growth factors, culture media 94
 guidelines 1110–1111
 mosaicism 64–65, 403
 near-ploidy **378**
 reporting 403
- hairy cell leukemia (HCL) 521
 Ham's F10 medium 140
 Hanks' balanced salt solution 138–139
 Hanks' trypsin solution 139
 haploid karyotypes 429
 haploinsufficiency 534
 HapMap Project 912
 haptens 779
 multicolor FISH **843**
 harvesting *see* cell harvesting
 Hazard Communication Standard (HCS) 981
 hazard identification symbol 981, 983
 helicase 664
Helicobacter pylori infection 524
 hemacytometer 969
 cell count determination 968–972, 969, 970
 cell viability determination 972–973
 hemangioma 664
 hematologic malignant disease 499
 common cytogenetic abnormalities **500–507**, 525
 cytogenetic methodology 525–533, **526–528**
 FISH 532
 ICD grouping **530**
 see also leukemia; lymphoma
 hematopoietic system 509
 heparin 92
 hepatitis precautions 975
 hepatoblastoma (HB) 604
 hepatocellular carcinoma 664
HER2 testing 418
 FISH analysis 824–831
 breast tissue pretreatment time 745–747, 747
 hereditary neuropathy with liability to pressure palsies (HNPP) **470**
Herlitz junctional epidermolysis bullosa 492
 heterochromatin 5, 10–11, 227, 494
 animal cytogenetics 1066
 constitutive 11, 227, 230–231, 494
see also C-bands
 facultative 11, 227, 494
 heteromorphic variation 301–302, 356, 403–404
 heteroplasmy 4
 heterozygosity 612
 hibernoma 593–594
- high-resolution banding 28
 histogenesis 613
 histones 10, 224, 494
 acetylation and deacetylation 229
 imprinting and 484
 HIV precautions 975
 Hodgkin lymphoma 514, 524–525
 Hoechst dye 249, 250
 homogeneously staining region (hsr) 399, 400
 homologous recombination (HR) 653, 665
 homologues 361–362
 homologue derivative 394, 395
 horse 1056, **1056**, 1071
 lymphocyte culture protocol 1087–1089
 horseshoe kidneys 665
 housekeeping genes 228
 human genome
 genomic variation 22
 sequencing project 21–22
 Human Structural Variation Database 912
 Huygens eyepieces 693
 hydatiform mole 133, 481, 482, 494–495
 hypertelorism 665
 hypogenitalia 665
 hypomethylation in ICF syndrome 660
 hypopigmentation 665
 hypoplasia 665
 hypospadias 665
 hypothyroidism 665
 hypotonic treatment 37–40, 39
 solutions **36**, **40**
 tumor cell culture 585
- idem 406
 idiogram 362
IGH-PAX5 rearrangement 525
 immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome **655**, 660, 660
 immunophenotype 534
 imprinting 481–486, 485, 494
 genomic disease and 488–493
 chromosomal syndromes 488–490
 germ cell tumors and 493
 growth regulation and 486, 486
 imprinted genes **483–484**
 imprinting control regions (ICGs) 495
 in vitro device (IVD) microarrays 914
 in vitro fertilization (IVF) 771
see also preimplantation genetic FISH
 incident reporting 998
 incomplete symbol 402
 incubators 124–125
 maintenance and safety 994–995
see also cultures
 indicator chromosomes 710
 insertions 385, 445, 464–466, 465
 direct 464
 interchromosomal 385, 464–465, 465

- insertions (*cont'd*)
 intrachromosomal 385, 464–466, 465
 inverted 464
- internal rate of return (IRR) 1042
- International System for Cytogenomic Nomenclature (ISCN) 359–426
 chromosome numbering system 304, 305
see also specific chromosomes
 language 359–364
 derivative chromosomes 394–397
 fluorescence in situ hybridization (FISH) 408–420
 karyotype 364–377, **423–425**
 microarray and region-specific assay 420–422
 multiple cell lines 404–408
 numerical events 378–380
 structural events 380–394
 symbols of uncertainty 397–402
 standards 359
 updates 426
- interphase 14
- interspersed repeats 228
- interstitial deletion 381, 449–450, 450
- interviews 1033–1034
- intronic mutations 665
- introns 8
- inventory 1042
- inverse beta distribution (betainv) 956–959
 multiplication rule 959–961
- inversions 356, 385–386, 445, 451–455
 paracentric 385–386, 445, 451–452, 452
 recombination models 453–455, 454
 pericentric 385–386, 386, 445, 451–453, 452
 pairing 454
see also specific chromosomes
- inverted microscope 701
- ISCN *see* International System for Cytogenomic Nomenclature (ISCN)
- isochores, G-bands and 227–228
- isochromatic gap 367
- isochromatid break 367, 371
- isochromosome gap 372
- isochromosomes 386–387, 387, 456–457, 456, 495
- isoderivative 395
- isodicentric chromosome 387–388, 388, 456
 pseudoisodicentric 387–388, 388
- Jacobson syndrome **469**
- JAK2* mutation 516
- job descriptions 1033
- job search 1112
- journals 1107
- jumping translocations 408
- junctional epidermolysis bullosa 492
- juvenile myelomonocytic leukemia (JMML) 516
- Kallmann syndrome **471**
 probe **731**
- Karnofsky performance score 613
- karyogram 66, 68, 354, 362–364
 abnormal 365, 366
 aneuploidies **432**
 arrangement 302
 banded 67
 karyotype 362, 364–377
 amending 377
 animal cytogenetics, standards and precedents 1068–1070
 chromosome count 365–367
 composite 402
 detailed 374
 interchromosomal/two breaks 374
 intrachromosomal/one break 374
 intrachromosomal/two breaks 374
 event symbols 367
 formulae **423–425**
 interphase FISH karyotype 416–417
 interphase fusion FISH karyotype 414, 415
 metaphase FISH karyotype 410–412
 abnormal results 411–412, 412
 metaphase fusion strategy 412–413, 415
 normal results 410–411
 order priority 374–376, 375
 repeated description 376–377
 short form 372–374
 interchromosomal/two breaks 372, 373–374
 intrachromosomal/one break 372, 372
 intrachromosomal/two breaks 372, 373
 spaces 372
 string of cytogenetic tests 411
 structural breakpoint 367–369, 371
 tetraploid 443–444, 444
 triploid 442–443, 442
- karyotyping 66–67
 analysis error 69
 animal cytogenetics 1066–1068
 final report 68–69
 laboratory procedure manual 69–70
 molecular 905
 multicolor karyotyping 612
see also karyotype
- kidney tumors 605–607, 606
- kinase 665
- kinetochore 235, 235
 staining 235–238
 Cd staining 235–236
- Klinefelter syndrome 382, **471**
 clinical consequences 430
 tiger 1059
- KMT2A (MLL)* rearrangements 513–514, 519
- Köhler illumination 695, 696, 706–707
- Laboratory Chemical Safety Summaries (LCSS) 981
- laboratory documents *see* documents
- laboratory events 1022–1023
- laboratory information system (LIS) 1045–1046, 1048, **1051**
 compliance and security 1048
 CytoGen system 1048–1051

- definition 1045
- future trends 1051–1052
- historical background 1045
- laboratory management 1049, **1050**
- software and hardware considerations 1046–1047
- validation and implementation 1047–1048
- workflow/LIS interfaces 1046
- laboratory management 1031–1032
 - management functions 1032–1033
 - leadership 1032
 - monitoring and control 1032–1033
 - planning (goal setting) 1032
 - staffing 1032
 - personnel management 1033–1036
 - productivity and workload 1038–1039
 - quality management 1036–1037
- see also* financial management; laboratory information system (LIS)
- laboratory procedure manual 69–70
- landmarks 304, 305, 362, 363
- Langer–Giedion syndrome **468**
- late replication analysis 295–298
 - G-band pattern 297–298
 - R-band pattern 297, 298
- lateral asymmetry 249
- leadership 1032
- Leber congenital amaurosis 492, 495
- Leishman's stain protocols 208–209, 266
 - restaining permanently mounted slides 300
- lenses *see* microscope
- leukemia 499, 508, 534
 - acute lymphoblastic *see* acute lymphoblastic leukemia (ALL)
 - acute myeloid *see* acute myeloid leukemia (AML)
 - adult T-cell leukemia/lymphoma 520
 - B-prolymphocytic (B-PLL) 521
 - Burkitt 523
 - chronic eosinophilic (CEL) 516
 - chronic lymphocytic (CLL) 521
 - chronic myelogenous (CML) 515
 - chronic myelomonocytic (CMML) 515–516
 - chronic neutrophilic (CNL) 516
 - common cytogenetic abnormalities **500–507**
 - cytogenetic methodology 525–533, **526–528**
 - FISH 532
 - mitogens used **529**
 - hairy cell (HCL) 521
 - incidences **508**
 - juvenile myelomonocytic (JMML) 516
 - presenting characters **510**
 - protocols 553–567
 - T-prolymphocytic (T-PLL) 520
 - leukocytopenia 534
 - leukocytosis 534
 - light band 357
 - LINES 22
 - lipids 3
 - lipoblastoma 593
 - lipoma 592
 - chondroid lipoma 594
 - cytogenetic abnormalities 592–593, 593, 594
 - liposarcoma (LPS) 594, 595
 - myxoid (MLS)/round cell (RC) liposarcoma 594
 - well-differentiated (WDLPS) 594, 595
 - liver tumors 604
 - locus-specific probes 722, 723, 735, 779
 - protocol 796–799
 - signal pattern variation 777
 - long interspersed elements (LINEs) 13
 - loss of heterozygosity (LOH) 394
 - low copy repeats (LCRs) 21
 - structural rearrangements and 446–449
 - deletions 449–450
 - duplications 450–451
 - inversions 451–455
 - low-grade astrocytomas (LGA) 602
 - lung cancer 603–604, **603**
 - lymph node biopsy 556
 - lymphadenopathy 534
 - lymphoblastoid cell lines 132
 - protocol 167–171
 - lymphocytes 90–91, **90**
 - animal lymphocyte culture protocols 1078–1084
 - avian lymphocytes 1078–1084
 - horse lymphocytes 1087–1089
 - using autologous plasma/buffy coat 1085–1087
 - using whole blood 1084–1085
 - G-banding protocol 284–285
 - late replication analysis protocol 295–298
 - separation from whole blood 675–676
 - see also* B cells; T cells
 - lymphoma 499, 522, **523**, 552
 - adult T-cell leukemia/lymphoma 520
 - anaplastic large cell (ALCL) 525
 - Burkitt 523
 - common cytogenetic abnormalities **500–507**
 - cytogenetic methodology 525–533, **526–528**
 - FISH 532
 - mitogens used **529**
 - diffuse large B-cell (DLBCL) 522
 - double-hit 524, 524
 - follicular (FL) 523
 - Hodgkin 514, 524–525
 - incidence **523**
 - MALT 524
 - mantle cell (MCL) 523
 - marginal zone B-cell (MZBCL) 524
 - non-Hodgkin 522
 - primary cutaneous (CTCL) 525
 - triple-hit 524
 - lynx, Siberian 1057
 - lysosome 4
 - M-TEL-FISH 835
 - macaque, Rhesus **1066**
 - macrocythemia 665
 - macroglobulin 534

- macroglobulinemia 534
 mainline clone 405–406
 malignant peripheral nerve sheath tumors (MPNSTs) 601
 MALT lymphoma 524
 management *see* laboratory management
 mantissa 937
 mantle cell lymphoma (MCL) 523
 marginal zone B-cell lymphoma (MZBCL) 524
 marker chromosomes 62, 401, 459–460, 918
 Marshall syndrome 921
 maternal age effect 439–440
 maternal cell contamination
 amniotic fluid culture 180, 760
 chorionic villus sample 186, 207
 mathematical symbols 367, **370–371**
 mBAND FISH 835
 mean 962–965
 mediastinum 534
 medical waste disposal 980
 medulloblastoma (MB) 602, 665
 meiosis 16–19, 17, 434, 839
 anaphase I 18
 diakinesis 18
 diplotene 18
 leptotene 17
 metaphase I 18
 metaphase II 19
 nondisjunction 433–435, 434, 435
 pachytene 18
 prophase I 17–18
 telophase I 18
 zygotene 18
 meiotic disjunction 61–62
 meiotic malsegregation 490, 495
 MEM complete medium 140, 148–149
 meningioma 602
 mental retardation 907–908
 alpha-thalassemia/mental retardation (ATR-16) syndrome **469**
 mesoblastic nephroma 607
 mesoderm 87
 mesothelioma 607–608
 messenger RNA (mRNA) 4
 metacentric chromosomes 357, 360
 metaphase finders 703
 metastasis 613
 methotrexate 46
 methyl green 240, *240*, 241
 staining protocol 277–278
 methylation 12, 484, 494, 495, 665
 assay *492*
 hypomethylation in ICF syndrome 660
 methyltransferase 665
 microarray studies 29, 420–422
 see also array comparative genomic hybridization (aCGH)
 microcephaly 665
 microdeletions 28, 381, 382, 449–450
 microphthalmia 666
 microsatellites 13, 724
 instability (MIN) 653
 microscope 687–695, 688
 cleaning 694–695, 992
 condensers 692–693
 confocal microscopes 703
 ergonomic considerations 996
 eyepieces 693
 focus 707
 homogenous system 694
 image capture 701–703
 inverted microscope 701
 lenses 690–692
 curvature of field 691
 depth of field 691
 lens magnification 691
 lens markings 692, 692
 objective lenses 690
 oil-immersion lenses 691–692
 parfocal lens 691
 working distance 691
 light path 688–689
 magnification 689, 689
 higher magnification use 707
 maintenance 992
 mechanical stages 694
 numerical aperture 689–690, 689
 resolution 689–690
 see also brightfield microscopy; fluorescence microscopy; phase
 contrast microscopy
 midline lethal carcinoma 604
 Miller–Dieker syndrome **470**
 probe **731**
 minisatellites 13, 724
 minute chromosomes 459, 460
 double minute 60, 402, 578
 mitochondria 4
 DNA (mtDNA) 4
 mitosis 15–16, *16*
 anaphase 16
 chromatid separation 63
 metaphase 16, 88
 nondisjunction 437–438, 437
 prophase 15
 telophase 16
 mitotic arrest 35, 37
 agents 25, 37
 tumor cell culture 584
 mitotic index 161–162
 mitotic recombination event 457, 493, 495
 modal number 965
 molar solutions
 dilution 947
 to a specific concentration 947–949
 preparation 943–946
 molarity 943
 conversion to percent 951
 percent conversion to molarity 951
 mole 943
 molecular karyotyping 905
 molecular weight (MW) 943

- monoblasts 534
 monoclonal gammopathy of uncertain significance (MGUS) 522
 monomorphism 1072
 monosomal karyotype 534
 monosomy 441
 monosomy rescue 490, 495
 monosomy X *see* Turner syndrome
 mosaicism 62–65, 64, 438–439
 amniotic fluid culture 180
 interpretation criteria 198–199
 chorionic villus sample 185
 CMA analysis 438–439, 920
 confined placental mosaicism (CPM) 87–88, 185
 constitutional 405
 detection 65
 dynamic 458
 exclusion 128
 Fanconi anemia 658
 FISH analysis 438, 765
 guidelines 64–65, 403
 marker chromosomes and 459
 mitotic nondisjunction and 437
 mosaic triploidy 443
 trisomy 9 **468**
 trisomy 21 920
 variegated translocation mosaicism 667
 motivation of staff 1035–1036
 mounting media 216–218
 mouse *1065*
 multichromosomal translocation 392–393, 393
 multicolor (multiplex) FISH (M-FISH) 612, 719, 722–724, 724,
 779, 834–849, **834**, 837
 applications **835**, 837–840
 3D nuclei 840
 animal models 840
 cancer 839
 meiosis 839
 preimplantation genetic diagnosis 839
 prenatal diagnosis 837–839
 constitutional disorders 837
 methods 840–849, **841**
 counterstain 848, 891–892
 denaturation 847, 886, 889–890
 image acquisition 849–850
 post-hybridization wash 847, 886, 890
 probes 842–846, **843**, **844–845**
 slide aging and storage 842
 slide preparation 841, 886
 slide pretreatment 846, **846**, 886, 889
 online databases 833
 protocols 884–891
 safety considerations 864
 theory 835–837, 836–837
 troubleshooting 849, **850**
 multilineage dysplasia 534
 multiple cell lines 404
 multiple events derivative 395–396
 multiple myeloma (MM) 521–522, 816
 multiplex-FISH *see* multicolor (multiplex) FISH (M-FISH)
 multiplication 939–940
 multiplication rule 959–961
 Mycoplasma testing 159–160
 mycosis fungoïdes (MF) 525
 myelodysplastic syndromes (MDSs) 514–515
 5q-syndrome 515
 cytogenetic abnormalities **500–501**
 pediatric 515
 therapy-related 514
 myelofibrosis 534
 myeloid metaplasia 534
 myeloproliferative neoplasms 515–516
 myxedema 666
 myxoid liposarcoma (MLS) 594
 N-banding 244
 Nalgene Cryogenic Controlled-Rate freezing container 166–167
 National Fire Protection Agency (NFPA) 984
 hazard identification symbol 984, 985
 near-ploidy 379
 guidelines **378**
 neocentromere 459, 1072
 neocentromeric chromosomes 398, 465
 neoplasm 534
 neoplastic clonal evolution 405, 406
 neoplastic polyploidy 407–408
 nephroblastoma 607
 nephroma, mesoblastic 607
 net present value (NPV) 1041
 net revenue budget 1040
 see also financial management
 neuroblastoma (NB) 601–602
 neurofibromatosis type 1 (NF1) 599–601
 probe **731**
 neurofibromatosis type 2 (NF2) 601
 neutropenia 666
 neutrophilia 556
 neutrophils 556, 666
 nevus 613
 nick translation 855, 894–895, 898
 Nijmegen breakage syndrome (NBS) **655**, 659, 661
 non-Hodgkin lymphoma 522
 non-small-cell lung carcinoma (NSCLC) 603–604
 nonallelic homologous recombination (NAHR) 21, 446–449,
 457, 460
 nondisjunction 63, 433–435, 434, 435
 mitotic 437–438, 437
 secondary to chromatid predision 433, 436
 nonhistone proteins 10–11
 nonhomologous end-joining (NHEJ) 448–449, 460
 repair (NEJR) 653
 NOR *see* nucleolus organizing regions (NORs)
 NOR probes 726
 Not 1 492, 493, 495
 nuclear envelope 5
 nuclear matrix 5
 nucleic acids 6, 781
 see also DNA; RNA
 nucleolus 5, 243

- nucleolus organizing regions (NORs) 5, 60, 358
 interstitial insertions 89
 N-banding 244
 silver staining (AgNOR) 243–246, 243
 protocol 287–288
 technical considerations 245–246
- nucleosomes 10
 core particle 223
- nucleotides 6
 instability (NIN) 653
- nucleus 5–10
 3D nuclei FISH 840
- nullisomy 379
- obligate heterozygotes 666
- Occupational Safety and Health Administration (OSHA) 975, 979
- oil-immersion lenses 691–692
- oligodendrogloma 602
- oligonucleotides 779
- one centric derivative 394
- operating budget 1040
see also financial management
- orangutans 1057
- osteosarcoma (OS) 599, 600
- ovarian tumors 607
 teratoma 481, 482
see also germ cell tumors (GCTs)
- ovens 992
- p-arm 357
- P1 bacteriophage 737
- p53 (TP53) gene 14, 666
 lung cancer and 603, 605
- Pallister–Killian syndrome 386, 387, 431, 469
- pancytopenia 666
- papillary renal cell carcinoma 606
- paracentric inversion 386, 453, 455, 469
 chromosome 11 469
 recombination models 453–455, 454
- paraffin-embedded malignant tissue 418–420, 420, 608
 FISH studies 810–811
 pretreatment 745–747, 749, 750–751
 scoring 764
- parental origin 388
- Pareto charts 1023, 1023
- parfocal lens 691
- Patau syndrome 469, 431
 karyogram 430
- patent ductus arteriosus 666
- payback period 1042
- PDGFRA* rearrangement 516
- PDGFRB* rearrangement 516–517
- pediatric myelodysplastic syndromes 515
- peer-reviewed journals 1107
- Pelizaeus–Merzbacher disease 471
- pepsin pretreatment for FISH 800–801
- percent solutions
 dilution 950
 molarity conversion to percent 951
- percent conversion to molarity 951
 preparation 946–947
- percutaneous umbilical blood sampling (PUBS) 186
- performance evaluation 1035
- pericentric area 357
- pericentric inversion 385–386, 386, 445, 451–455, 452
- chromosome 2 309
- chromosome 8 468
- chromosome 9 468
 pairing 454
 Y chromosome 471
- peripheral blood 87–88
 chromosome analysis 95
 chromosome instability syndrome studies 88–89
 constituents 89–91
 culturing 92–93
 animal cytogenetics 1063–1064, 1078–1084
 equipment and supplies 93–94
 harvesting 95
 troubleshooting 97
- fragile site studies 89
- protocols 98–117
 hematologic disorders 553–567
 microarray confirmation studies 115–117
 rhino blood culture 1089–1090
- specimen handling 91–93
 anticoagulants 92
 stimulants 91
 storage of fixed specimens 95
- peripheral nerve sheath tumors (PNSTs) 599–601
 malignant (MPNST) 601
- peroxisome 4
- permanganate effect on pyrimidine residues 224
- personal protective equipment (PPE) 977, 979
- personnel management 1033–1036
 competency 1034–1035
 job descriptions 1033
 motivation and retention 1035–1036
 performance evaluation and appraisals 1035
 policy manual 1036
 staff level assessment 1033
 team selection 1033–1034
 training 1034
- pH meter 994
- phase contrast microscopy 699–701, 700, 706
- Phelan–McDermid syndrome 471
- Philadelphia chromosome (Ph) 514
 acute lymphoblastic leukemia 519
 acute myeloid leukemia 514
 chronic myelogenous leukemia 515
see also BCR/ABL1 translocation
- phosphate buffer solution 141
- phospholipids, cell membrane 1
- phytohemagglutinin (PHA) 91
- pipettes 993–994
 Eppendorf pipette calibration 1004–1006
- placenta 1092
 biopsy procedure 1091
- planapochromatic lens 690

- planning 1032
 calendar 1039
- plasma cells 816
 dyscrasias 816
 FISH studies 567–571, 814–818
 separation and harvest procedures 567–571, 815–818
- plasma membrane *see* cell membrane
- plasmacytosis 534
- plasmids 19
- pleomorphic adenoma 604, 605
- pleural effusions 133
- ploidy 378
 near-ploidy 379
 guidelines 378
- PML-RARA* oncogene 511
- poikiloderma 666
- policy manual 1036
- polycythemia vera (PV) 516
- polyhydramnios 490, 495
- polymerase chain reaction (PCR) 21, 666
 degenerate oligonucleotide-primed (DOP-PCR) 842–843
 reverse transcriptase PCR (RT-PCR) 611
- polymorphisms 404, 915
- polyploidy 378
 neoplastic 407–408
- polysaccharides 3
- polysomes 4
- population doubling number 119
- population doubling time 119
 growth rate estimation 162–163
- positional cloning 666
- potassium chloride solution preparation 943–944
- Potocki–Lupski syndrome 470
- Potocki–Shaffer syndrome 468
- powers of 10 937, 938
- Prader–Willi syndrome 469, 488–490, 490
 clinical features 489
 CMA analysis 922
 FISH analysis 762–763
 probes 731, 732, 733
 methylation assay 492
 uniparental disomy 489–490, 491
- preimplantation genetic FISH 771–775, 839
 aneuploidy screening 771, 818–821
 methods 774–775
 obtaining samples 772–773
 preimplantation abnormalities 773–774
 probe design 773
 protocols 818–823
 scoring 775
 technical issues 773, 774
 translocation status screening 771, 772, 821–823
- prenatal diagnosis 28, 173, 837–839
 CMA 911
 cystic hygroma fluid 209–210
 fetal blood sampling 186
see also amniotic fluid; chorionic villus sampling (CVS)
- primary colony 119
- primary constriction *see* centromere
- primary cultures 119
- primary myelofibrosis (PMF) 516
- primitive neuroectodermal tumor (PNET) 599
- PRINS (primed in situ labeling) 775
- printers 703
- printing 715
- Privacy Rule 998
- probes *see* fluorescence in situ hybridization (FISH)
- procedure manual 69–70
- process management 1013–1015
- productivity 1038–1039
- products of conception 134
 transport medium 137
- professional organizations 1112
- proficiency testing 1021
- progenitor cells 666–667
- programmed freezing 131
- prostate cancer 605
- proteins 3
- proximal direction 361, 361
- pseudodicentric chromosomes 383
- pseudodiploid karyotype 380
- pseudoisodicentric chromosomes 387–388, 388
- pulmonary stenosis 667
- pycnotic sottoses 492, 495
- q-arm 357
- Q-banding 27, 28, 58–59, 215, 220–222, 220
 chromosome 13 330
 clinical significance 221–222
 historical and theoretical perspectives 220–221
 protocol 266–268
 technical considerations 222
 variations
 chromosome 3 311
 chromosome 4 313
 chromosome 5 315
 chromosome 13 333
 Y chromosome 353
see also banding techniques
- 5q– syndrome 515
- qh region 357
- quadruplication 384
- quality control overview document 1025–1029
- quality indicators 1019–1020
- quality plan 1019
- Quality System Essentials (QSE) 1011–1013
- quartz lenses 690
- questions 1034
- quinacrine 220
 structure 221
see also Q-banding
- R-bands 27, 28, 58, 215, 228, 238–240
 clinical significance 238
- DAPI/distamycin A staining 241–243, 241, 242
 protocol 275–276
- fluorescent R-banding 238–241, 241
 acridine orange 238–239
 chromomycin A3/methyl green 240, 240,
 277–278

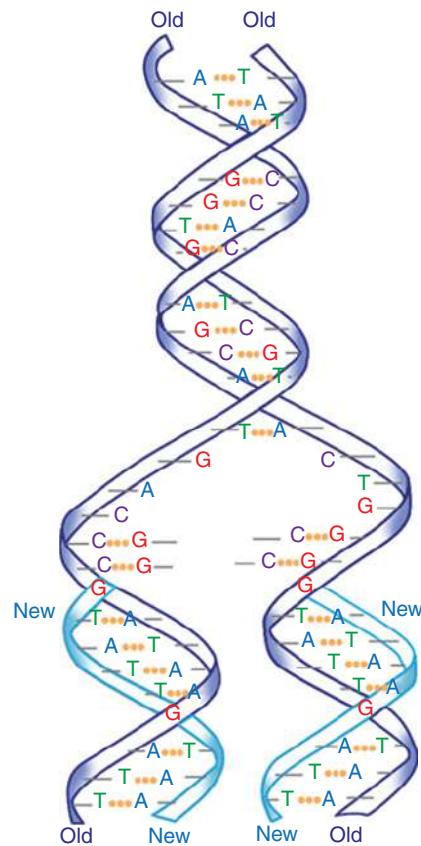
- R-bands (*cont'd*)
- Giemsa R-banding 238
 - protocol 277–278
 - replication timing 228
 - late replication analysis 27, 298
 - see also* banding techniques
 - radial ray defects 667
 - random chromosome loss 62
 - RARA* rearrangements 511
 - RBI* mutations 14
 - reagents 138–140
 - labeling 126
 - reciprocal chromosome painting 1070
 - reciprocal translocation 391, 392, 445, 460–462, 460, 461, 462
 - BCR/ABL1 391, 412
 - chromosome 4 467
 - chromosome 11 469
 - recombinant chromosome 389
 - formation of 389
 - recombination 18, 440
 - allelic 446
 - banding pattern associations 229
 - homologous (HR) 653, 665
 - mitotic recombination event 457, 493, 495
 - nonallelic homologous (NAHR) 446–448, 446, 460
 - nonhomologous end joining (NHEJ) 448–449, 460
 - records 1018
 - animal cytogenetics 1062
 - corrections 1019
 - errors 1018, 1018
 - reference range management 1015
 - refrigerators 991–992
 - region-specific assay (RSA) 420–421
 - regulatory agencies 1000–1001
 - renal cell carcinoma (RCC) 605
 - clear cell 606, 606
 - papillary 606
 - renal hypoplasia 667
 - renaturation *see* DNA
 - repetitive DNA 13
 - replication banding 14, 229, 248, 249, 250–251
 - clinical significance 250–251
 - technical considerations 251
 - replication plating 160–161
 - reporting 68–69
 - CMA results 915
 - final report 68–69
 - FISH results 765
 - guidelines 403
 - incident reporting 998
 - laboratory information system 1049, 1050
 - sources of error 69
 - representative oligonucleotide microarray analysis (ROMA) 910
 - restriction endonuclease banding 252
 - restriction enzymes 19
 - restriction fragment length polymorphisms (RFLPs) 19
 - retinoblastoma 14
 - revenue projection 1040
 - reverse banding 238
 - see also* R-bands
 - reverse transcriptase PCR (RT-PCR) 611
 - rhabdomyosarcoma (RMS) 596
 - alveolar 596–597, 596, 611
 - embryonal 596
 - rhino blood culture 1089–1090
 - ribonucleic acid *see* RNA
 - ribosomal RNA (rRNA) 9
 - ribosomes 4
 - endoplasmic reticulum 3
 - mitochondria 4
 - ring syndrome 458–459
 - rings 390, 390, 445, 457–459, 458, 578
 - derivative involving more than one chromosome 396
 - of known centric origin 390
 - of unknown origin 400–401
 - well-differentiated liposarcoma 594, 595
 - RNA 9–10
 - messenger (mRNA) 4
 - ribosomal (rRNA) 9
 - transfer (tRNA) 9
 - translation 9
 - RNase treatments 750
 - Roberts syndrome 655, 661
 - Robertsonian translocation 62, 396, 445, 462–464, 463
 - gazelle 1058
 - robotic harvester 995–996
 - Romanowsky staining 219
 - see also* staining
 - Rothmund–Thomson syndrome (RTS) 655, 662
 - round cell liposarcoma (MLS) 594
 - Rubinstein–Taybi syndrome 469
 - Russell–Lyon hypothesis 12
 - Rx-FISH 769, 770, 834
 - safety considerations 975, 987
 - biological hazard safety 975–979
 - engineering controls 977–979
 - housekeeping procedures 979
 - personal protective equipment 977, 978
 - spill management 979
 - universal (standard) precautions 977
 - work practice controls 977
 - chemical safety 979–986
 - chemical emergencies 985–986
 - chemical hazards 984
 - Chemical Hygiene Plan (CHP) 981
 - chemical labels 982
 - chemical storage 983
 - fume hoods 984
 - Hazard Communication Standard (HCS) 981
 - hazard identification symbol 981, 983
 - Laboratory Chemical Safety Summaries (LCSS) 981
 - spill management 985
 - working with chemicals 984–985, 985
 - disaster plan 988

- electrical safety 986–988
- fire safety 986, 988
- FISH 757, 790
 - multicolor FISH 864
 - regulatory aspects 998–1001
 - compliance 999–1000
 - incident reporting 998
 - inspections/drills/training 999
 - Privacy Rule 998–999
 - regulatory and accreditation agencies 1000–1001
 - see also* equipment operation, maintenance and safety
- salivary gland tumors 604–605, 605
- Sanger DNA sequencing 19
- sarcoma 535, 578
 - alveolar soft part (ASPS) 598
 - clear cell (CCS) 598
 - Ewing (ES) 599, 601, 610, 730
 - granulocytic 534
 - synovial (SS) 496, 597
- satellite DNA 13, 13, 301
 - probes 724–726, 725, 777, 779
- satellites 357
- scaffold proteins 224
- scales 994
- schwannoma 601
- scientific journals 1112
- scientific notation 937–939
 - addition 939
 - division 939–940
 - multiplication 939–940
 - subtraction 939
- scleroderma 236, 667
 - CREST variant 236, 237, 286
- scoliosis 667
- secondary constriction 357
- segmental isodisomy 495
- segregation errors 63, 432–435, 434, 435
 - meiotic 490, 495
 - mitotic 437–438, 437
- selective permeability 2
- semi-apochromatic lens 690
- seminoma 607
- sequential staining 253
 - protocol 298–299
- serial dilutions 952–956, 952, 953, 954
- serum 94, 125
 - plating efficiency 160
- sex chromosomes 303
 - aneuploidy 379–380, 430
 - constitutional karyotype 379
 - constitutional notation in a neoplastic karyotype 380
 - neoplastic karyotype 379–380
 - see also* X chromosome; Y chromosome
- sex vesicle 18
- sex-reversed XY males 471
- Sézary syndrome (SS) 525
- short interspersed elements (SINES) 13
- shoulder band 357
- sickle cell disease 19
 - detection 19–21
- sideline clone 406–407, 406
- sign out 1027–1028
- silver staining, nucleolus organizing regions 243–246, 243
 - protocol 287–288
 - technical considerations 245–246
- Silver–Russell syndrome 486, 486, 487
- simple sequence repeats (SSR) 3
- SINES 22
- single nucleotide polymorphisms (SNPs) 21
- single-fusion FISH
 - interphase 414, 415
 - metaphase 412–413, 415
- sister chromatid exchange (SCE) 658
 - clinical significance 251
 - testing 246, 248, 249, 250, 251
 - protocols 289–294
 - technical considerations 251
- skin biopsy 128, 134
 - animal 1090–1091
 - transport medium 136
- slide-making 49–58, 51, 52
 - amniotic fluid 178–179
 - automation 49
 - FISH studies 58, 748, 754, 755, 792–794
 - multicolor FISH 841
 - preimplantation FISH 774
 - historical background 49–50
 - in situ* cell drying 58
 - mounting 215–216
 - media 216–218
 - protocols 75–85, 80, 792–794
 - blood cells 107
 - quality control 1026
 - slide aging 58, 218–219, 219, 749, 842
 - theory of 50–53
 - tumor cells 586–587
 - variables 53
 - air flow 57
 - ambient humidity and temperature 55–56
 - angle of the slide 55
 - cell and culture type 57
 - culturing and harvesting techniques 57
 - dilution factor 57
 - height from which cells are dropped 56
 - slide cleaning and labeling 57
 - slide type 57
 - wet versus dry slides 53
 - wicking effects 56
 - see also* fixation
 - small-cell lung carcinoma (SCLC) 603, 603
 - smears, FISH analysis 806–808
 - Smith–Magenis syndrome 382, 470
 - probe 731
 - solid tumors
 - chromosome analysis 587–592
 - culture of tumor cells 579, 582, 582

- solid tumors (*cont'd*)
- cell dilution and culture initiation 583
 - cell synchrony 585
 - culture maintenance 583–584
 - culture media 581
 - culture vessels 581
 - fixation 586
 - growth patterns 584
 - harvest time determination 585, 586
 - hypotonic treatment 585
 - mitotic arrest 584
 - protocols 631–644, 646–651
 - tissue dissociation 581–583
 - malignant behavior 579
 - slide-making 586–587
 - specimen receipt 580
 - specimen requirements 579–580
 - specimen transport 580
 - techniques
 - comparative genomic hybridization (CGH) 611–612, 853, **854**
 - FISH 608–610, **609**
 - multicolor karyotyping 612
 - reverse transcriptase PCR (RT-PCR) 611
 - tumor classification 578–579
- see also specific tumors*
- solutions 942
- definitions 942
 - dilution 947–956
 - dilution ratios 949
 - molar solutions 947–949
 - percent solutions 950
 - serial dilutions 952–956, 952, 953, 954
 - to a specific concentration 947–949
 - fixative solution preparation 950
 - molar solution preparation 943–946
 - molarity conversion to percent 951
 - moles and molarity 943
 - percent conversion to molarity 951
 - percent solution preparation 946–947
 - stock solution 947–949
- Sotos syndrome **467**
- probe **731**
- Southern blotting 19–21, 20, 495
- species integrity 1057
- specimen logging 32–33
- quality control 1025–1026
- specimen procurement 29–32
- specimen quality monitoring from off-hill sites 1030
- spectral karyotyping (SKY) 612, 719, 780, 833, **834**
- applications **835**
 - comparative cytogenetics 840
 - probes 842–846, **843, 844–845**
- procedures **841**
- image acquisition 849
 - slide pretreatment **846**
- protocols 864–884
- theory 835–837
- troubleshooting 849, **850**
- see also* multicolor (multiplex) FISH (M-FISH)
- sperm FISH 775, 776
- sphingolipids 3
- spindle 18
- spindle fibers 4–5
- splenic lymphoma with villous lymphocytes (SLVL) 524
- SSC solution 780
- 2x SSC treatment 749
- staff *see* personnel management
- staining 58–59, 213–215, 214
- 5-bromo-2'-deoxyuridine (BrdU) methodologies 246–251
 - amniotic fluid 179
 - animal cytogenetics 1064
 - CENP staining 236–238, 237
 - protocol 286–287
 - centromeres 235–238
 - chromosome length affect 706
 - destaining slides 252
 - protocol 298–299
 - fluorescent dyes 215, **218**
 - G-11 staining 233–235, 234
 - protocol 274–275
 - kinetochores 235–238
 - restaining permanently mounted slides 299–300
 - sequential 253
 - protocol 298–299
 - solid staining methods 219–220
- see also* banding techniques
- standard deviation 962, 963
- Stark Law 1000
- stemline clone 406–407, 406
- multiple stemlines 408
- sterilization procedures 123–124
- autoclave sterilization protocol 1003
- steroids 3
- stock hypotonic solution 139
- prenatal specimens 139
- stock solution 947
- dilution to a specific concentration 947–949
 - preparation 944–945
 - streptavidin 753–754
 - studbooks 1058–1059
 - subcultures 119
 - amniotic fluid 195–197
 - dispersion of monolayer cells for subculture 127
 - fibroblast cultures 129, 155–156
 - protocol 155–156
 - submetacentric chromosomes 357, 360
 - subtelomere-specific probes 732–733, 734, 780
 - signal pattern variants 779
 - subtelomeric rearrangements 907
 - subtraction 939
 - suppliers 1109–1110
 - supraneuronal isochromosome 456
 - synapsis 18
 - synchronization
 - fibroblast cultures 130, 163–164
 - tumor cell cultures 585
 - synchrony chemicals 43, **46, 49**
 - mechanism of action 46–48

- synovial sarcoma (SS) 496, 597
 synteny 1072
 synthetic oligonucleotides 21
- T cells 90–91
 phytohemagglutinin effect 91
see also lymphocytes
- T-bands 228, 252
 protocol 294–295
see also banding techniques
- T-lineage acute lymphoblastic leukemia (T-ALL) 520
- T-prolymphocytic leukemia (T-PLL) 520
- tapir, lowland 1071, 1071
- technology assessment and implementation 1036–1037
- telocentric chromosomes 357
- telomere 357
 acquisition of 450
 probe 733, 779
- telomeric association (tas) 391
 well-differentiated liposarcoma 594, 595
- teratoma 481, 482, 495
- terminal deletion 373, 380–381, 449–450, 450
- test broth medium 140
- testicular tumors 607
see also germ cell tumors (GCTs)
- tetraphocomelia 667
- tetraploidy 61, 429, 443–444, 444
- tetrasomies 386, 387, 469, 456, 457
- textbooks 1107
- The Joint Commission (TJC) 999
- thermometers 991, 991
 calibration protocols 1006–1008
- thiazin dyes 226, 227
- thrombocytopenia 667
- thymidine 46–48
 chemical structure 246
 solution preparation 944
- thymus tumors 604
- tiger, Siberian 1057, 1059
- timers 994
 calibration protocol 1008–1010
- tissue cultures *see* cultures
- tissue piecing 1094–1095
- tissue transport 128, 132, 134–135
 solid tumor specimens 580
 transport media 135–138
- topoisomerase inhibitor 535
- TP53 mutations 14
- training 1034
 programs and courses 1111–1112
- transfer RNA (tRNA) 9
- translocation 61, 391–393, 445
 gazelle 1058
 jumping 408
 multichromosomal 392–393, 393
- preimplantation translocation status screening 771, 772, 821–823
- reciprocal 391, 392, 445, 460–462, 460, 461, 462
- BCR/ABL1 391, 412
- Robertsonian 62, 396, 445, 462–464, 463
 whole arm 366, 391–392
- transport
 amniotic fluid specimen 175
 chorionic villus sample 182
 tissue samples 128, 132, 134–135
 solid tumor specimens 580
 transport media 135–138
- trifunctional protein deficiency 496
- triplication 384, 384
- triploidy 61, 429, 442–443, 442, 481, 482, 496
 mosaic triploidy 443
- triradial chromosome 578
- trisomies 430, 439–441
 maternal age effect 439–440
 sex chromosomes 440
 trisomy 8 468
 trisomy 9 468
 trisomy 13 469, 432, 433
 trisomy 18 470, 432, 433
 trisomy 21 *see* Down syndrome
 trisomy X 471
- trisomy rescue 62, 490, 496
- trypsin 2
 cell dispersion 127, 129
 G-banding role 223, 226, 229–230, 230
 overtrypsinized G-bands 229, 230
 protocols 280–284, 571–575
 Hanks' trypsin solution 139
- tumor cells *see* solid tumors
- tumor classification 578–579
see also solid tumors
- turnaround times 1028
 delays in test results 1028
- Turner syndrome 380, 471, 441
 clinical consequences 430, 433
 karyogram 432
- UBE3A gene 496
- unbalanced translocation 61
- uncertainty 397–398
 additional material 399
 approximation range 399–400
 rings of unknown origin 400–401
 symbols of 396
 or 399
- unequal crossing over 447
- uniparental disomy (UPD) 61–62, 394, 438, 496, 780
 Angelman syndrome 489–490
 maternal 490–492, 492
 partial (segmental) 492–493, 495
 paternal 490, 492
- Prader–Willi syndrome 489–490, 491
- ring chromosomes and 458
- Robertsonian translocation and 462
 whole chromosome 490–492
- unit conversion 940–942
- units of service (UOSs) 1040
- unmasking of a recessive allele 449, 490, 496
- urothelial carcinoma 610

- validation 1015–1014
 CMA 914–915
 data analytical evaluation 1015
 FISH probes 408–409, 765–768
 laboratory information system 1047–1048
 reference range maintenance 1015
 requirements **1013**
 specimen number for 1014
 summary of results 1015
 validation plan 1015
- variable number tandem repeats (VNTRs) 780
 variance analysis 1042–1043
 variegated translocation mosaicism 667
 velocardiofacial (VCF) syndrome 21, 382, **470**
 ventricular septal defect 667
 volume projection 1039
 von Hippel–Lindau syndrome 606
 VP2000 automated slide processor 811–814
- WAGR syndrome **468**
 Waldenstrom macroglobulinemia (WM) 522
 wallaby, Parma *1065*
 waste disposal 980
 water baths 992
 weighted average cost of capital (WACC) 1041
 well-differentiated liposarcoma (WDLPS) 594, 595
 Werner syndrome (WS) **655**, 661
 whole arm derivatives 396–397, 398
 acrocentric derivative 396–397
 nonacrocentric derivative 397
 whole arm translocation *366*, 391–392
 whole chromosome paints *see* chromosome paints
 whole chromosome uniparental disomies 490–492
 whole-genome shotgun (WGS) sequencing 1071
- wildlife conservation 1059
 Williams syndrome *382*, **467**
 probe **731**
 Wilms tumor (WT) 607, 667
 window 357
 window frame 357
 Wolf–Hirschhorn syndrome **435**
 probe **731**
 workflow chart *1017*
 laboratory information system interfaces *1046*
 workload 1038–1039
 Wright stain *214*, 230
 protocol 281–284, 572–575
 Wt1 gene 496
- X chromosome *15*, **350**
 abnormalities 246, 250
 banding variation *351*
 c region 12
 deletions **470**
 duplications **471**
 inactivation 11, 12–13, 246
 monosomy X *see* Turner syndrome
 replication 250
 trisomy X **471**
 X-linked ichthyosis **471**
 probe **731**
- Y chromosome *352*
 banding variations *353*
 pericentric inversion **471**
- yeast artificial chromosomes (YACs) 737
- Zoo-FISH 1070



DNA replication illustrating two new helices being replicated semiconservatively.

Figure 1.3 The new helices being replicated semiconservatively (see p. 7 for full caption).

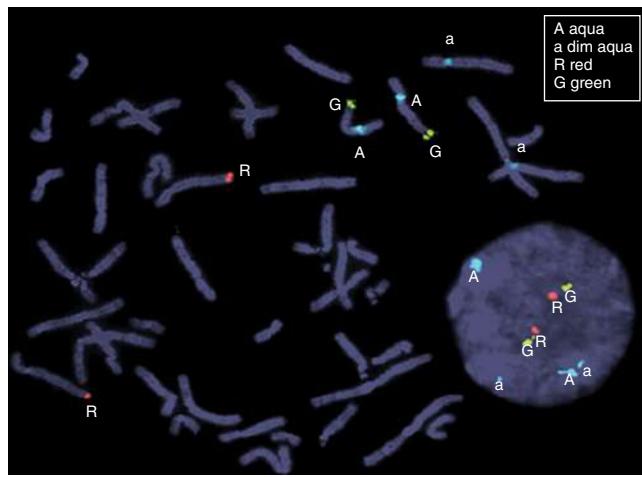


Figure 2.4 Fluorescence in situ hybridization on metaphase and interphase cells (see p. 28 for full caption).

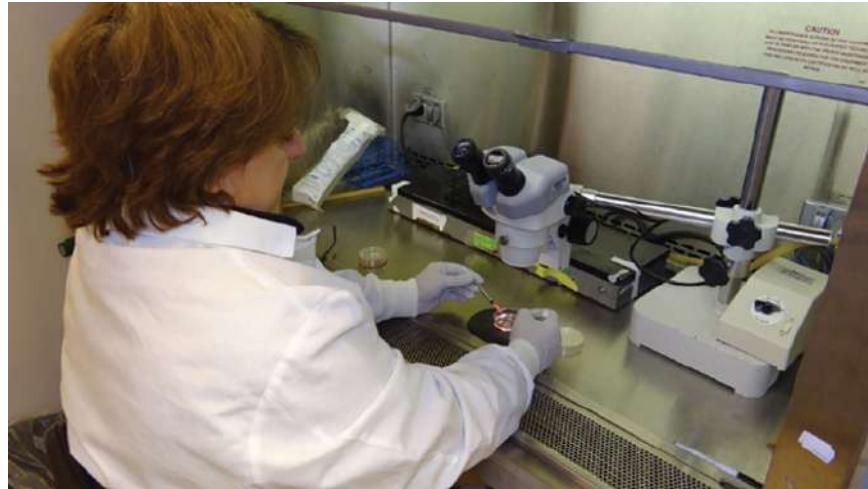


Figure 4.2 Sterile technique (see p. 121 for full caption).



Figure 4.3 Distinguishing a villus frond from maternal decidua (see p. 122 for full caption).

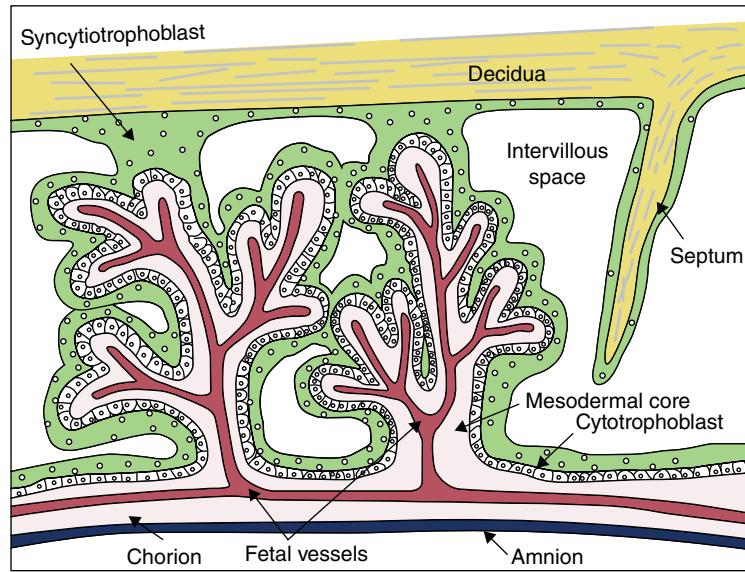


Figure 5.2 Diagram of first trimester chorionic villi within placenta (see p. 181 for full caption).



Figure 5.3 Removing maternal decidua from villus frond (see p. 182 for full caption).

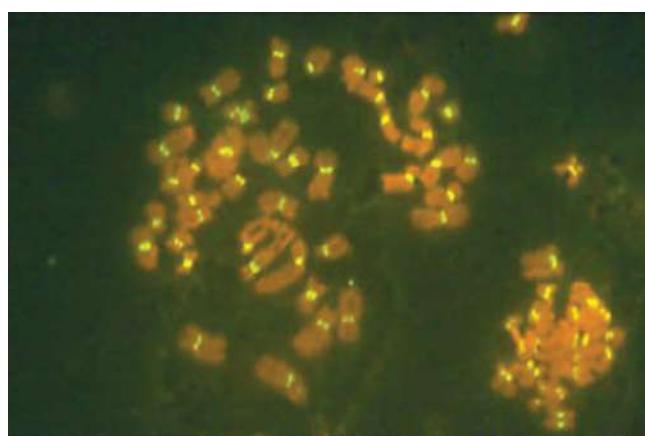


Figure 6.14 CENP-C antibody staining (see p. 237 for full caption).

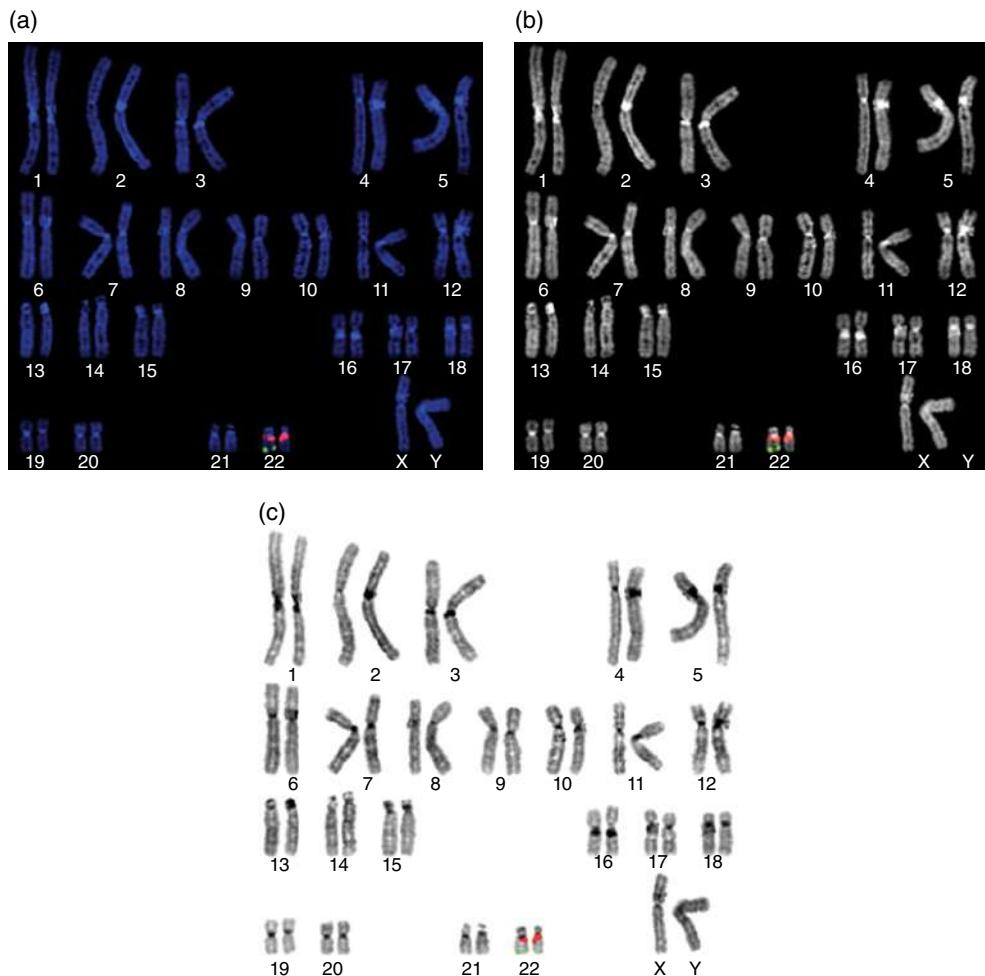
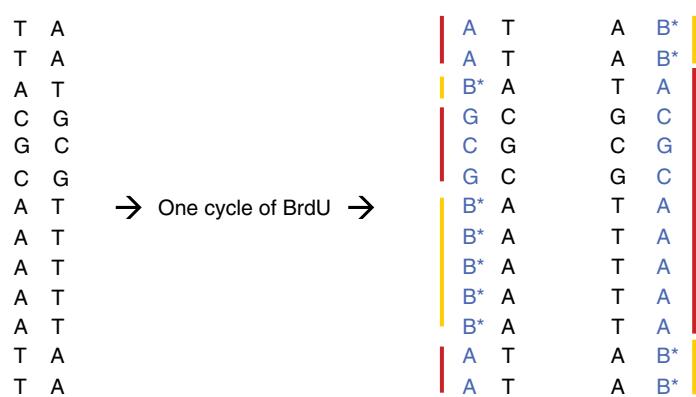


Figure 6.17 Cells from a FISH slide (TUPLE1/ARSA probes) are stained with DAPI to show a deletion of the TUPLE1 region (see p. 242 for full caption).



Black = original strands (inside strands). A-T regions are synthesizing, and G-C regions are not.
 Blue = newly replicated strands (outside strands)
 B^* = BrdU substitution

Figure 6.20 Incorporation of BrdU (B) into DNA during synthesis (see p. 247 for full caption).

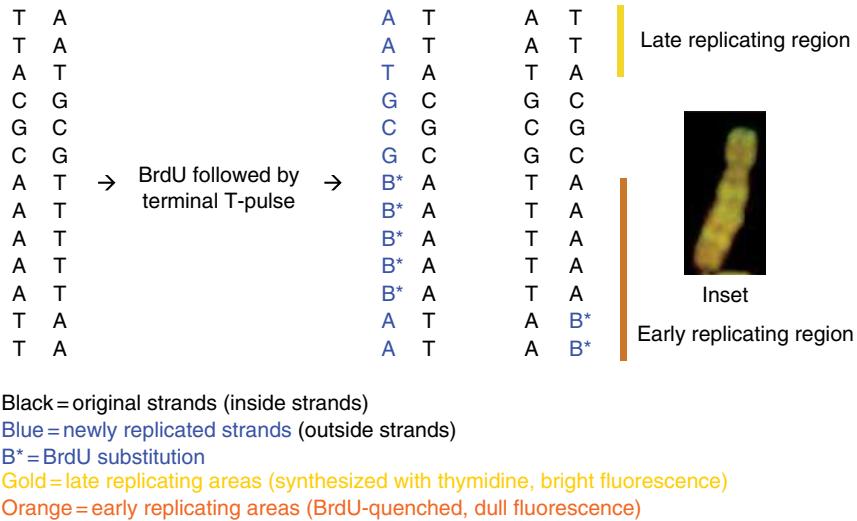


Figure 6.21 Incorporation of BrdU (B^*) into early replicating DNA, followed by a terminal thymidine (T) pulse (see p. 247 for full caption).

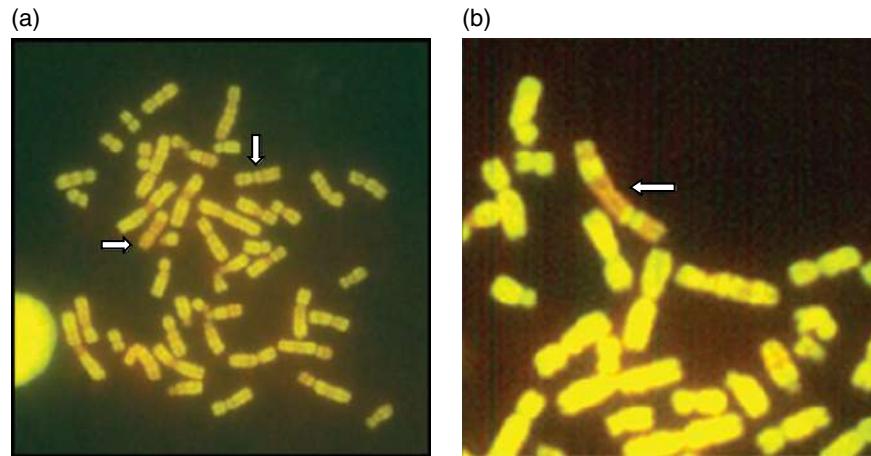


Figure 6.22 Replication banding (see p. 248 for full caption).

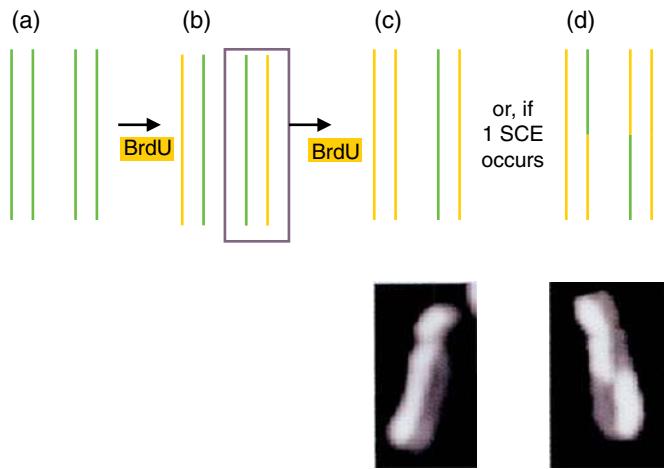


Figure 6.23 Sister chromatid exchange (SCE) (see p. 248 for full caption).

(d)

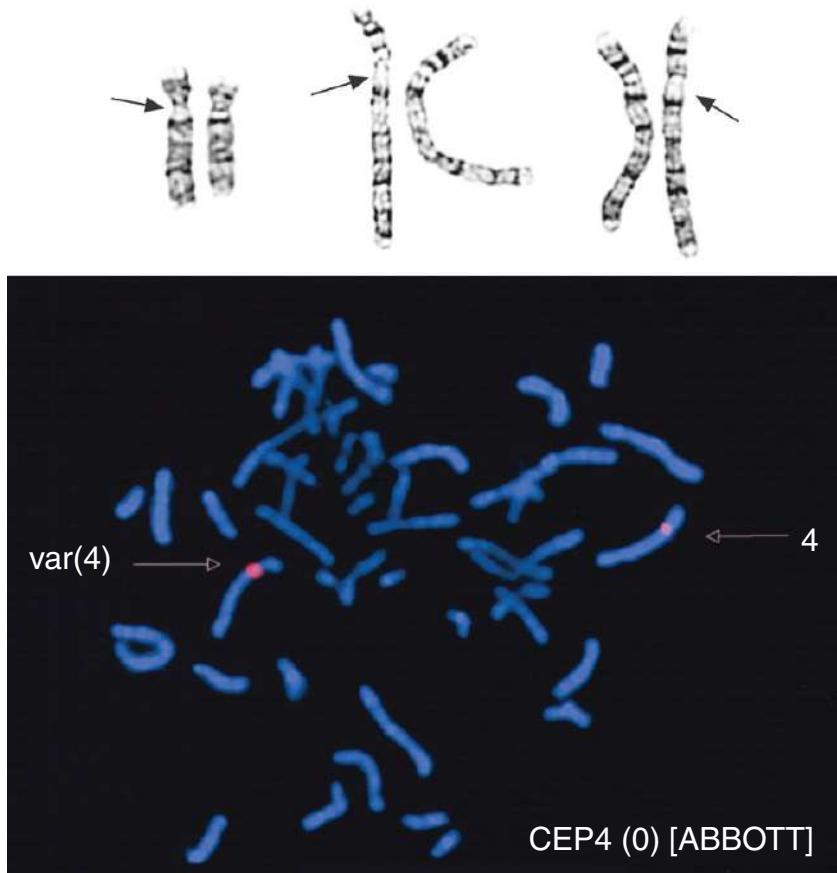


Figure 7.6 (d) Common G-banding and FISH signal variations for chromosome 4 (see p. 313 for full caption).

(c)

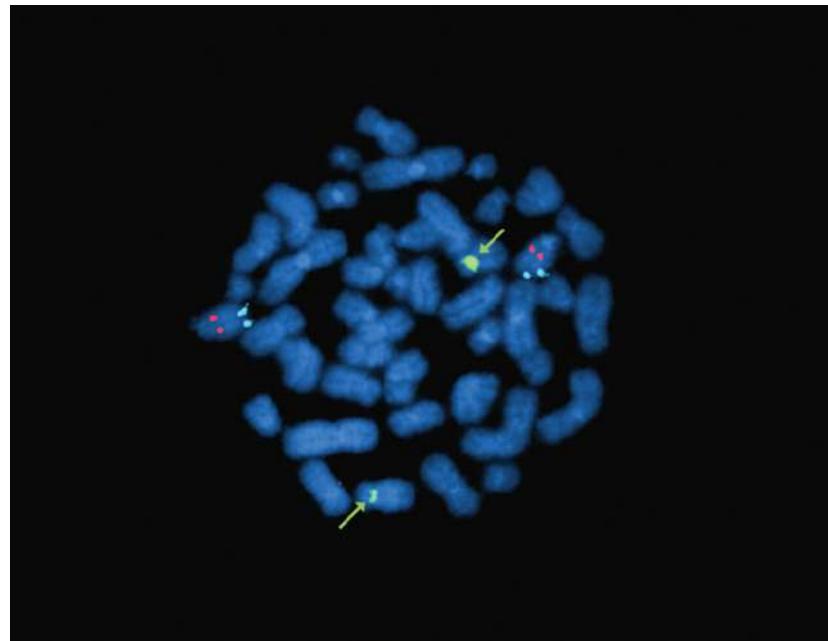


Figure 7.14 (c) Common variants for chromosome 12 (see p. 329 for full caption).

(d)

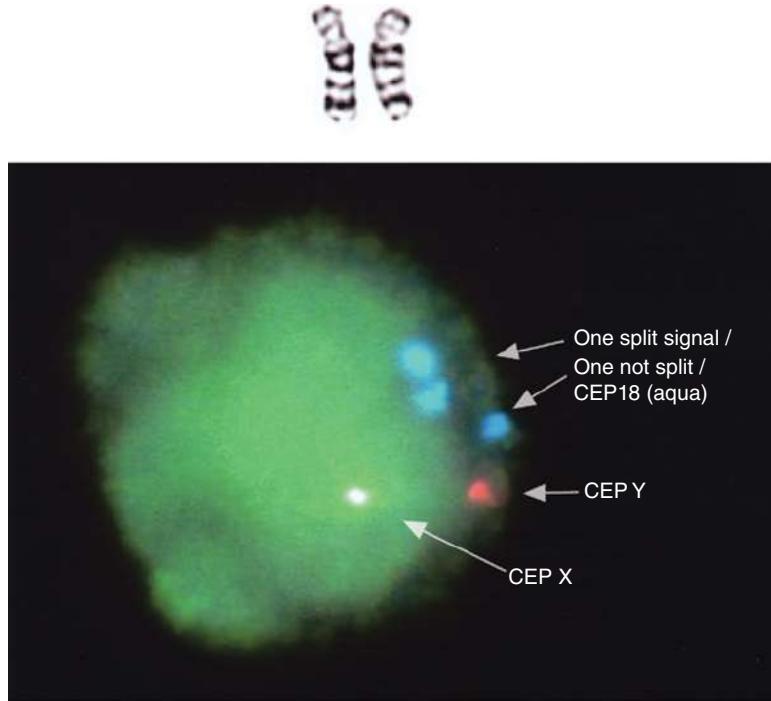


Figure 7.22 (d) FISH variation: The pericentromeric region of chromosome 18 may show variations with FISH probes for the alpha satellite region (see p. 343 for full caption).

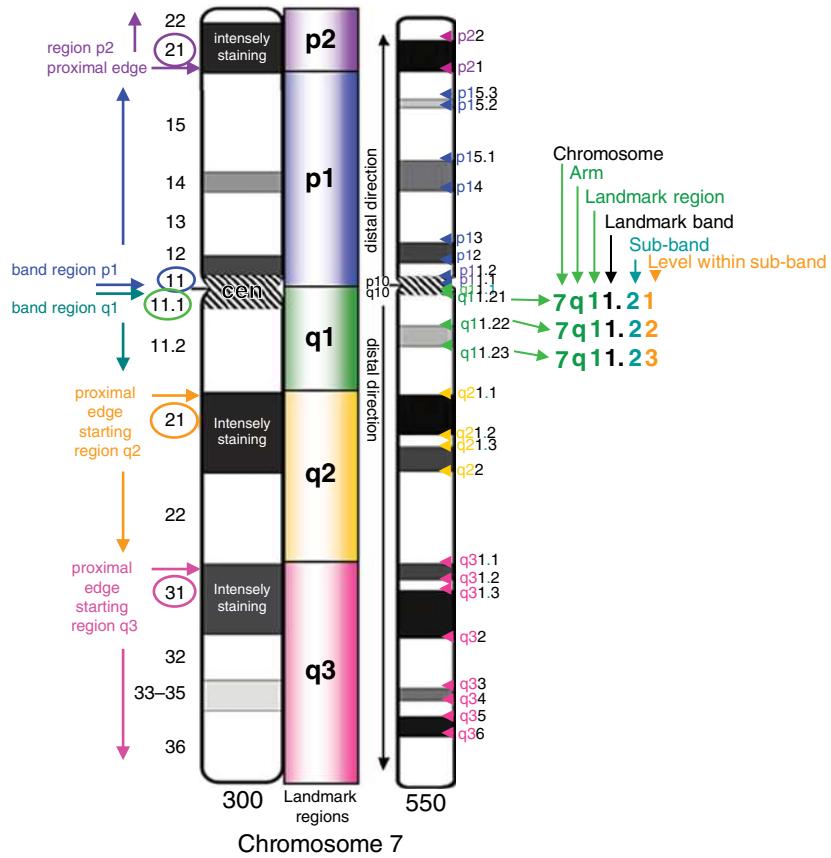


Figure 8.3 Band derivation (see p. 363 for full caption).

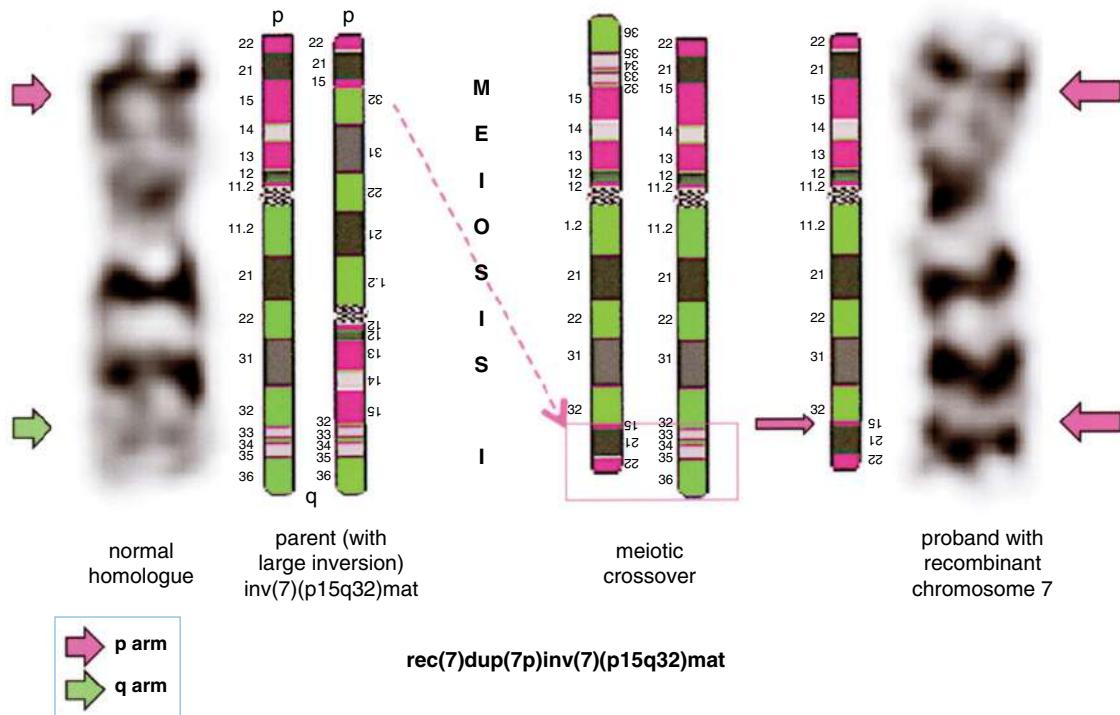


Figure 8.19 Forming a recombinant chromosome (see p. 389 for full caption).

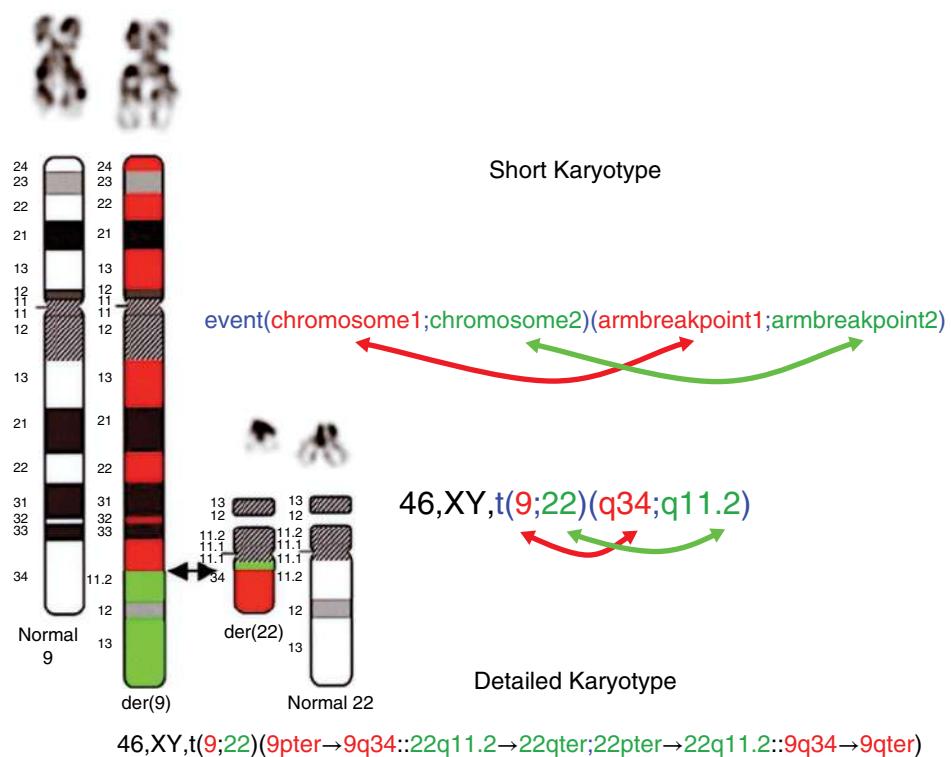
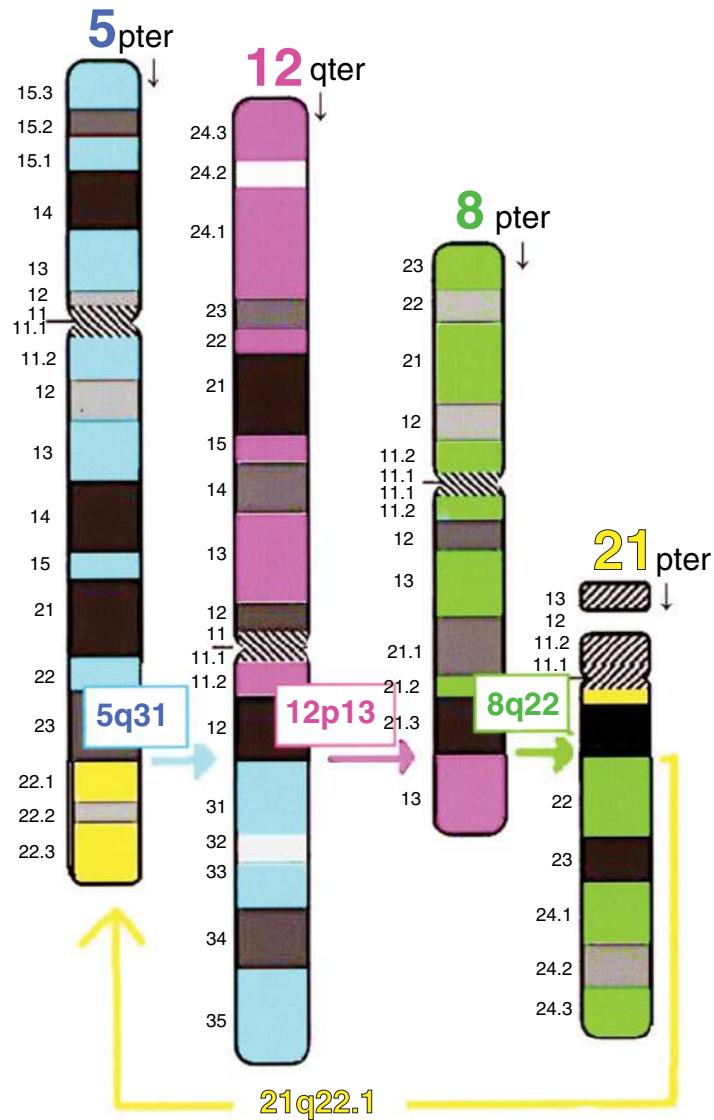


Figure 8.21 Reciprocal translocation (see p. 392 for full caption).



46,XY,t(5;12;8;21)(q31;p13;q22;21q22.1)

Figure 8.22 Four-break translocation (see p. 393 for full caption).

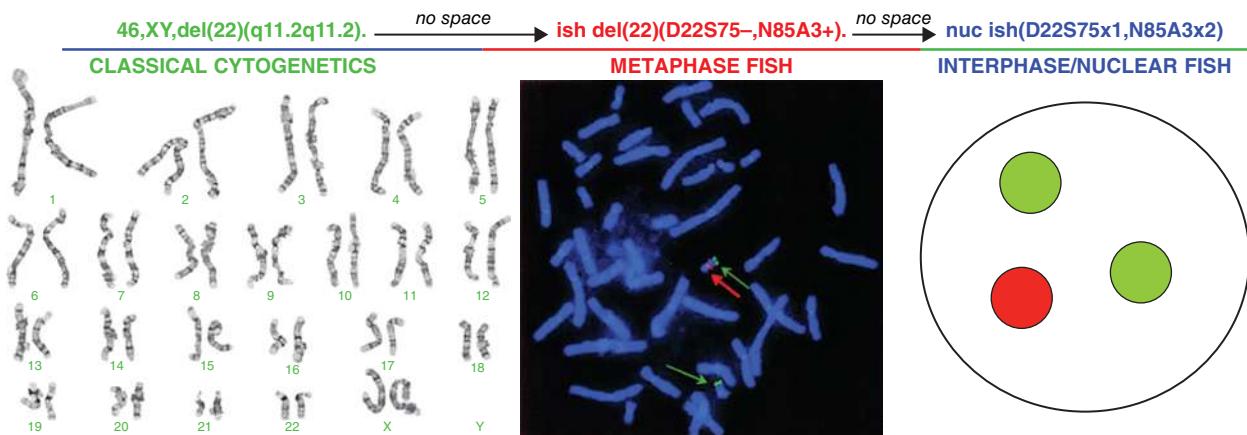
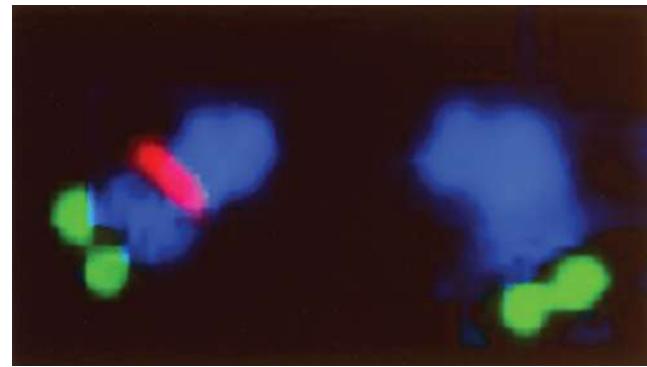


Figure 8.27 Karyotype string of cytogenetic tests (see p. 411 for full caption).



ish del(D22S75-, N85A3+)

Figure 8.28 Metaphase detection for gene locus deletion (see p. 412 for full caption).

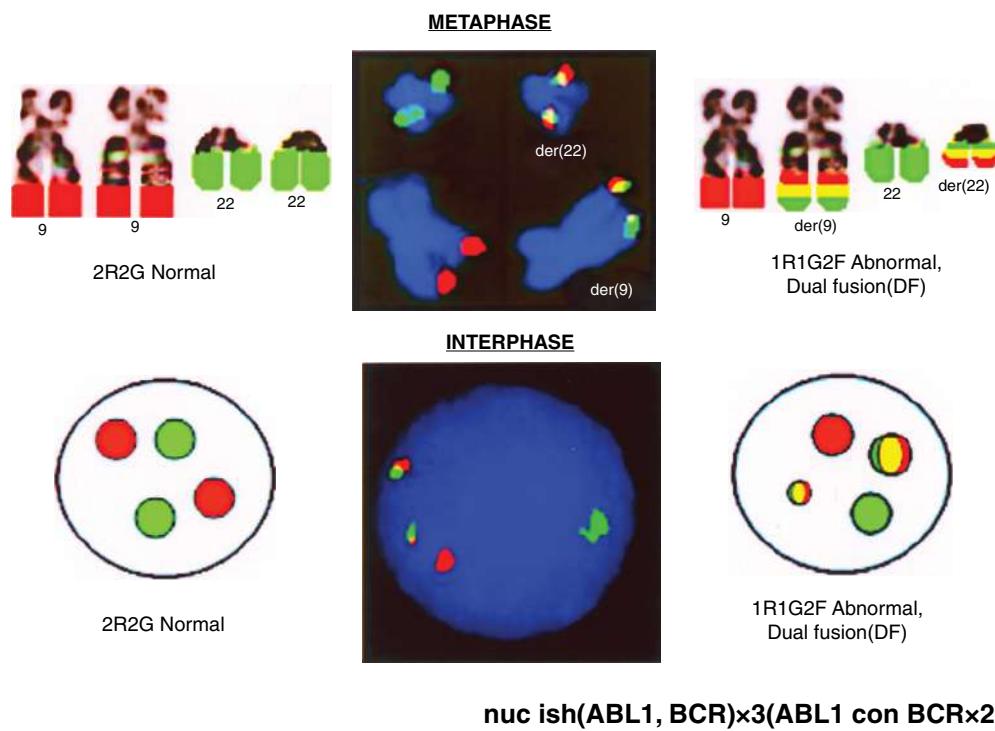


Figure 8.29 Fusion probe strategy (see p. 415 for full caption).

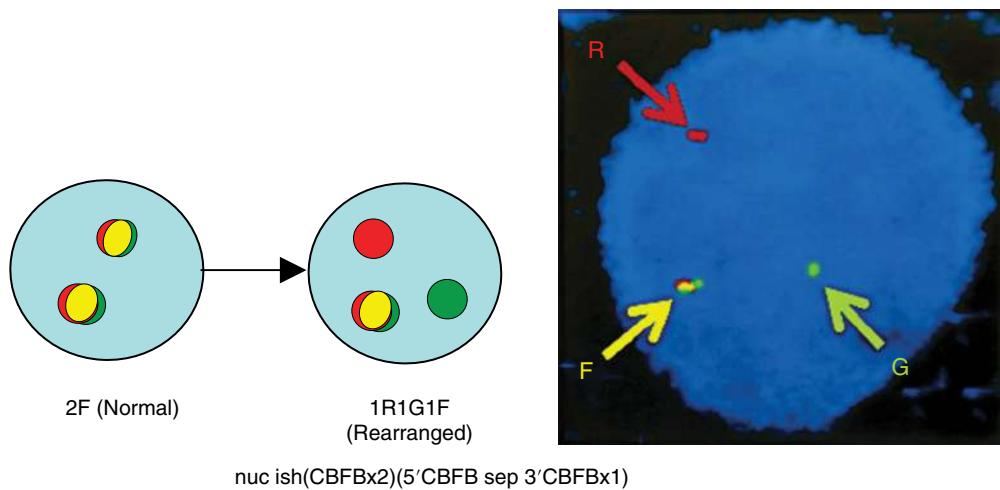
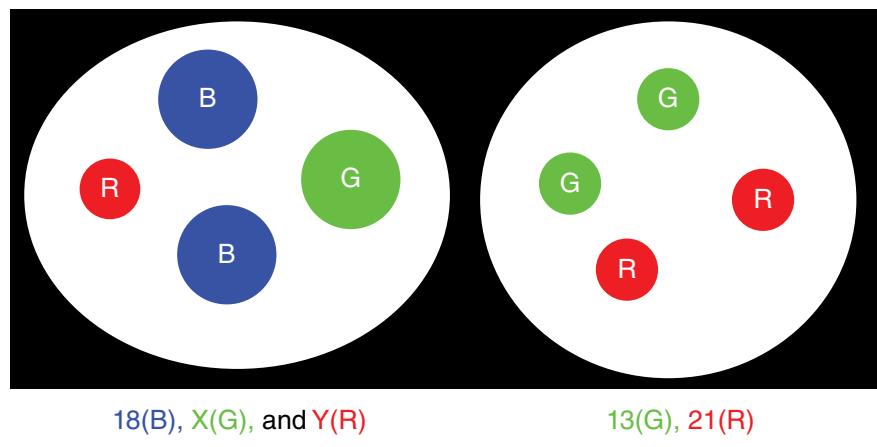


Figure 8.30 Break-apart strategy (see p. 416 for full caption).



nuc ish(DXZ1x1,DYZ3x1,D18Z1x2),(RB1,D21S259/D21S341/D21S342)x2

Figure 8.31 Centromeric and gene locus strategy (see p. 417 for full caption).

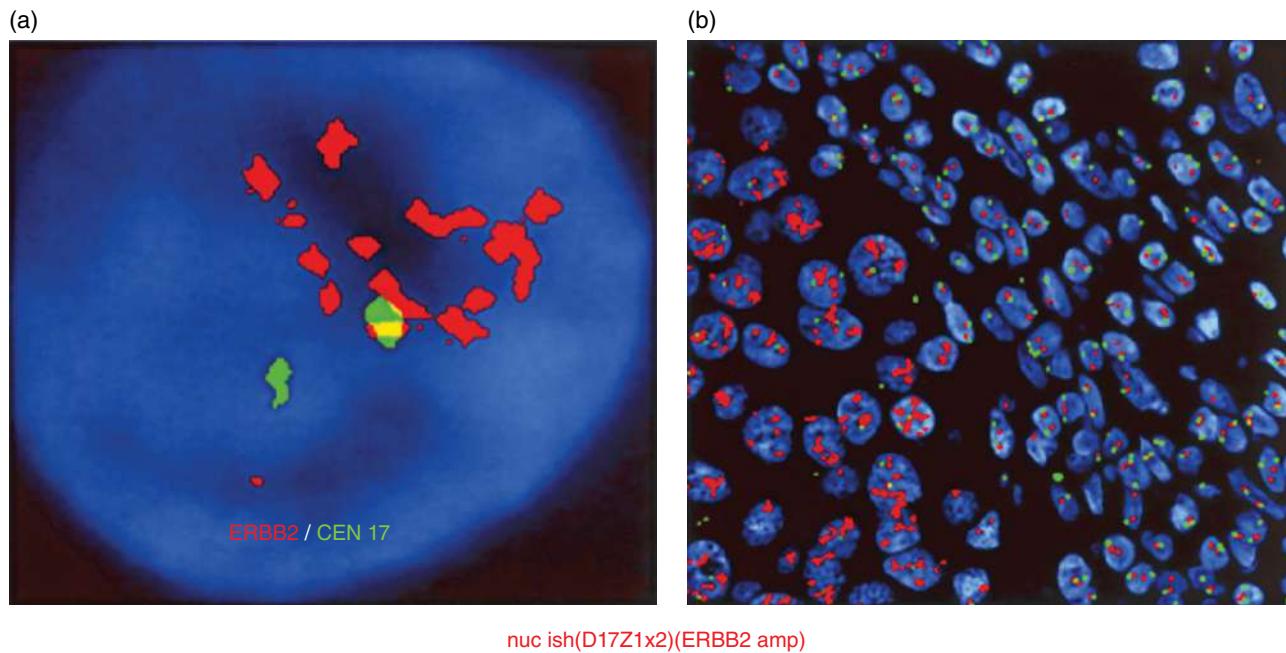


Figure 8.32 Paraffin-embedded breast tissue (see p. 419 for full caption).

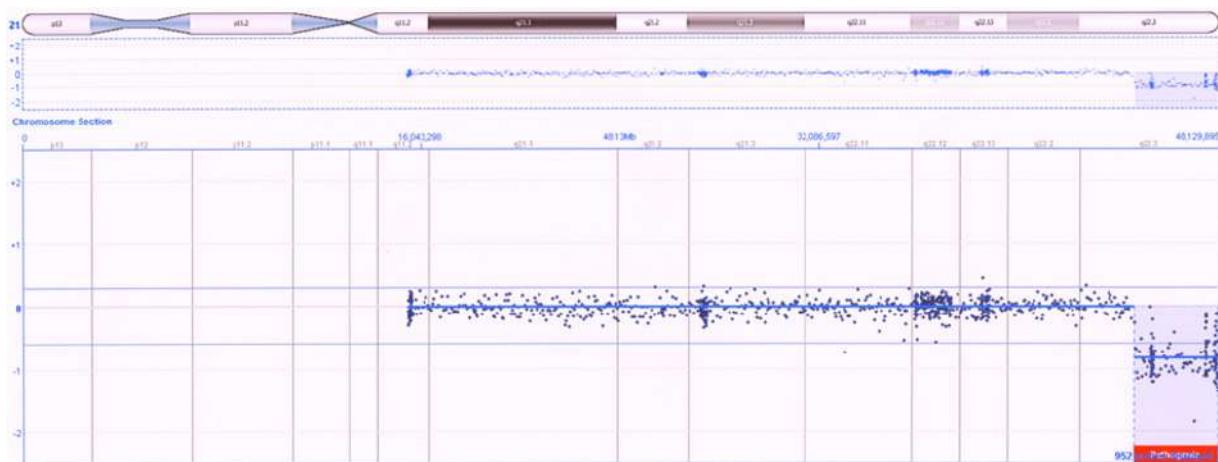


Figure 8.33 Microarray identifies a 3.3 Mb deletion of the subtelomeric region of chromosome 21 at q22.3 (see p. 422 for full caption).

(b)

Trisomy 21 (Down syndrome)

Although described as early as 1838, Down syndrome is named after John Langdon Down who documented the syndrome in 1866. Jérôme Lejeune discovered the extra small acrocentric, "G" group, chromosome in 1959.

Flat nasal bridge,
epicanthal folds (fold
of eyelid skin on the
inner side of the eye),
upturned palpebral
fissures (eye openings)
& protruding tongue¹



Single
palmar crease,
short fingers,
and 5th finger
clinodactyly
(incurving)²



Small, low-set,
malformed ears²



Brushfield spots¹
(white specks in the iris)



b-1

Trisomy 18 (Edwards syndrome)

Trisomy 18, also called Edwards syndrome,
was first reported in 1960 by John H. Edwards.



Clenched hands with
overlapping fingers²

Rocker bottom feet²
or a prominent
calcaneous (heel bone)

b-2

Monosomy X (Turner syndrome)

Turner syndrome is named after Henry Turner,
who described this disorder in 1938.



Fetal cystic hygroma²
(fluid filled sac) (above left)
may cause a webbed
neck² (above right)



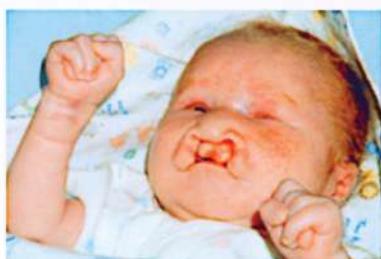
Edema² (swelling of
the hands and feet)

Short fourth
metacarpal²

b-4

Trisomy 13 (Patau syndrome)

Trisomy 13, also called Patau
syndrome, was first described by
Thomas Bartholinus in 1656. Its
chromosomal cause was reported
by Klaus Patau in 1960.



Cleft lip/palate and microphthalmia³
(small eyes)



Scalp defect²



Clenched fists
and polydactyly³
(extra finger)

b-3

Figure 9.2 (b) First aneuploidies identified (see p. 433 for full caption).

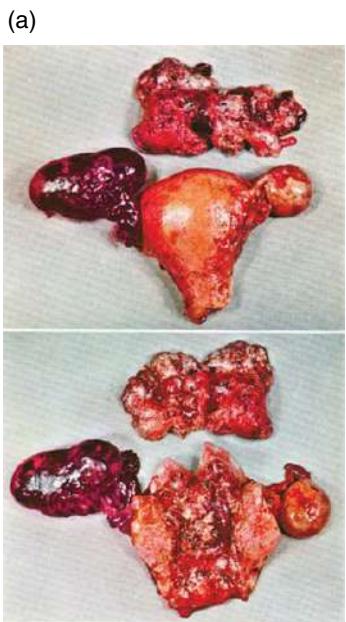


Figure 10.2 (a) Hydatidiform mole (see p. 482 for full caption).

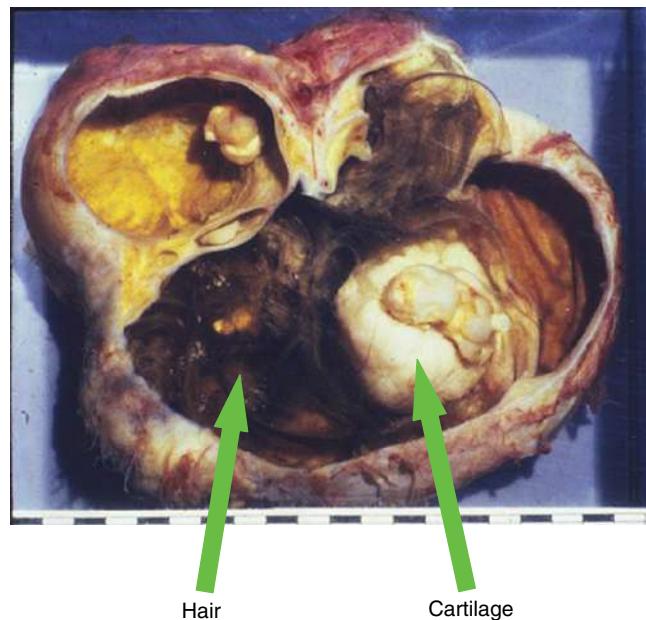


Figure 10.3 Teratoma (see p. 482 for full caption).

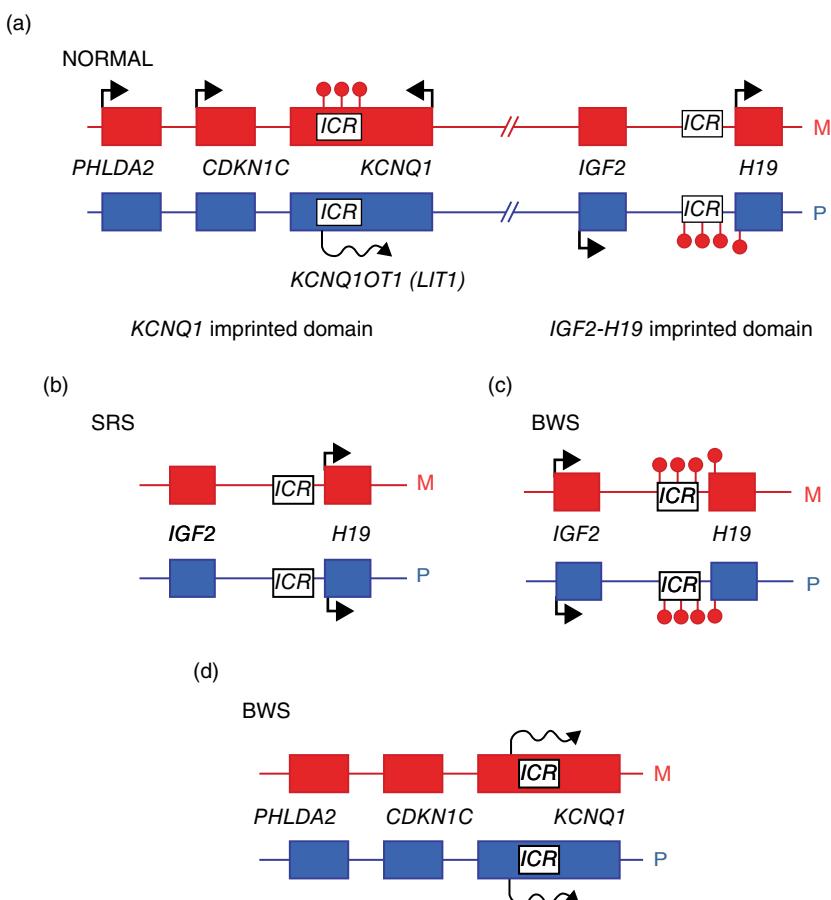


Figure 10.5 *IGF2* normal maternal/paternal gene regulation of *IGF2* (see p. 486 for full caption).

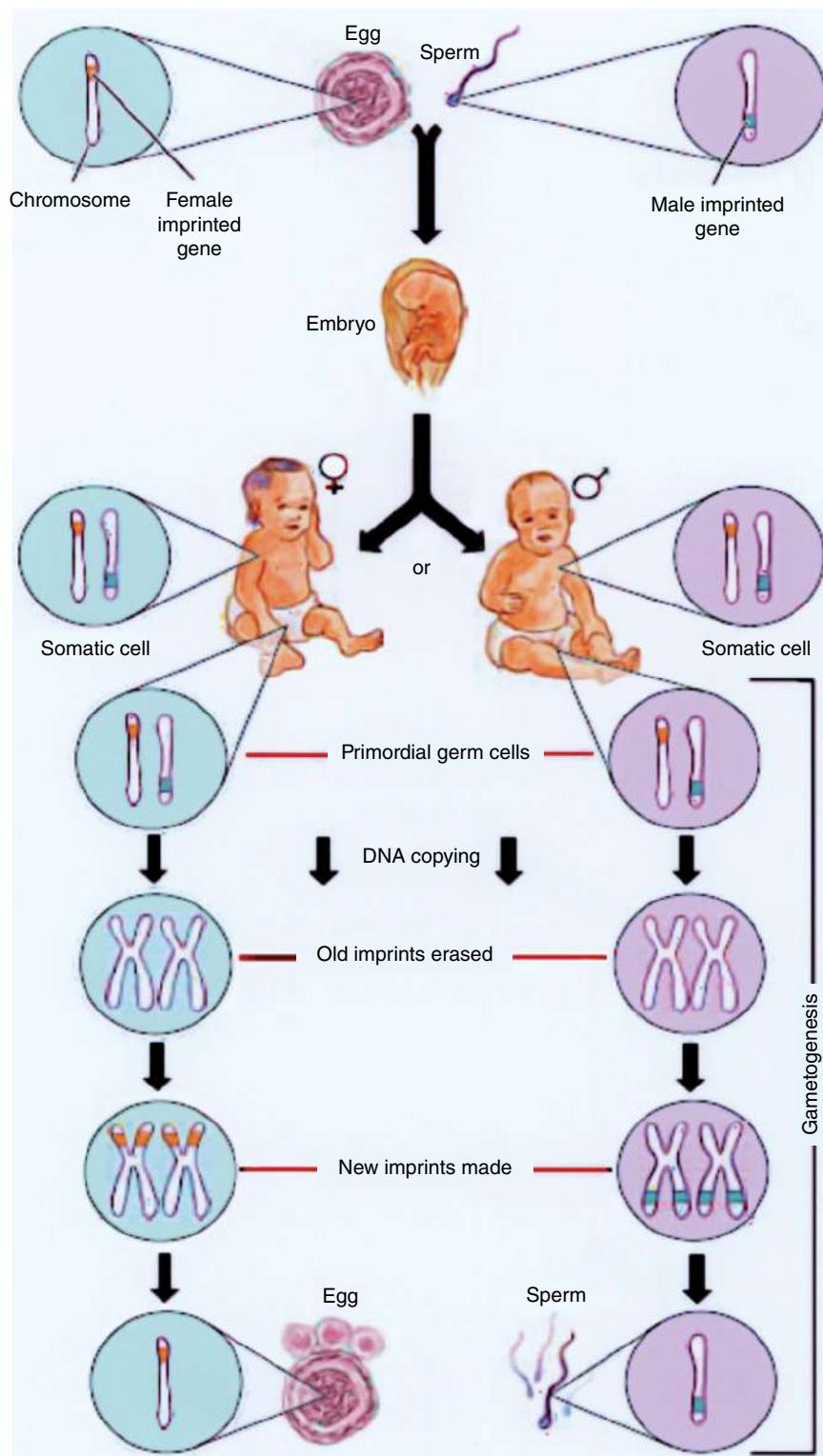


Figure 10.4 Germline imprint resetting process (see p. 485 for full caption).

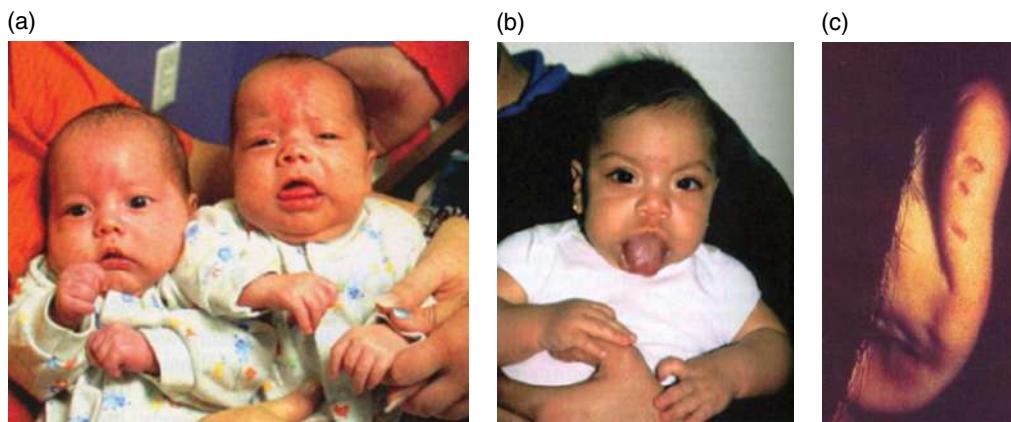


Figure 10.6 Newborn monozygotic twins discordant for Beckwith–Wiedemann syndrome (see p. 487 for full caption).



Figure 10.7 Female newborn infant (see p. 487 for full caption).

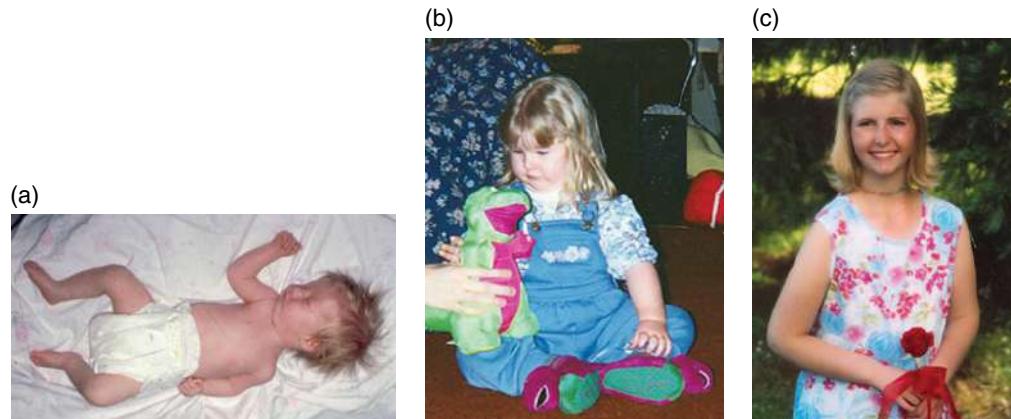


Figure 10.8 Female with Prader–Willi syndrome (see p. 488 for full caption).

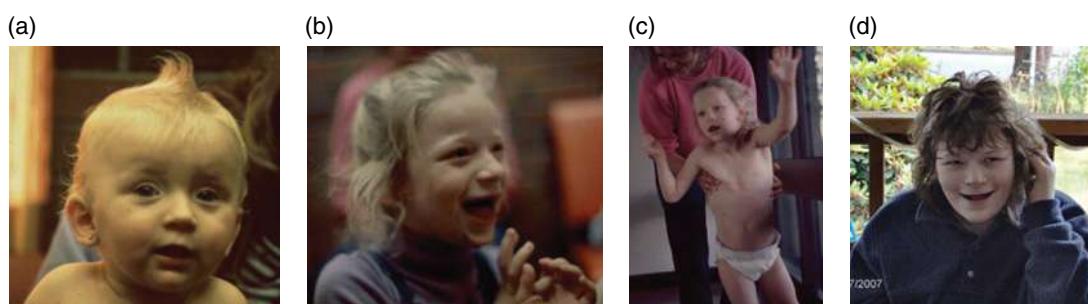


Figure 10.9 Female with Angelman syndrome (see p. 488 for full caption).



Figure 10.12 (a) Male with Prader–Willi syndrome due to uniparental disomy (see p. 491 for full caption).

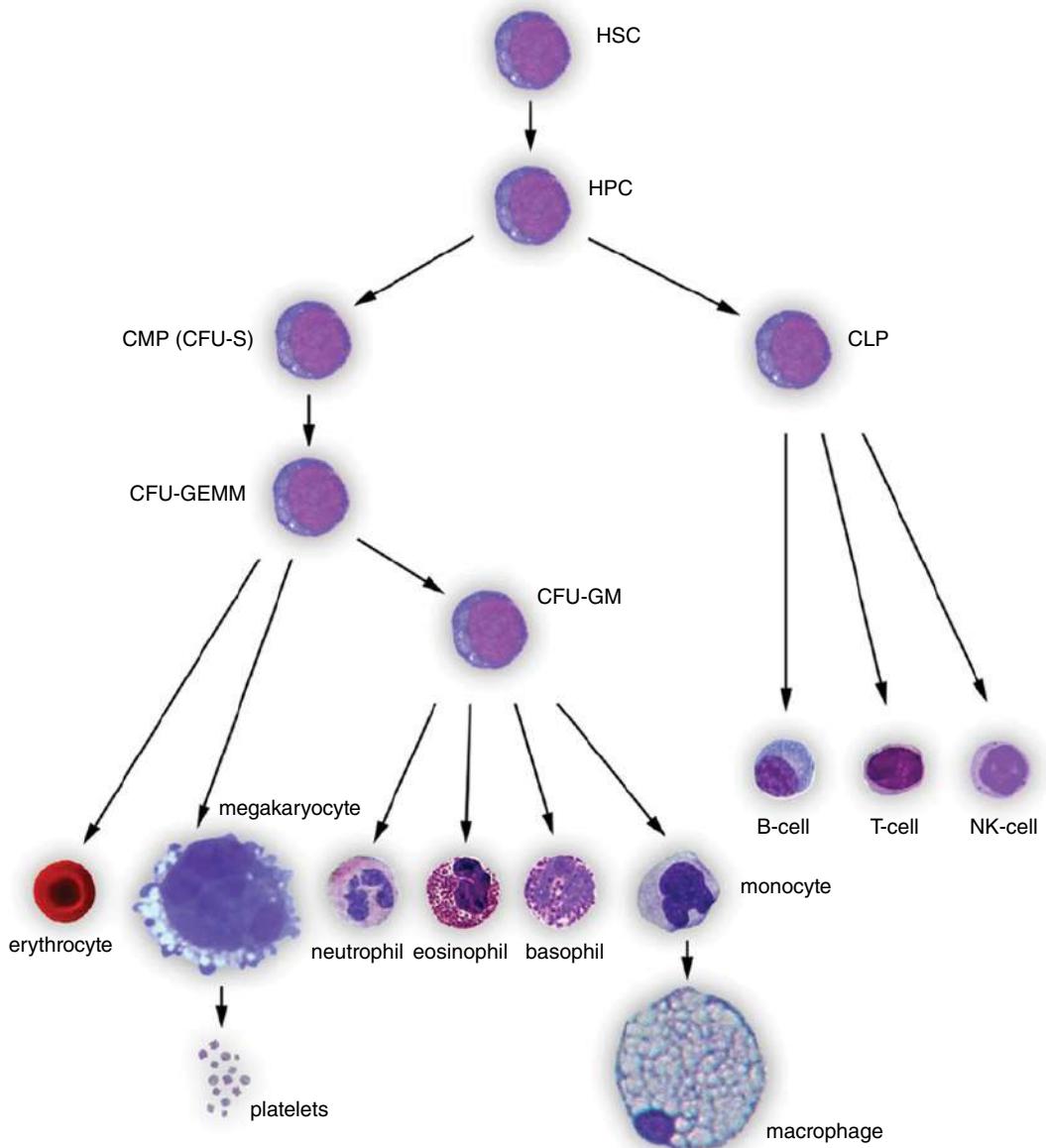


Figure 11.1 The hematopoietic system (see p. 509 for full caption).

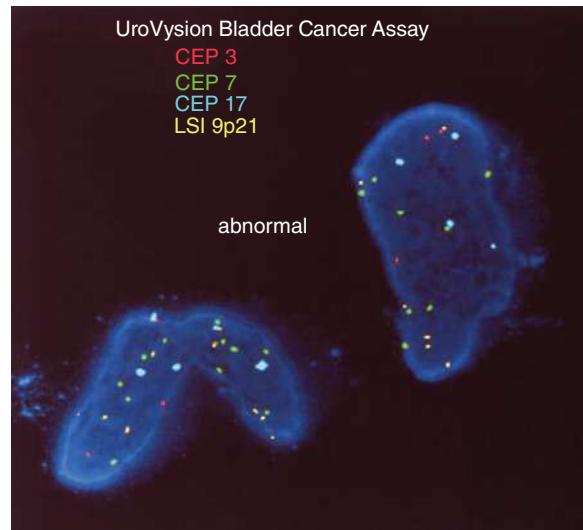


Figure 12.16 Urothelial carcinoma (see p. 610 for full caption).

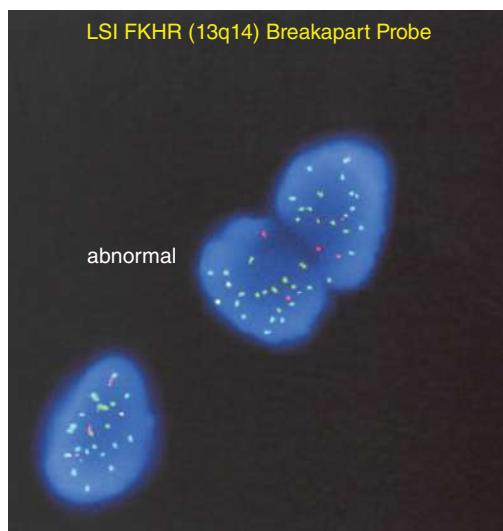


Figure 12.17 Disruption of *FKHR* (13q14) gene in alveolar rhabdomyosarcoma (see p. 611 for full caption).



Figure 13.1 DNA repair (see p. 654 for full caption).

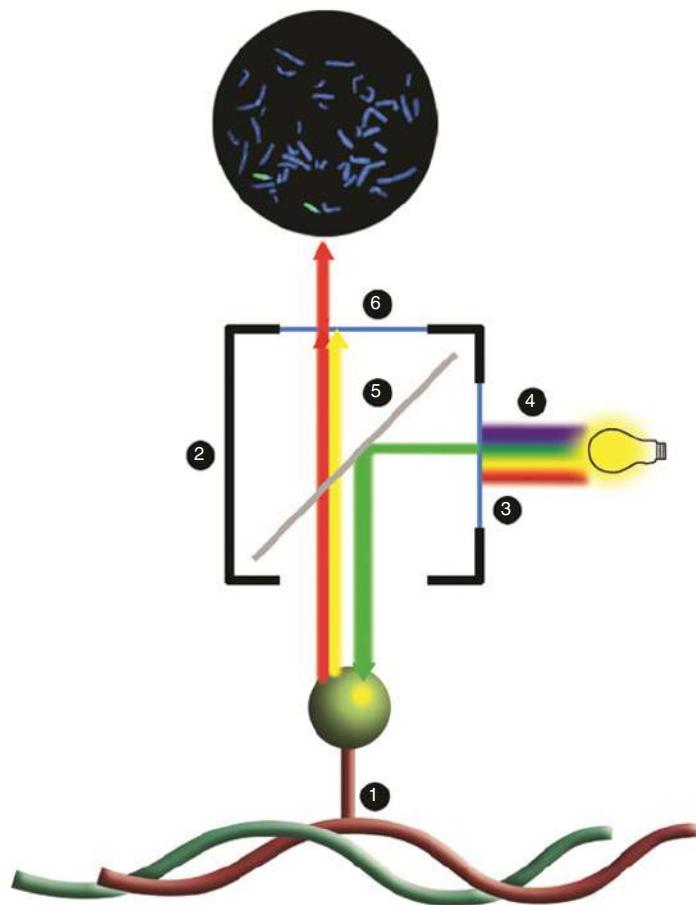


Figure 14.6 Fluorescence *in situ* hybridization microscopy (see p. 698 for full caption).

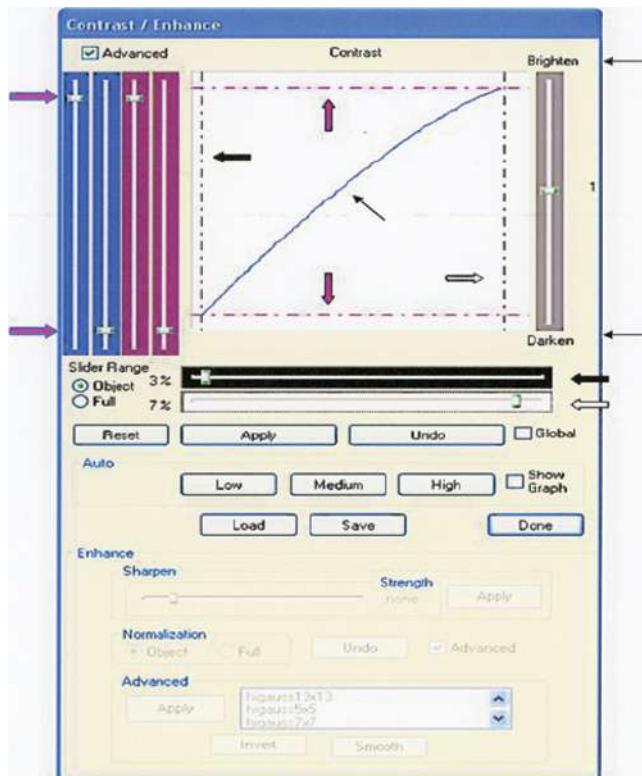


Figure 15.7 Advanced contrast feature of the CytoVision® computer imaging system (see p. 711 for full caption).

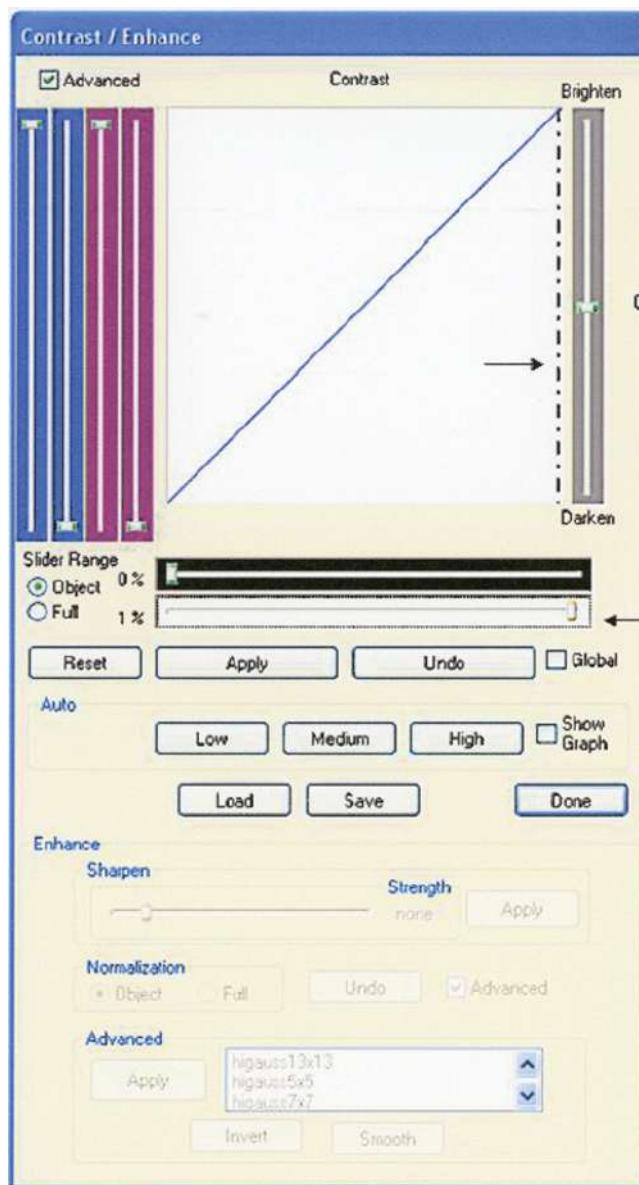


Figure 15.9 An example of a macro program (see p. 713 for full caption).

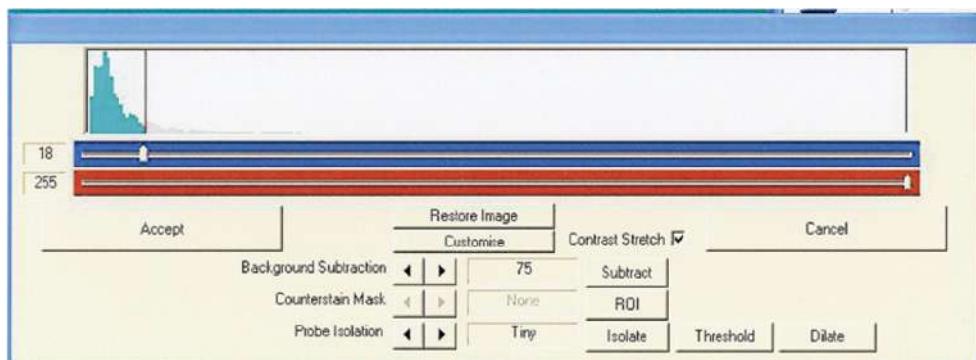


Figure 15.10 Thresholding tools (see p. 714 for full caption).

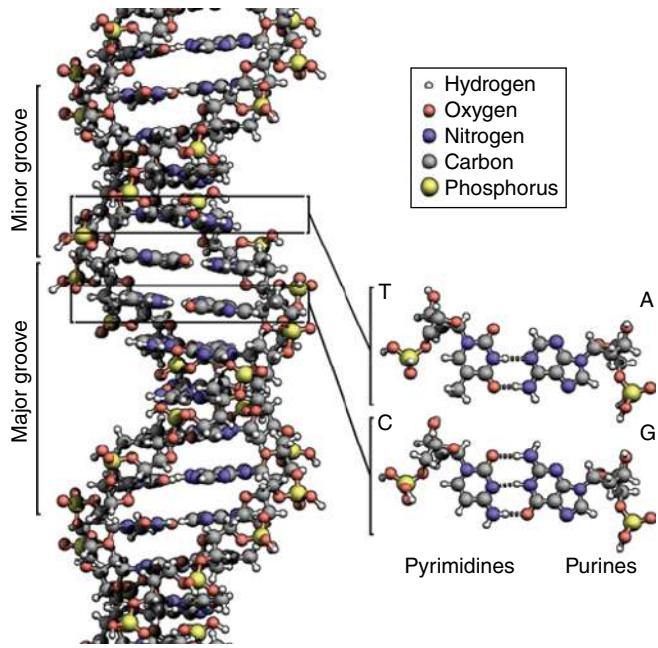


Figure 16.1 An element replica of DNA (see p. 718 for full caption).

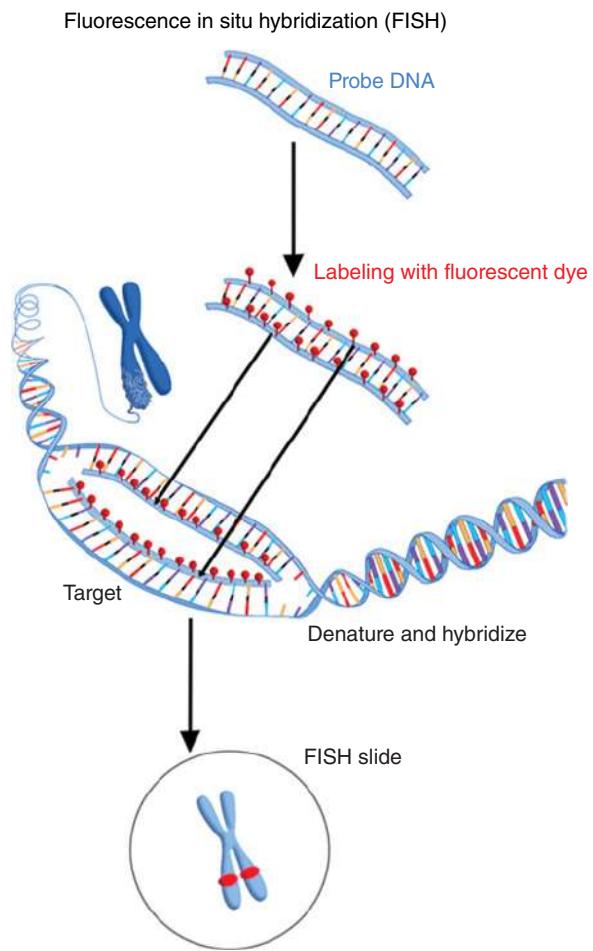


Figure 16.2 FISH process (see p. 718 for full caption).

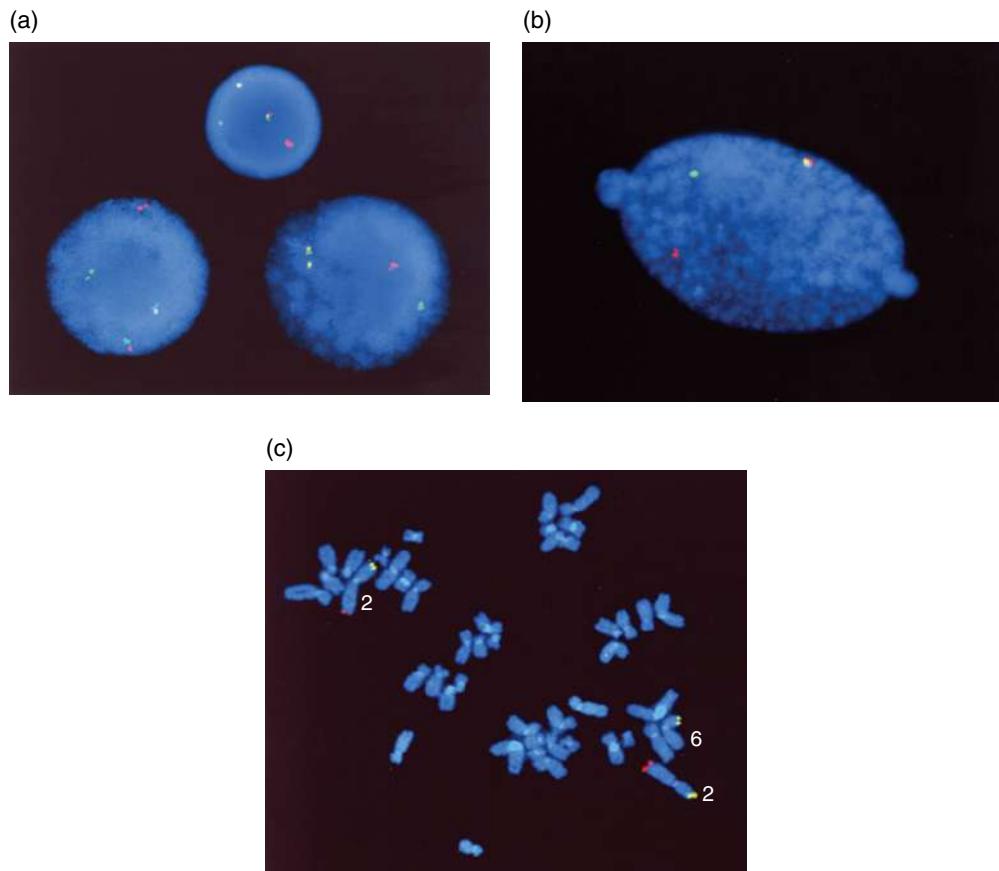


Figure 16.3 Single copy locus probes of several types (see p. 723 for full caption).

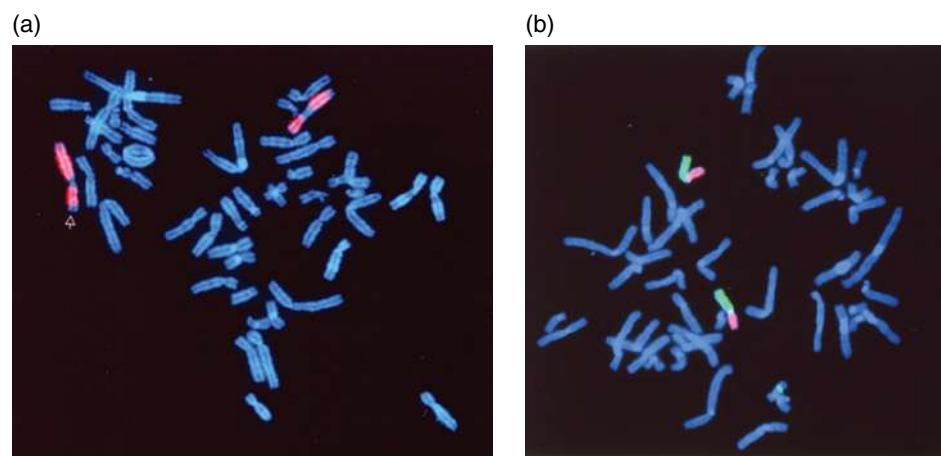


Figure 16.4 Chromosome paints (see p. 723 for full caption).

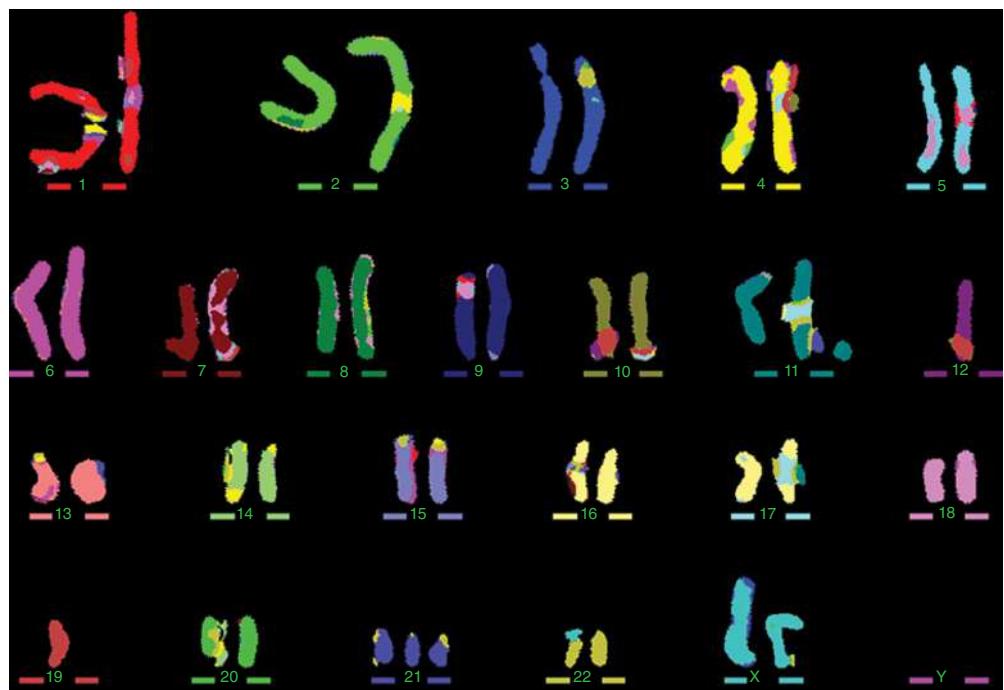


Figure 16.5 Multicolor FISH (see p. 724 for full caption).



Figure 16.6 Alpha satellite DNA probes (see p. 725 for full caption).

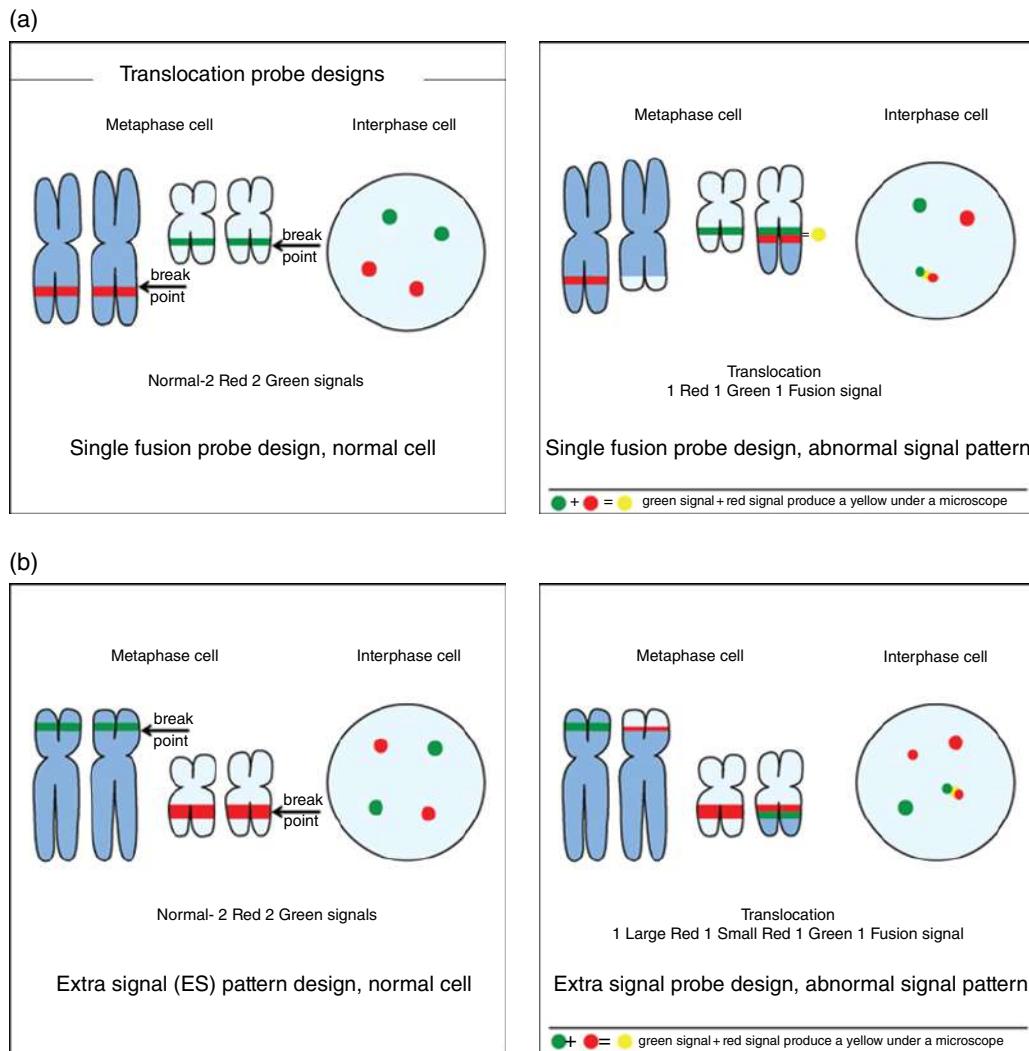


Figure 16.7 Translocation probe strategies (see p. 727–728 for full caption).

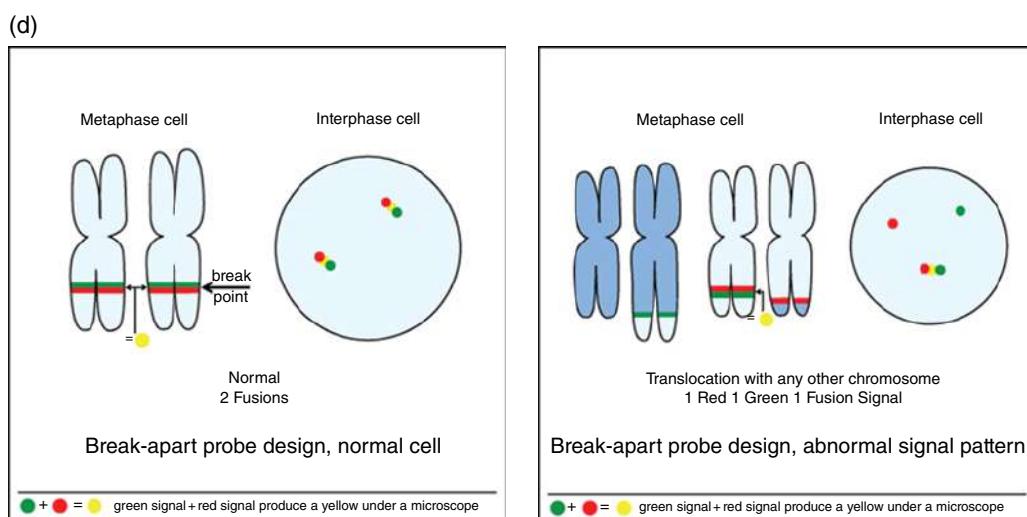
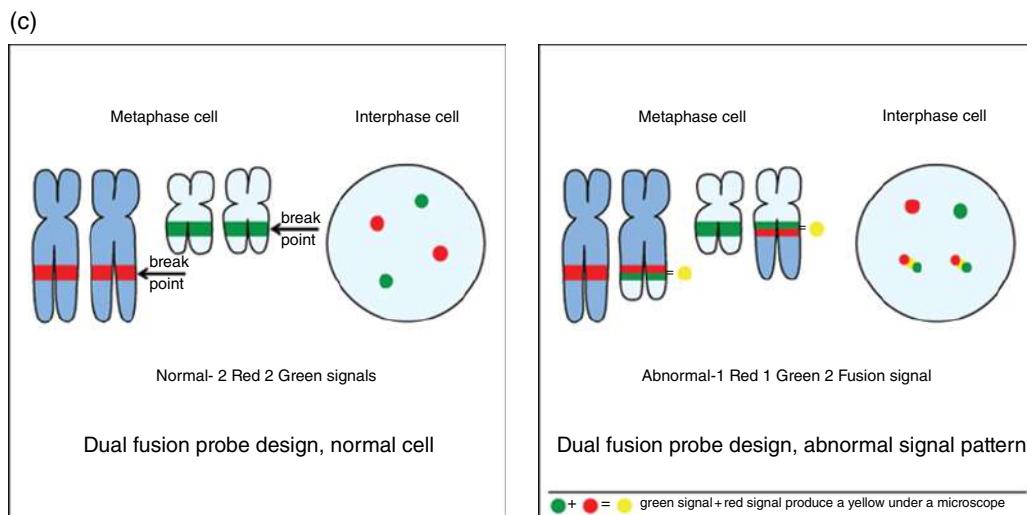


Figure 16.7 (Continued)

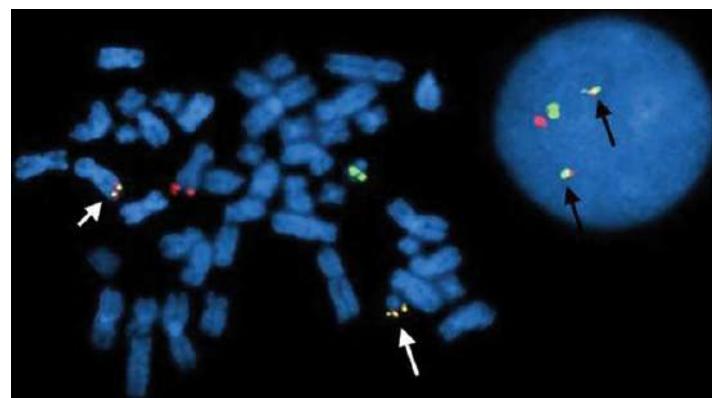


Figure 16.8 Fusion probe for *BCR/ABL1* t(9;22) FISH (see p. 729 for full caption).

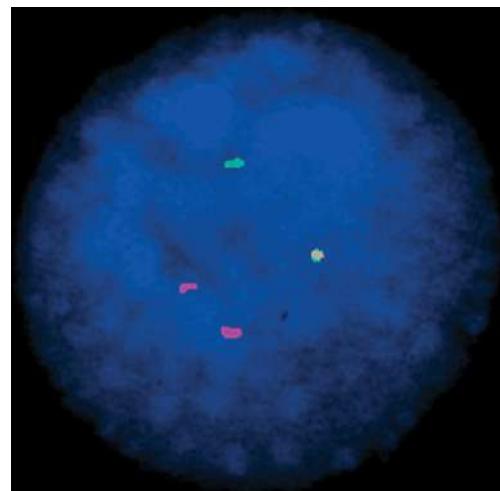


Figure 16.9 Extra-signal design probe for *TEL (ETV6)/AML1 (RUNX1T1)* fusion t(12;21) (see p. 729 for full caption).

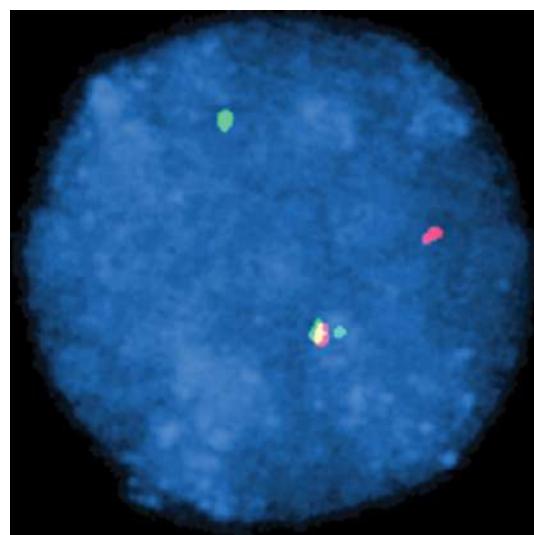


Figure 16.10 Break-apart probe for *MLL* (see p. 730 for full caption).

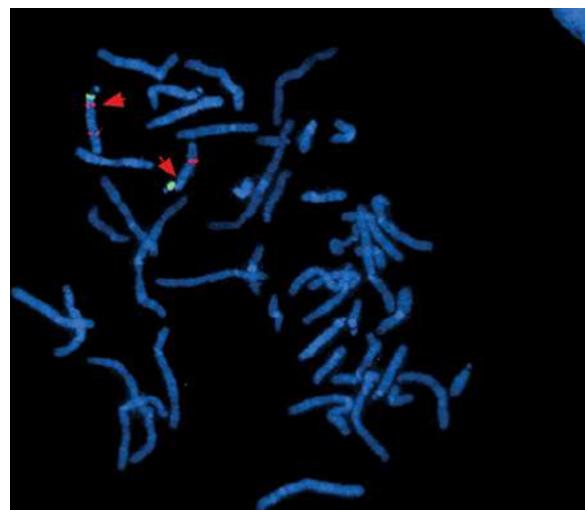


Figure 16.11 Constitutional deletion of chromosome 15q13 (see p. 732 for full caption).

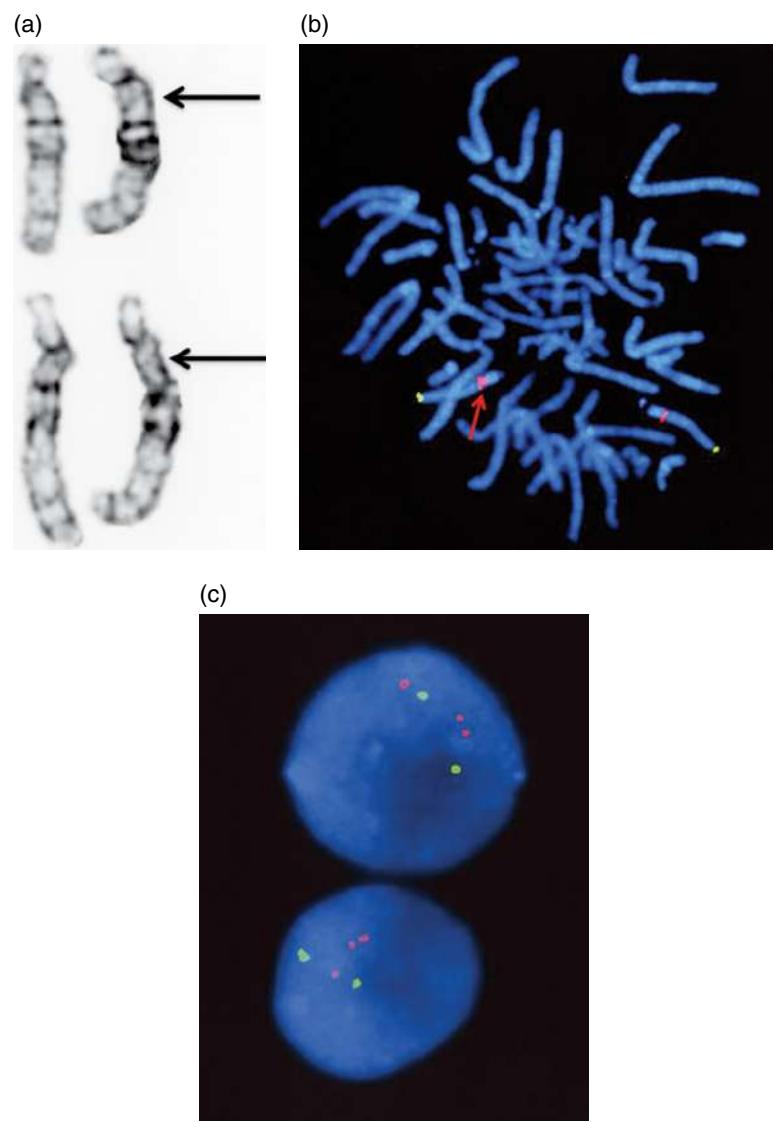


Figure 16.12 Locus-specific probes for duplication (see p. 733 for full caption).

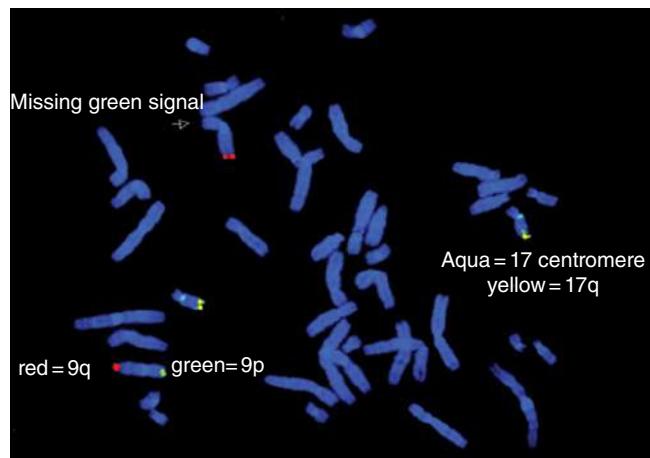


Figure 16.13 Subtelomere-specific panels (see p. 734 for full caption).

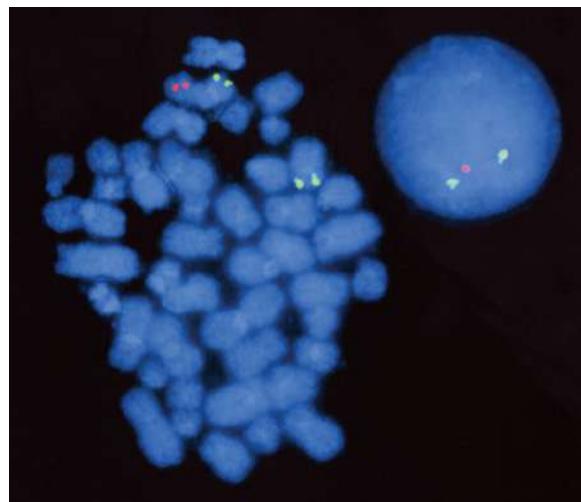


Figure 16.14 Acquired deletion of *EGR1* (5q) (see p. 735 for full caption).

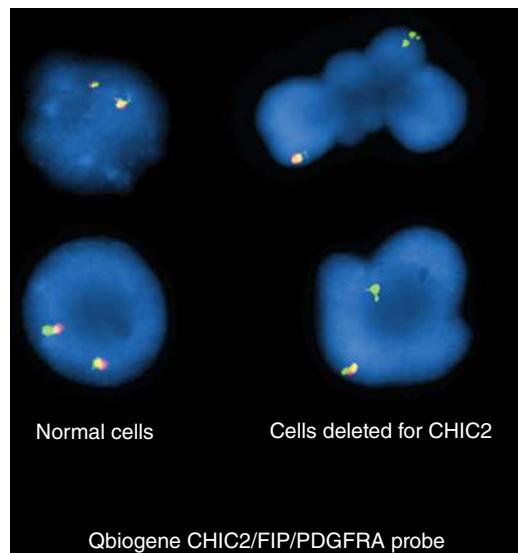


Figure 16.15 *CHIC2* deletion probe (see p. 736 for full caption).

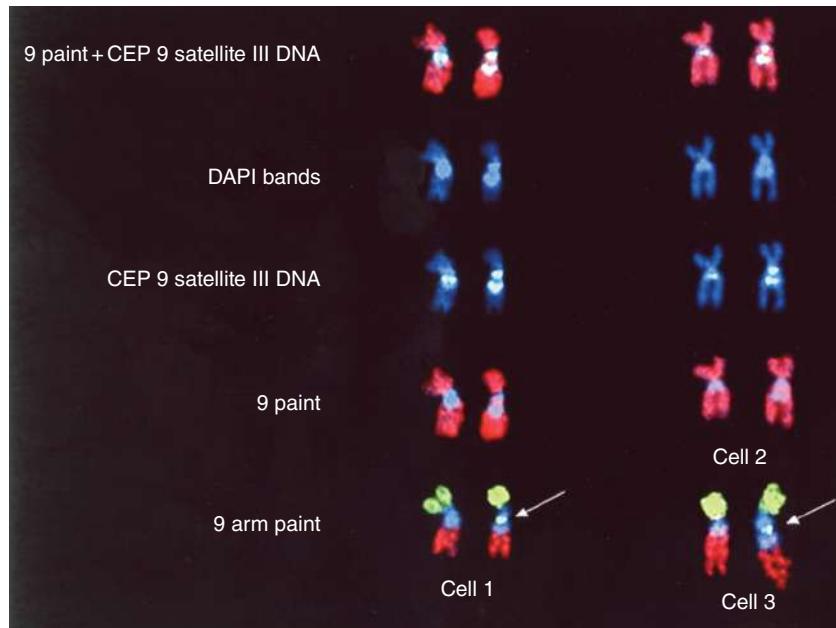


Figure 16.16 Probe mixtures and sequential hybridization to delineate a chromosome 9 abnormality (see p. 739 for full caption).

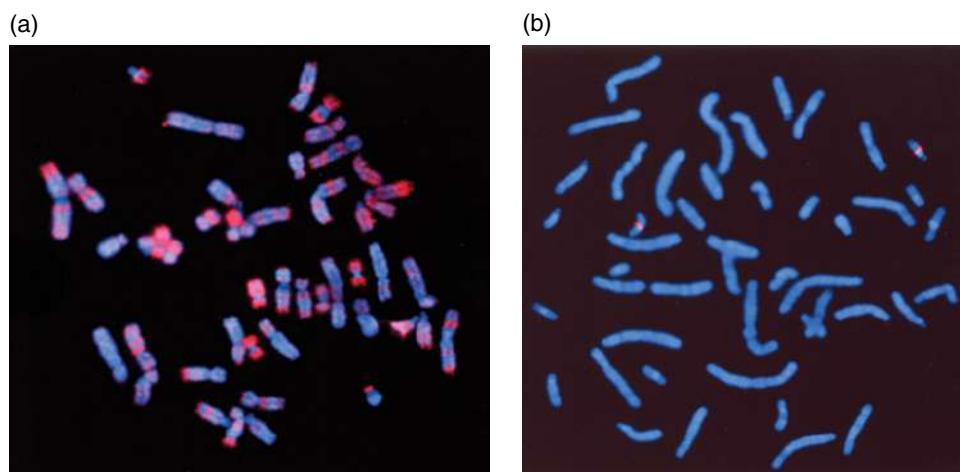


Figure 16.17 Alu banding (see p. 740 for full caption).

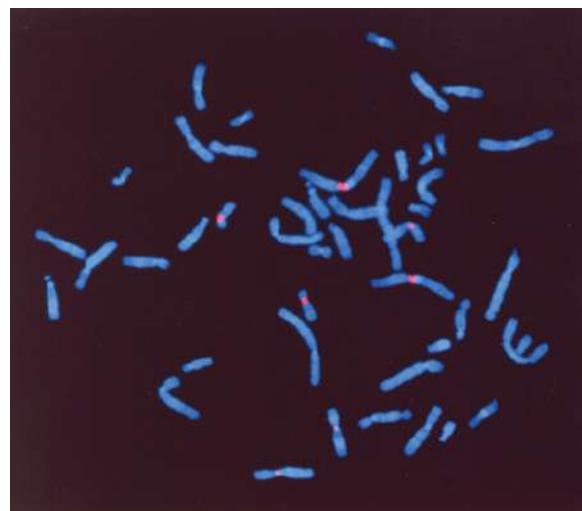


Figure 16.18 Effect of low stringency buffer and post wash (see p. 743 for full caption).

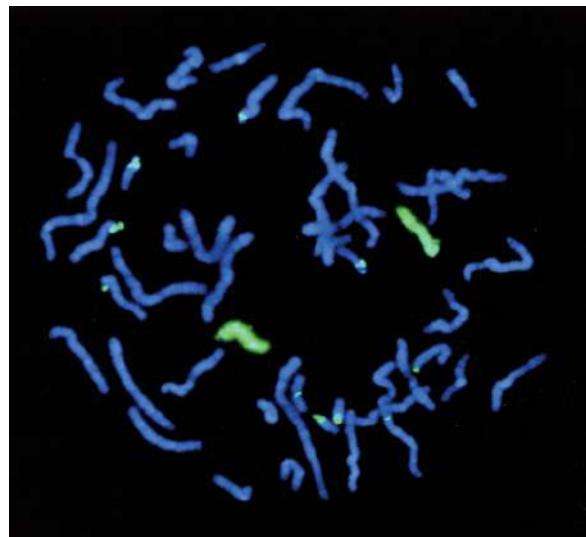


Figure 16.19 Acrocentric paint cross-hybridizes in short arm regions (see p. 744 for full caption).



Figure 16.20 Whole arm paints (see p. 745 for full caption).

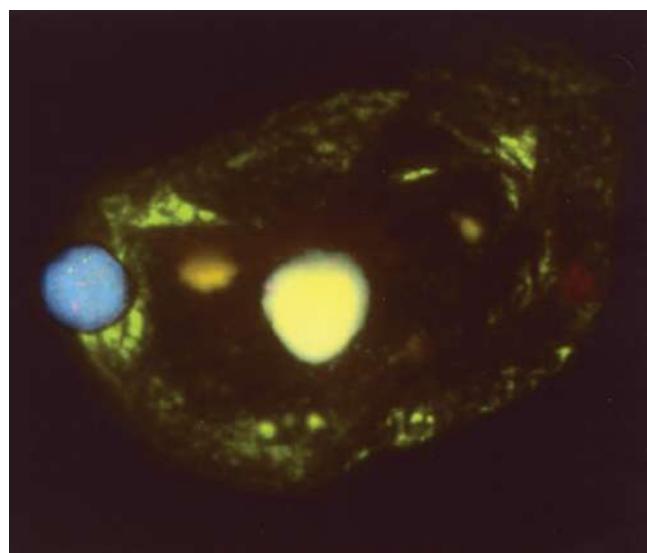


Figure 16.22 Comparison of amniocytes for FISH aneuvysion (see p. 746 for full caption).

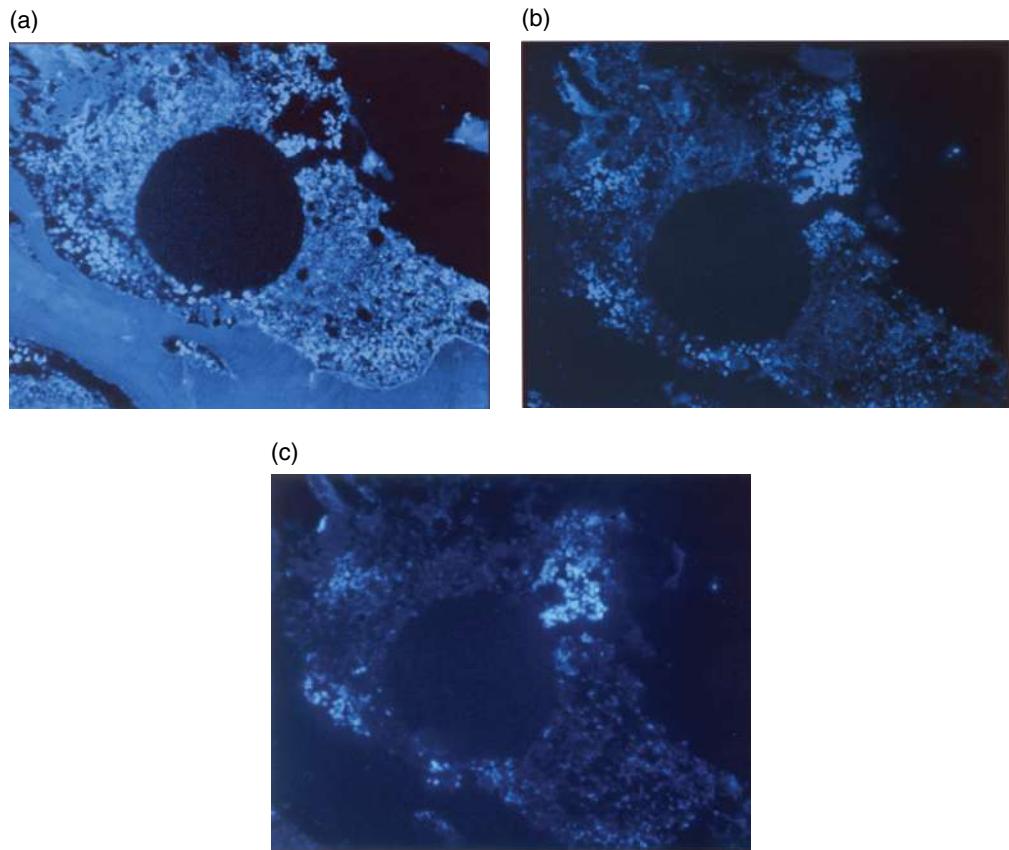


Figure 16.23 Comparing pretreatment time for *HER2/neu* FISH on breast tissue (see p. 747 for full caption).

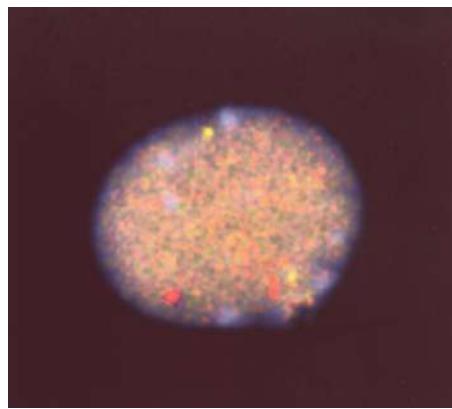


Figure 16.24 Effect of probe mixture drying out (see p. 752 for full caption).

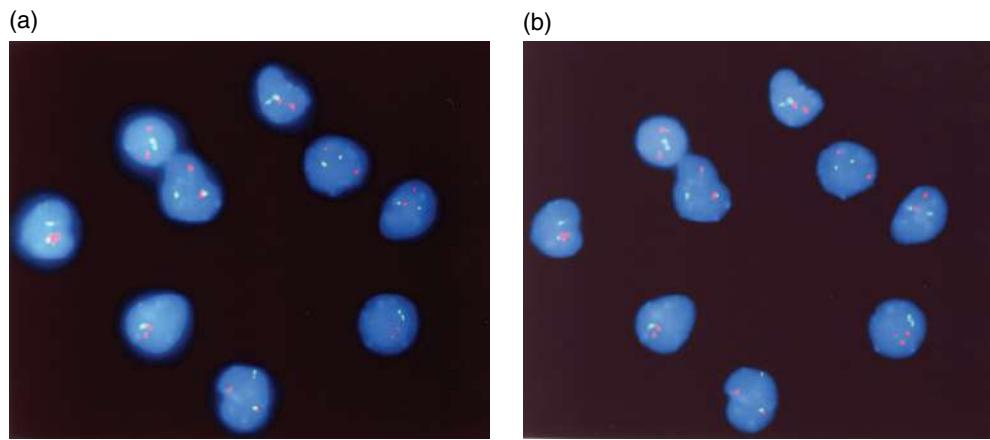


Figure 16.27 Proper centration of the microscope lamp (see p. 757 for full caption).

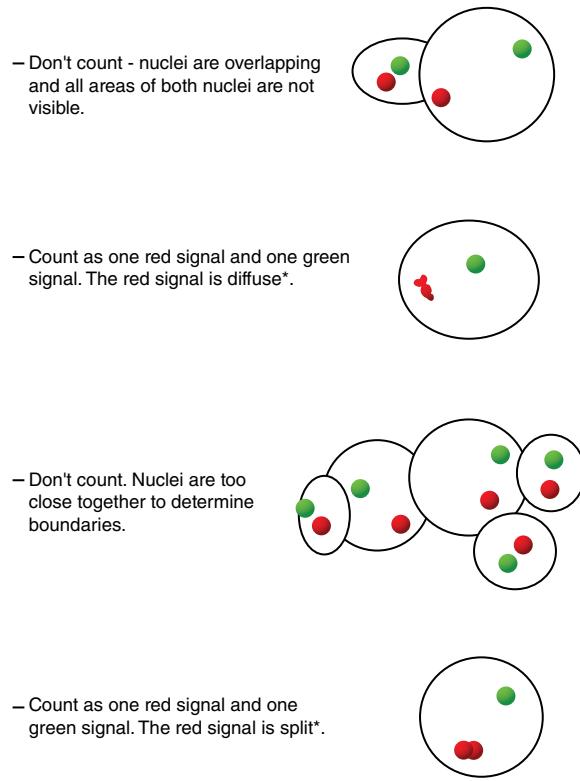


Figure 16.29 Two color counting guidelines (see p. 760 for full caption).

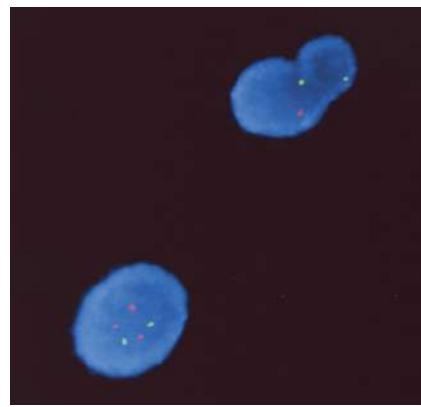


Figure 16.30 Maternal cell contamination in amniotic fluid interphase FISH study (see p. 760 for full caption).

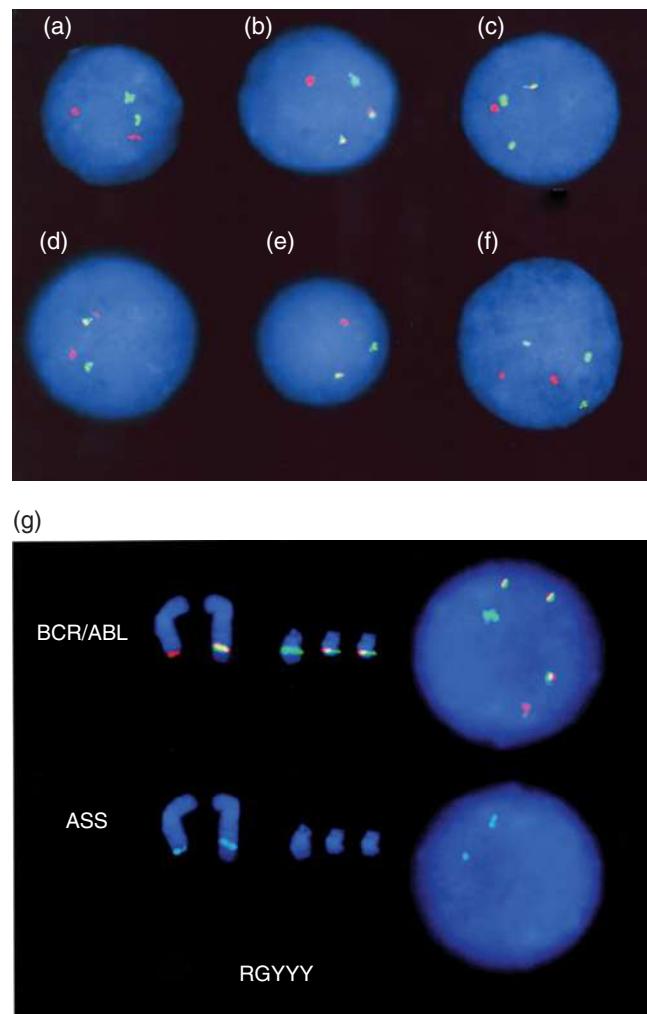


Figure 16.31 *BCR/ABL1/ASS* metaphase and interphase variations (see p. 761 for full caption).

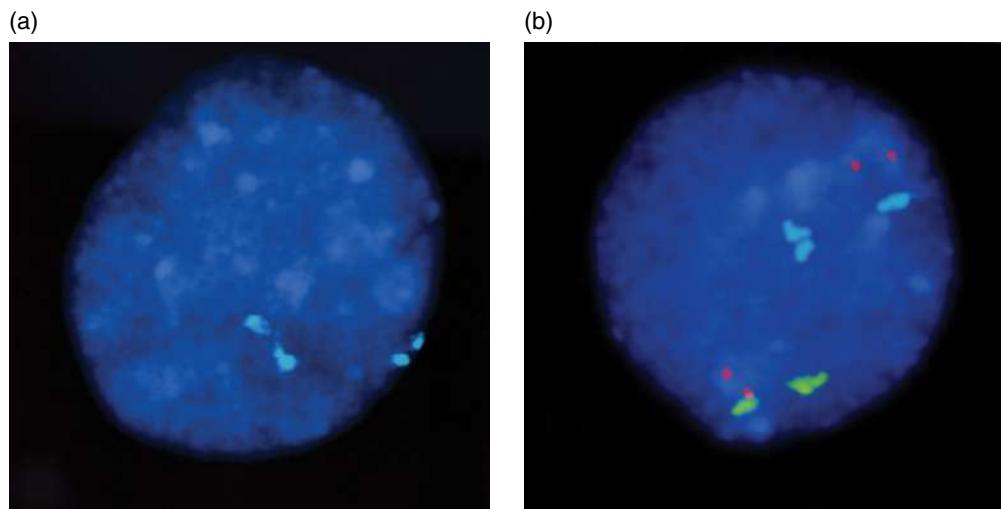


Figure 16.32 Scoring replicated signals (see p. 762 for full caption).

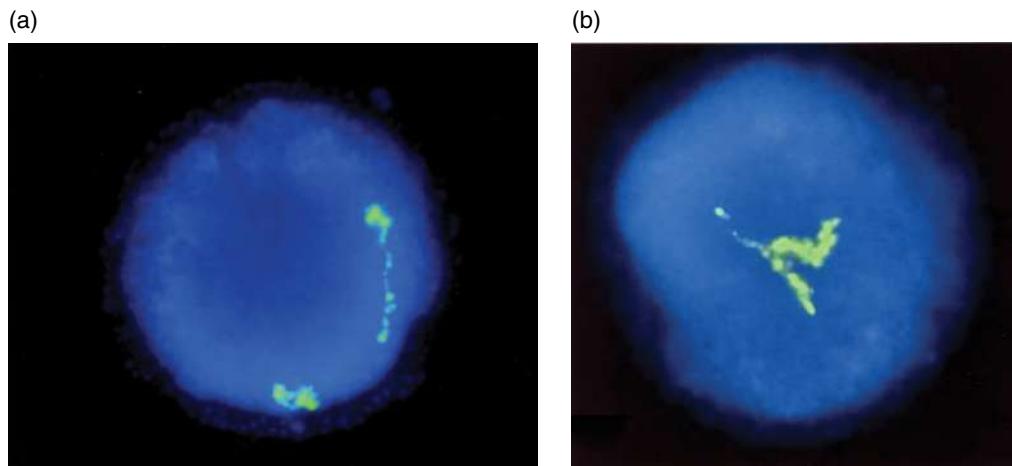


Figure 16.33 Scoring repetitive DNA signals (see p. 763 for full caption).

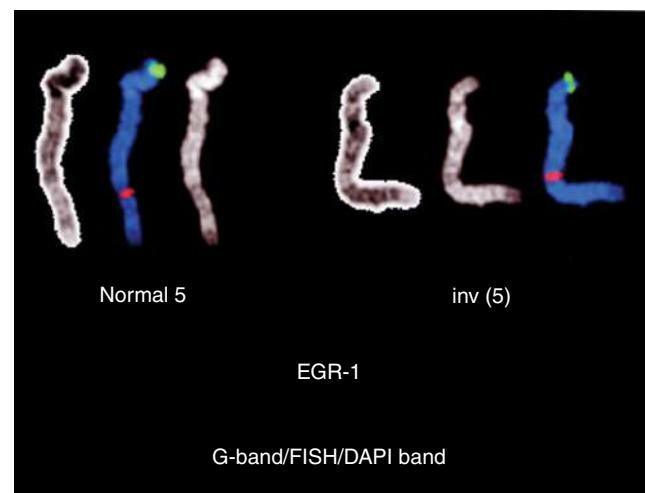


Figure 16.34 Using DAPI bands to determine location of a probe (see p. 763 for full caption).

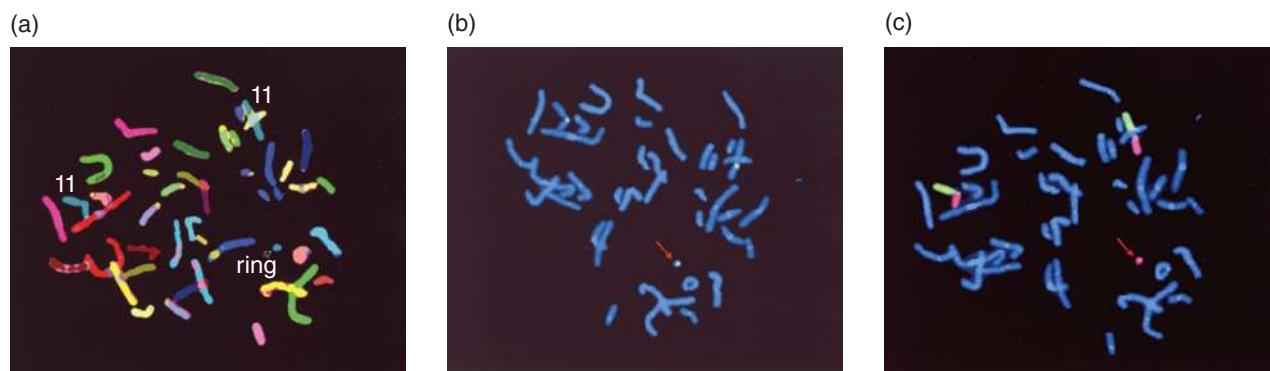
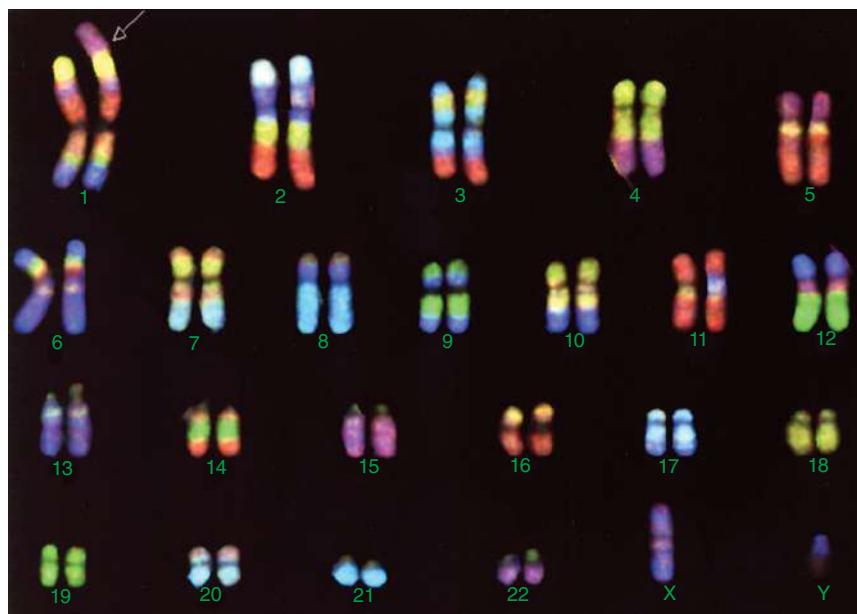


Figure 16.35 Sequential hybridizations of the same cell to identify an unknown (see p. 764 for full caption).

(a)



(b)



Figure 16.36 Rx FISH (see p. 770 for full caption).

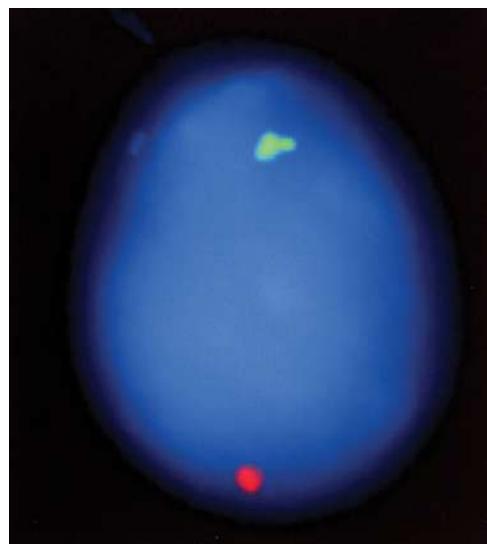


Figure 16.38 Sperm FISH (see p. 776 for full caption).

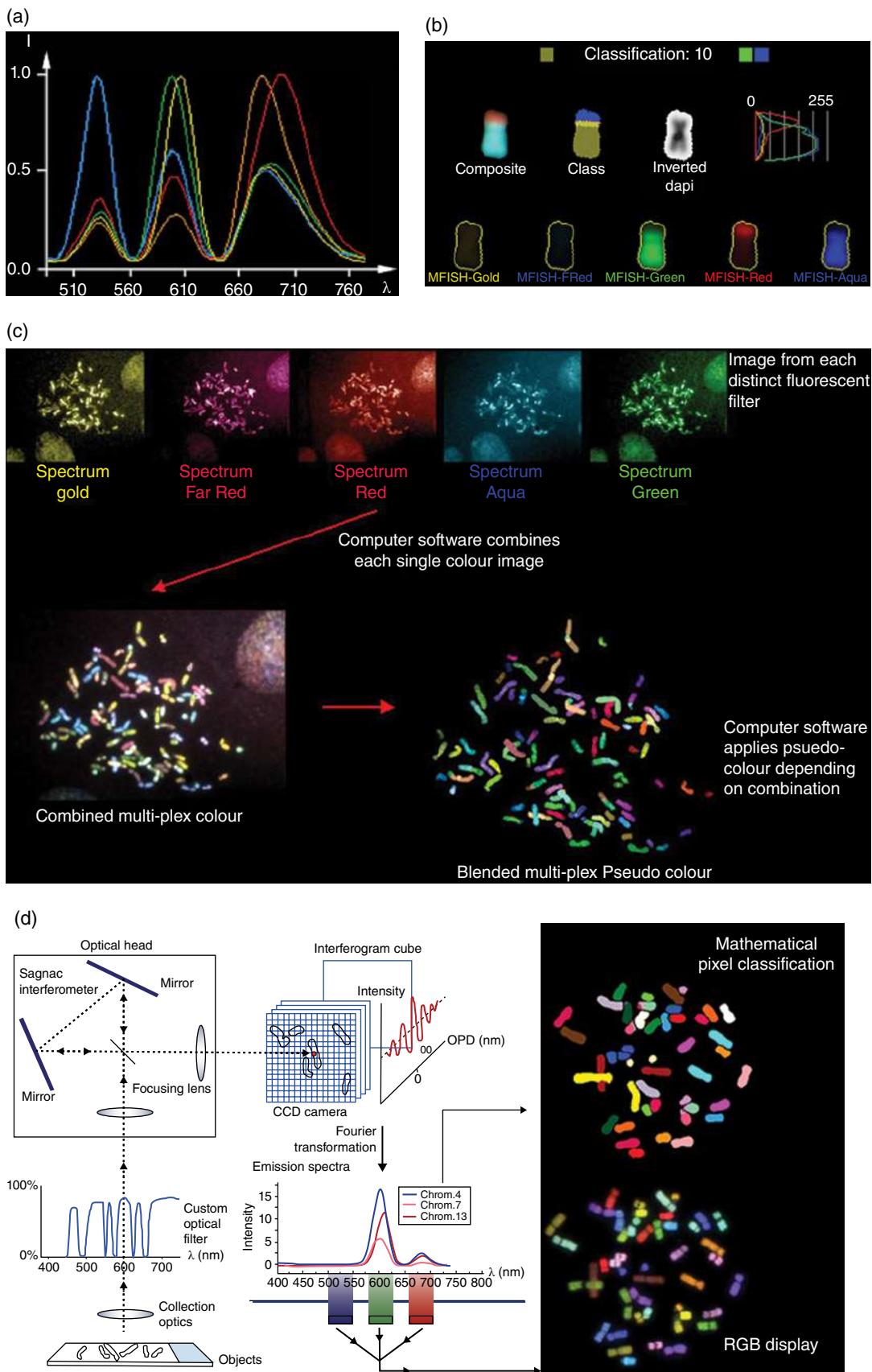


Figure 17.1 Spectral imaging (see p. 836–837 for full caption).

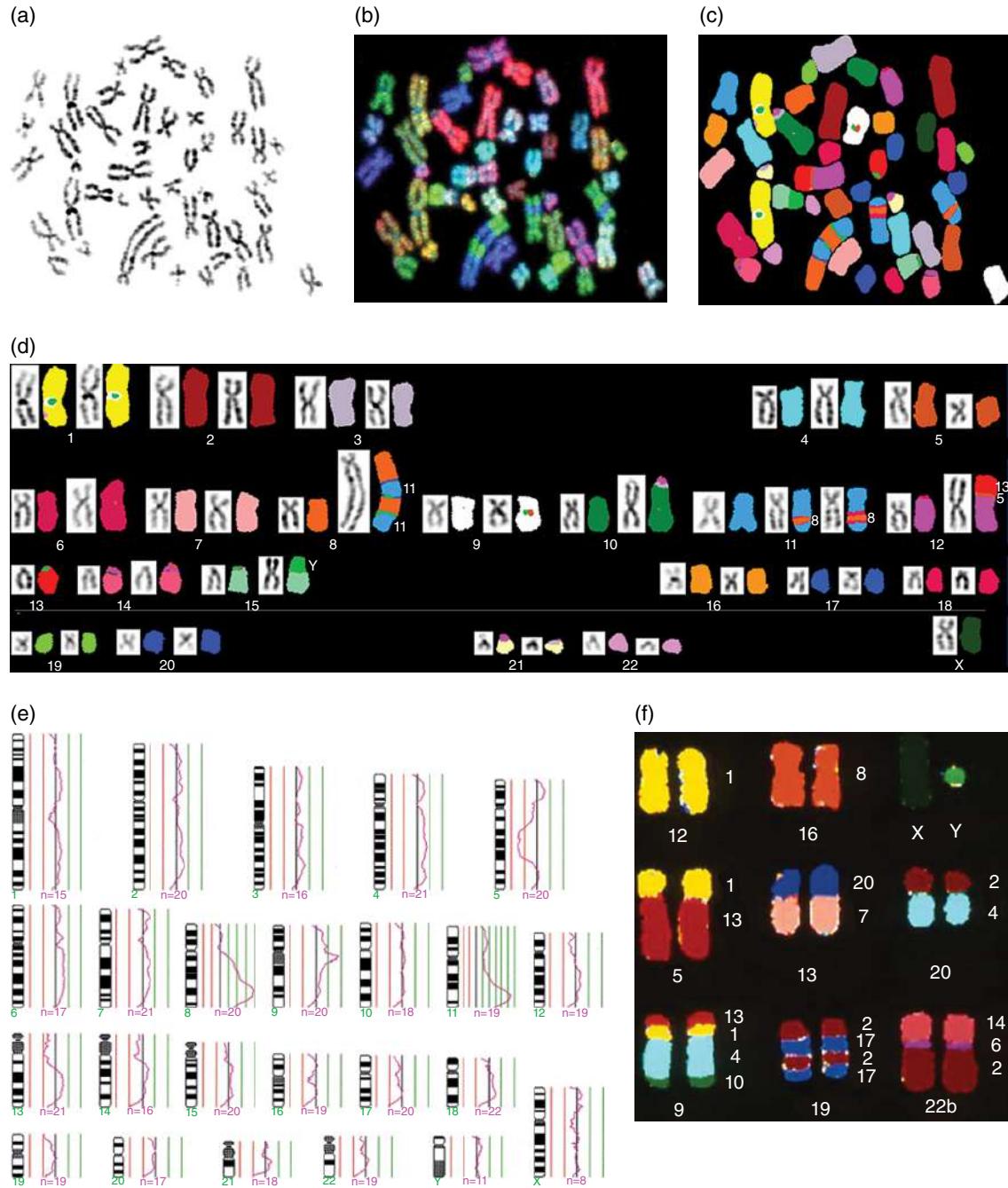


Figure 17.2 DAPI (see p. 838 for full caption).

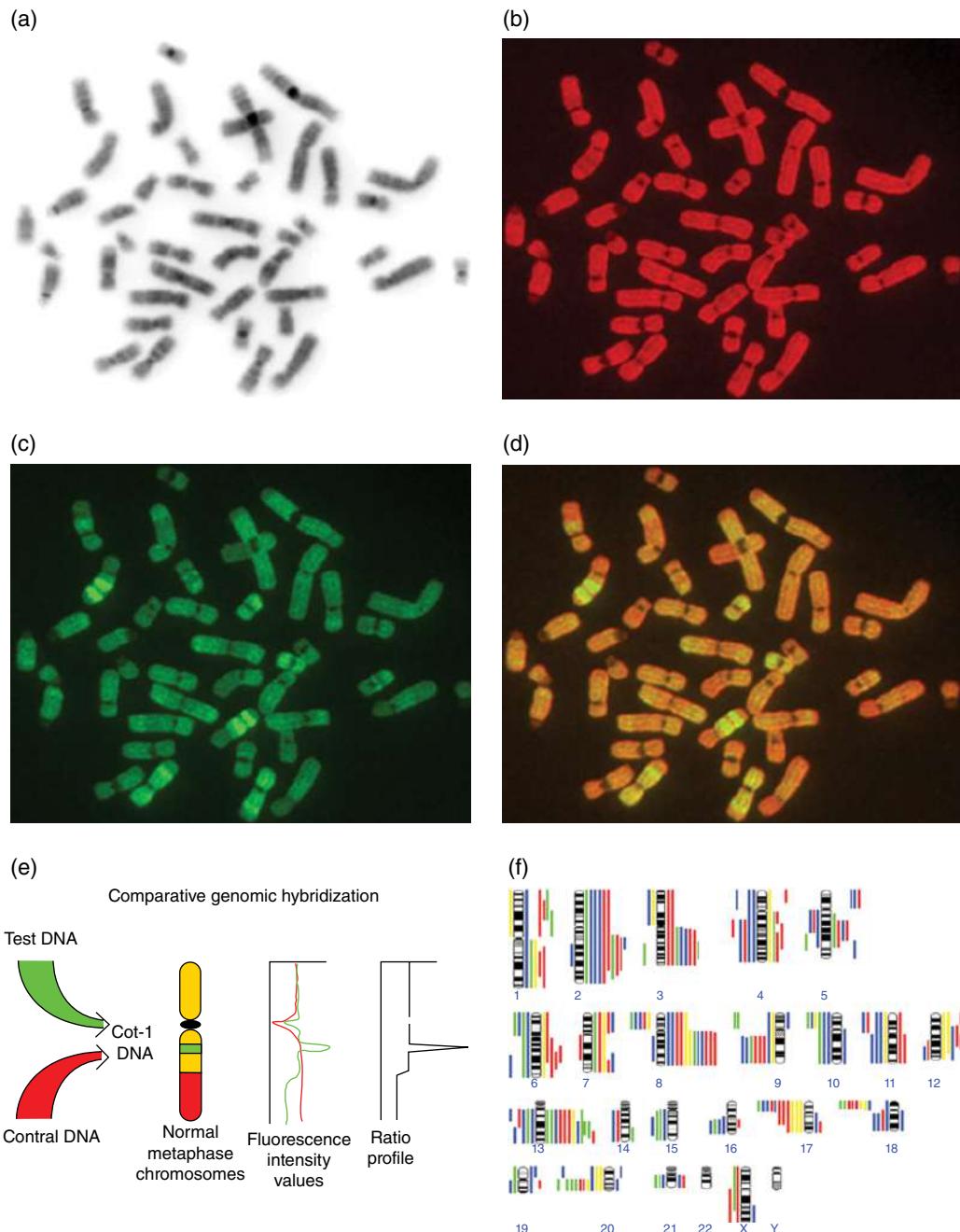


Figure 17.3 Acquired metaphase images from CGH of breast cancer cell line SKBR3 (see p. 852 for full caption).

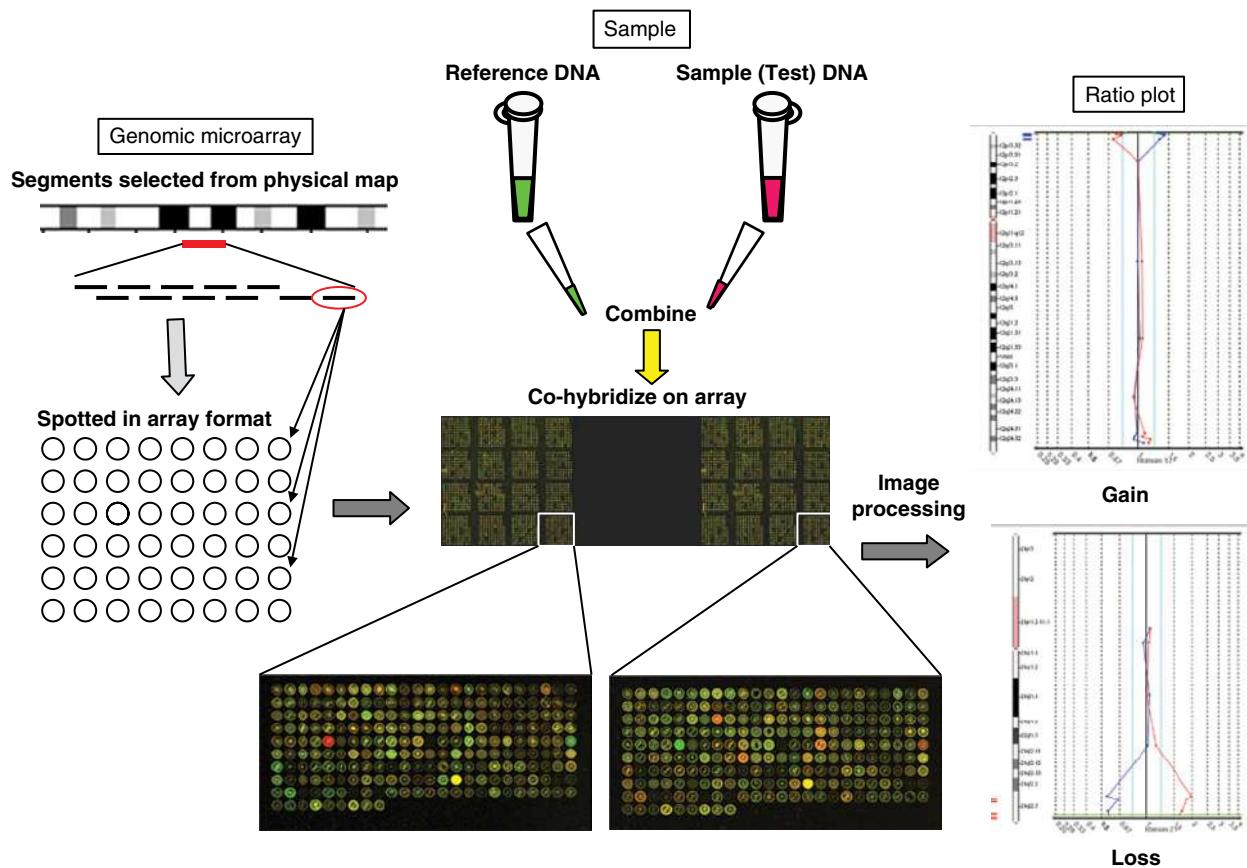


Figure 18.1 Genomic microarray hybridization profile construction (see p. 904 for full caption).

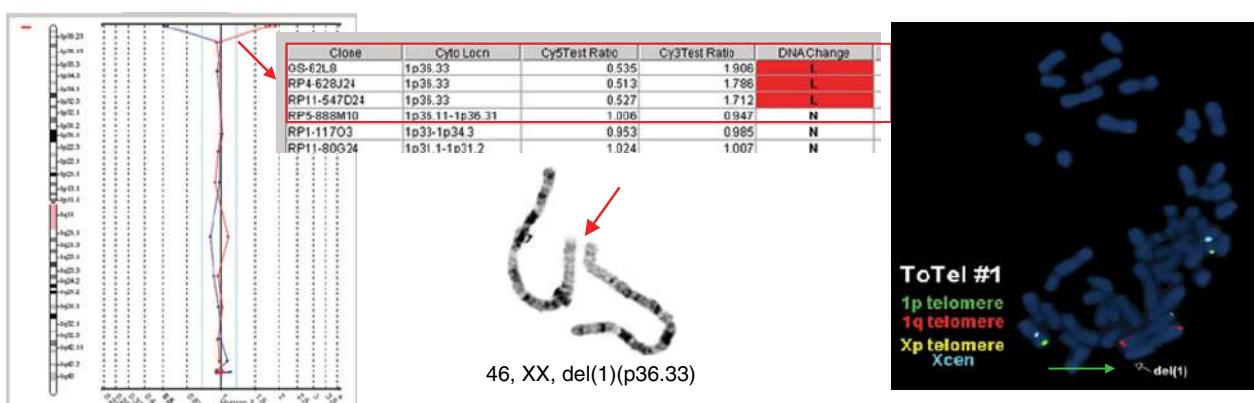


Figure 18.3 Case study 1 (see p. 916 for full caption).

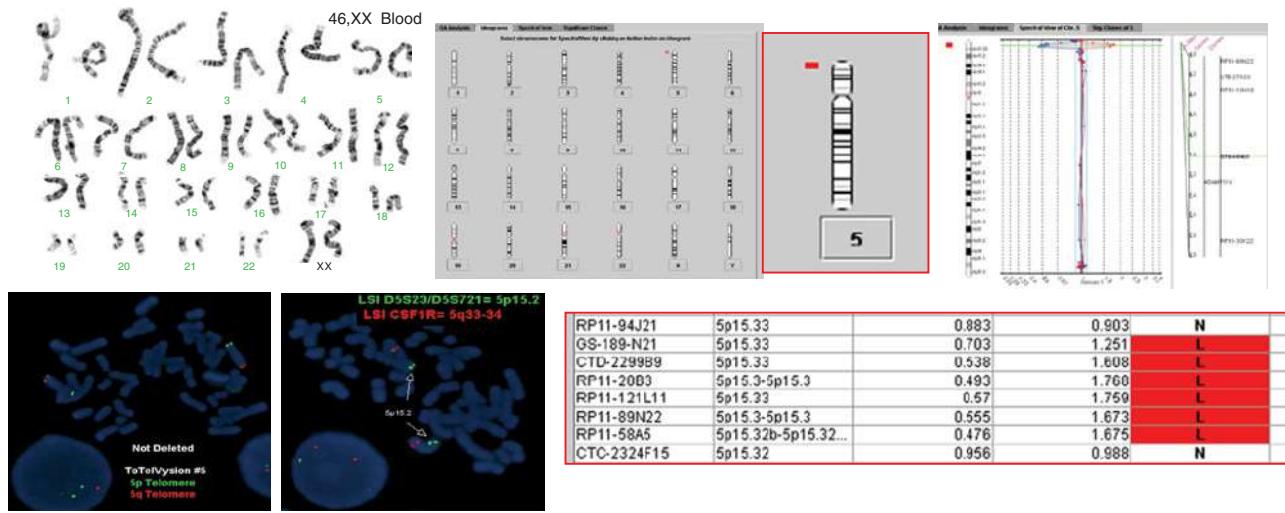


Figure 18.4 Case study 2 (see p. 917 for full caption).

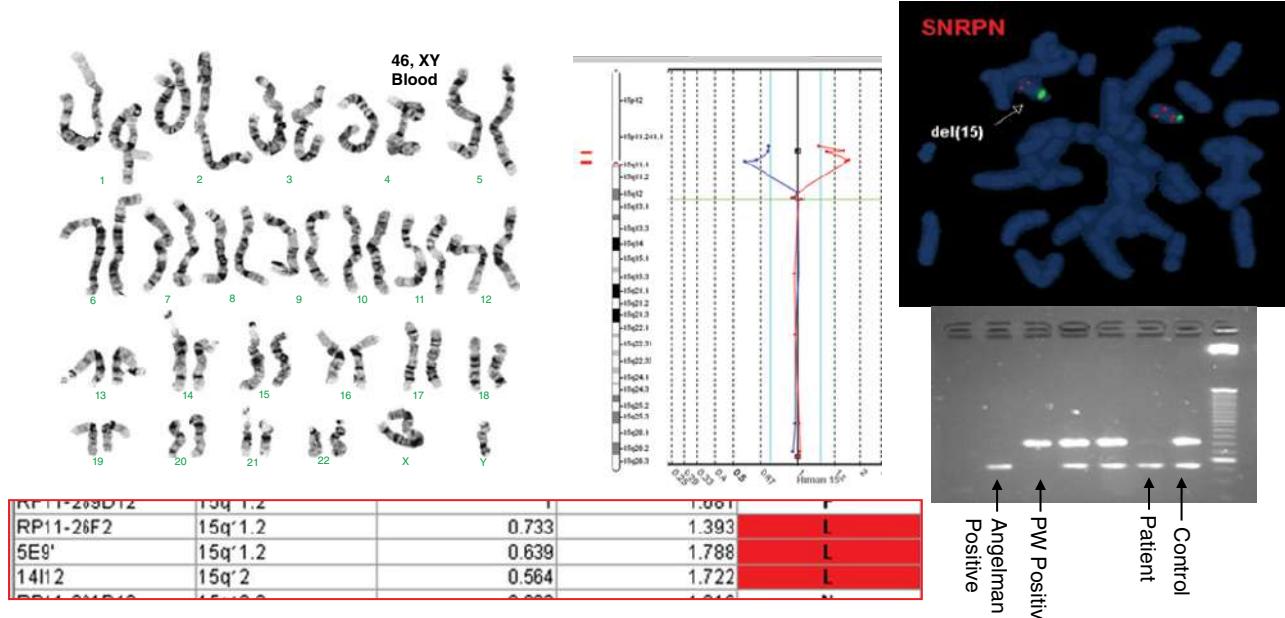


Figure 18.5 Case study 3 (see p. 917 for full caption).

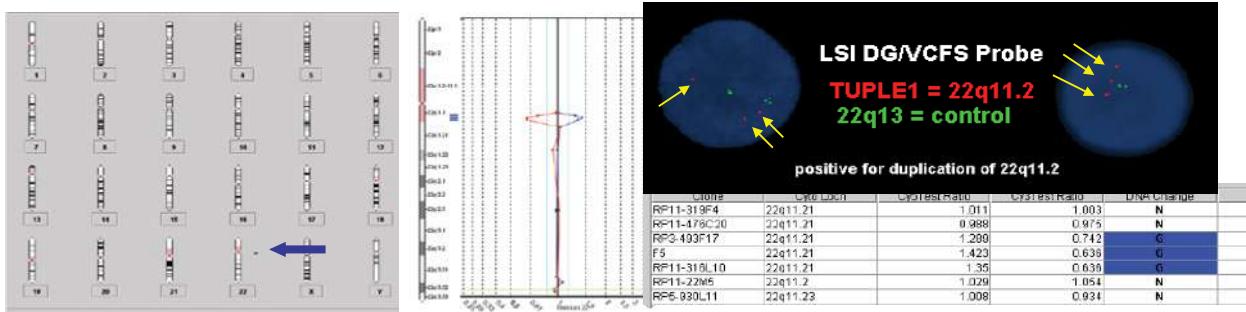


Figure 18.6 Case study 4 (see p. 918 for full caption).

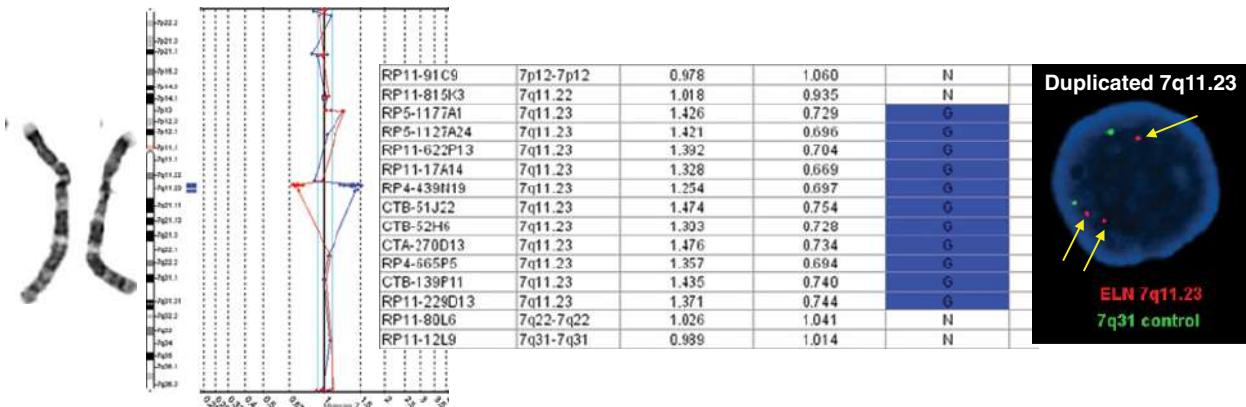


Figure 18.7 Case study 5 (see p. 918 for full caption).

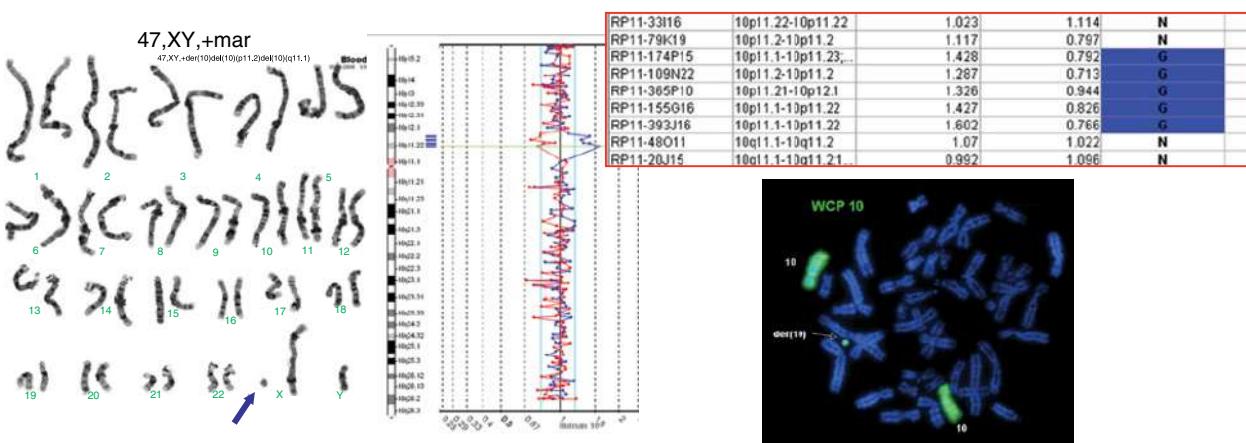


Figure 18.8 Case study 6 (see p. 918 for full caption).

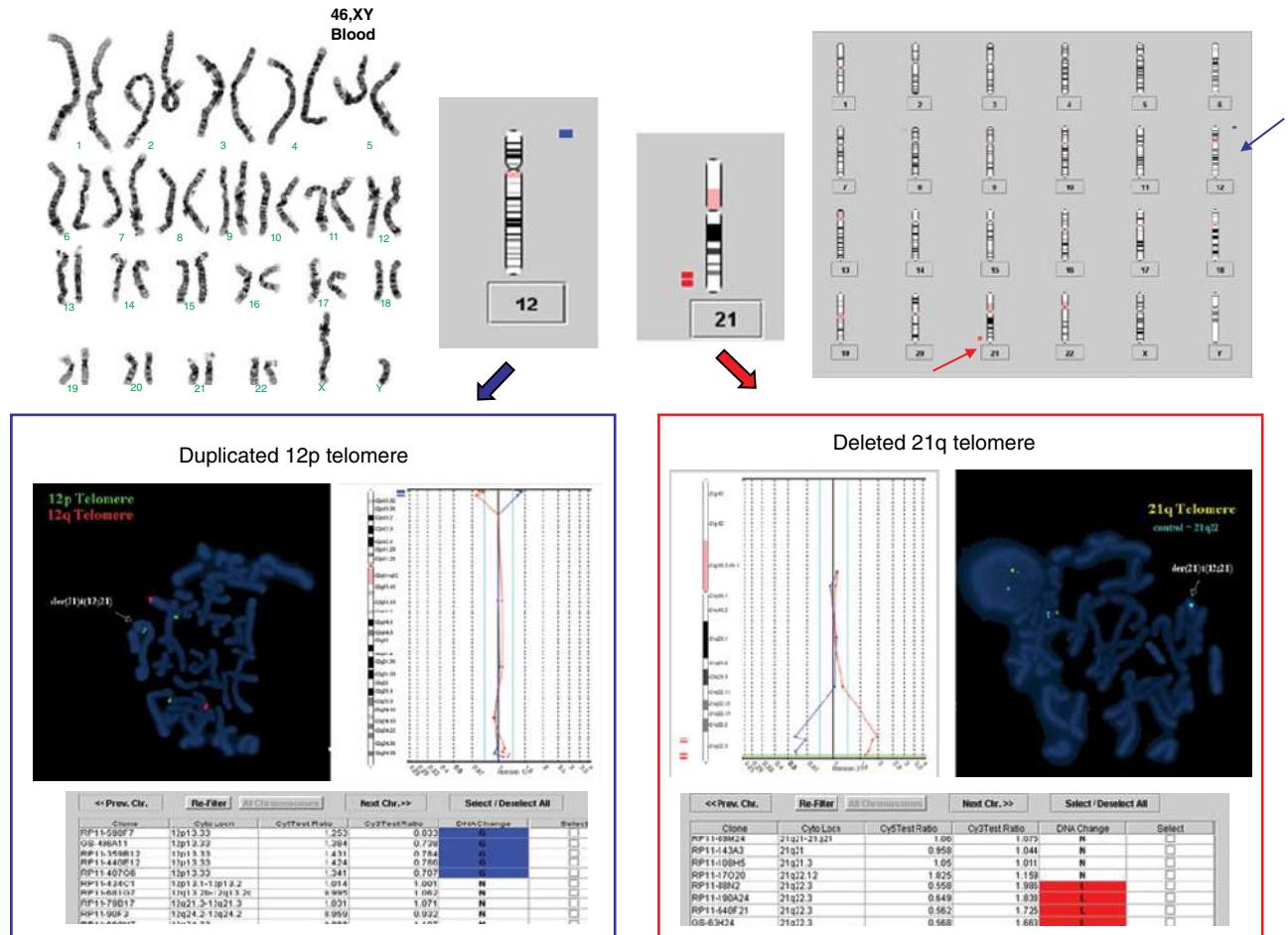


Figure 18.9 Case study 7 (see p. 919 for full caption).

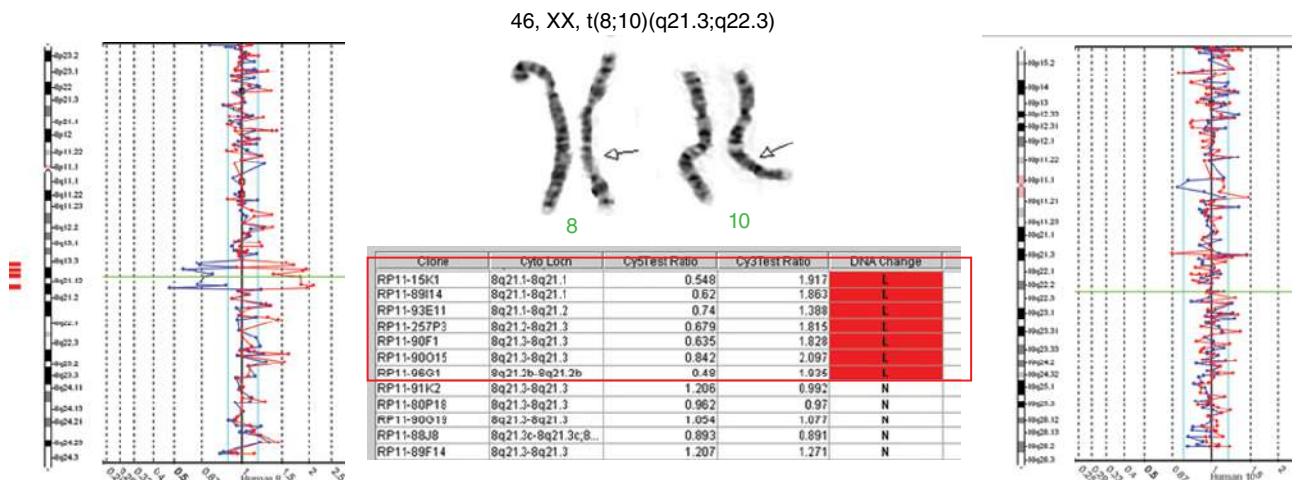


Figure 18.10 Case study 8 (see p. 919 for full caption).

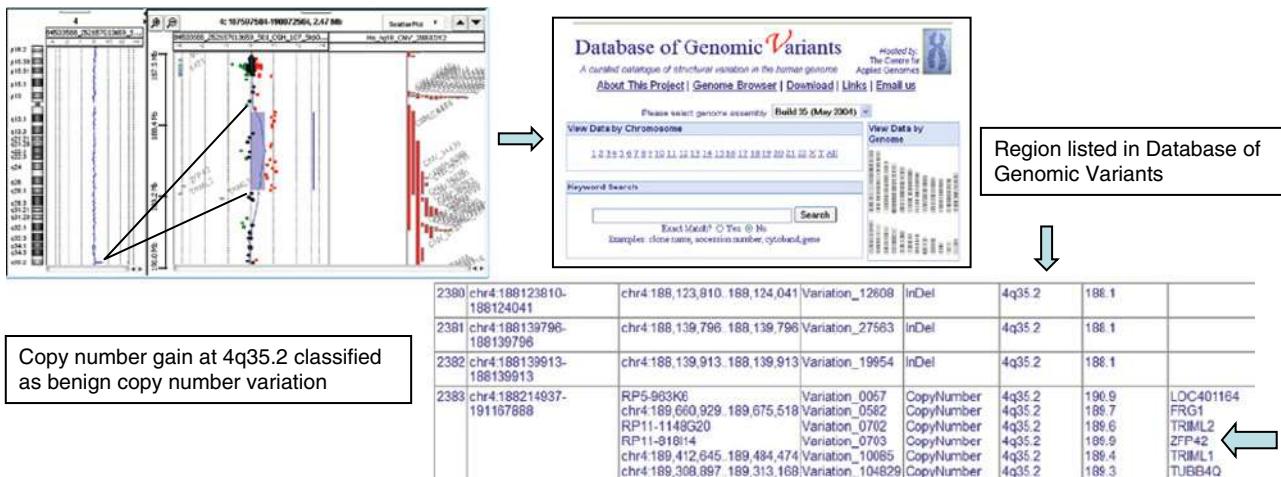


Figure 18.11 Case study 9 (see p. 920 for full caption).

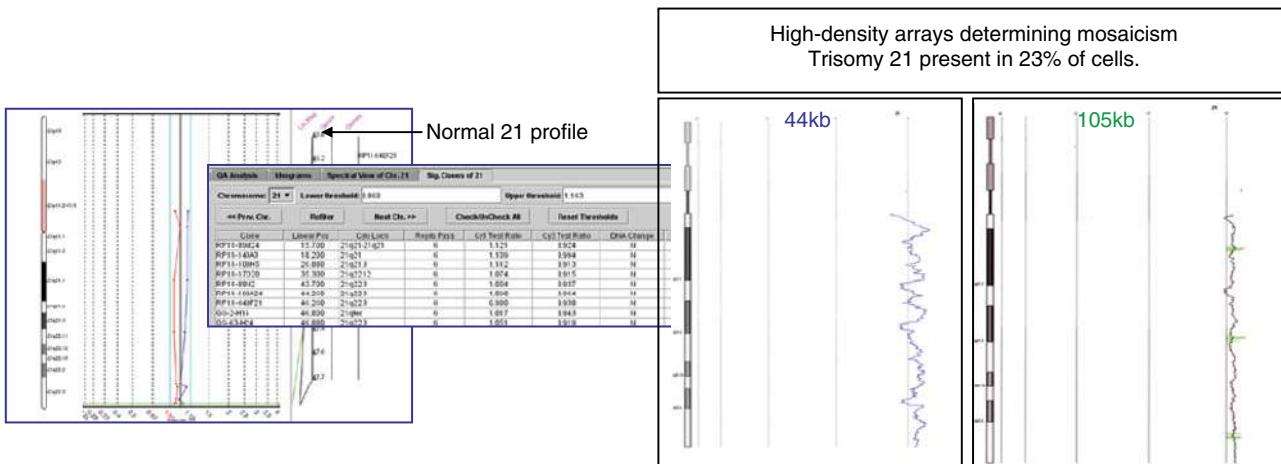


Figure 18.12 Case study 10 (see p. 920 for full caption).

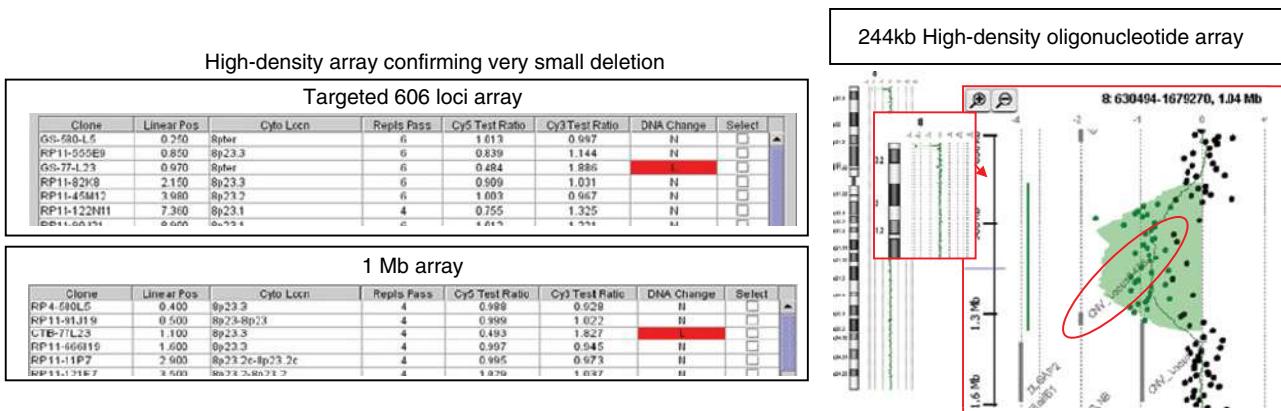


Figure 18.13 Case study 11 (see p. 921 for full caption).

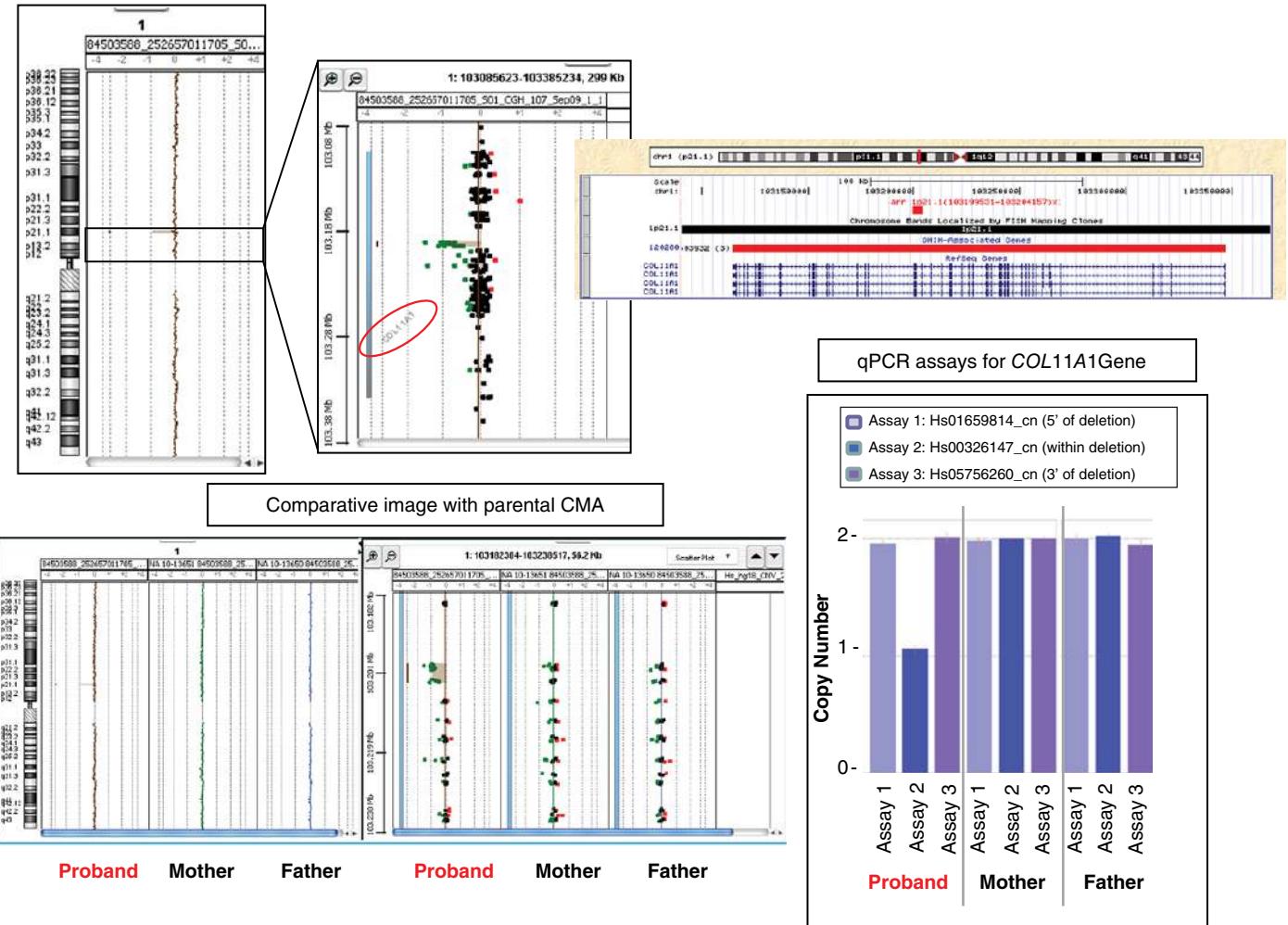


Figure 18.14 Case study 12 (see p. 921 for full caption).

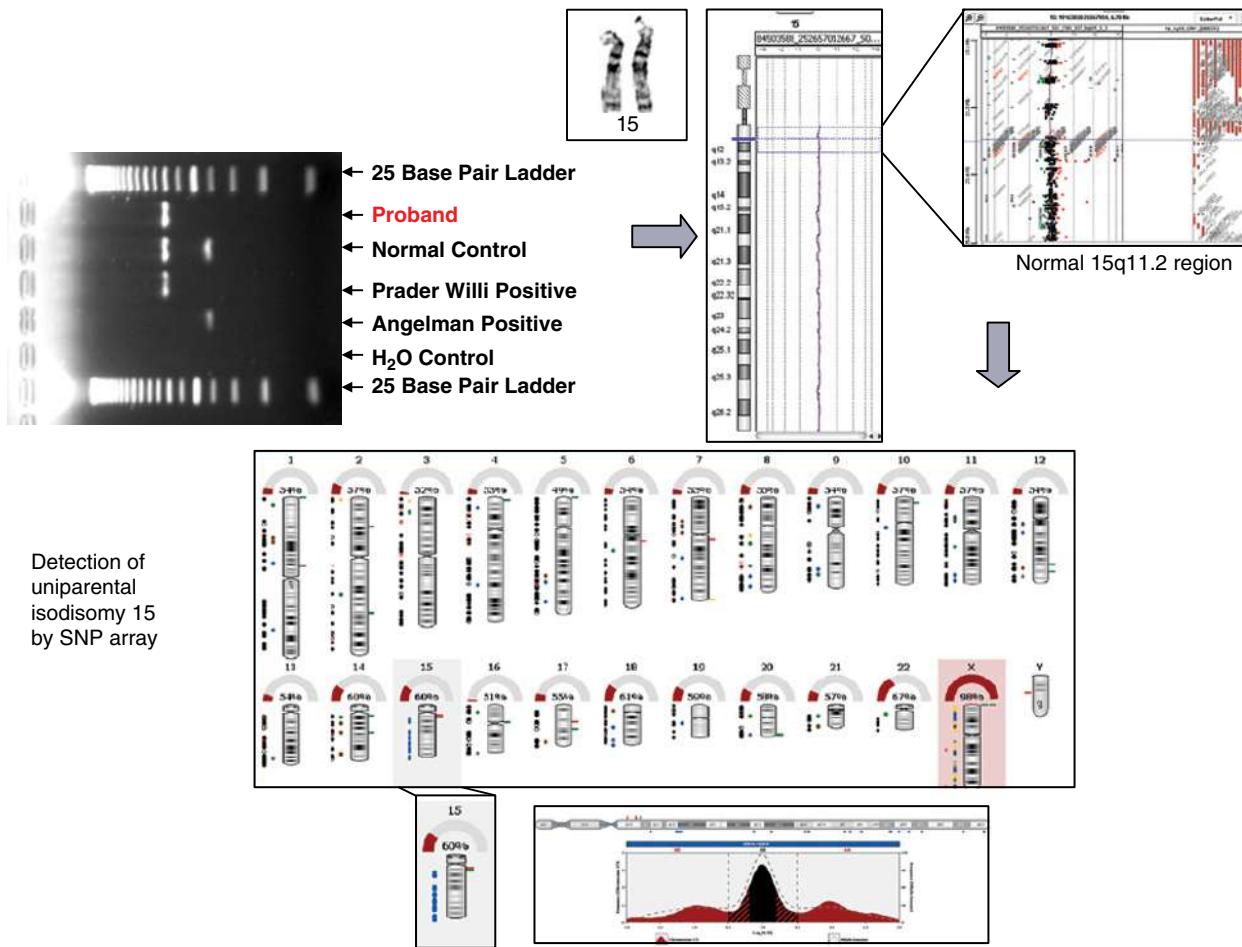


Figure 18.15 Case study 15 (see p. 922 for full caption).

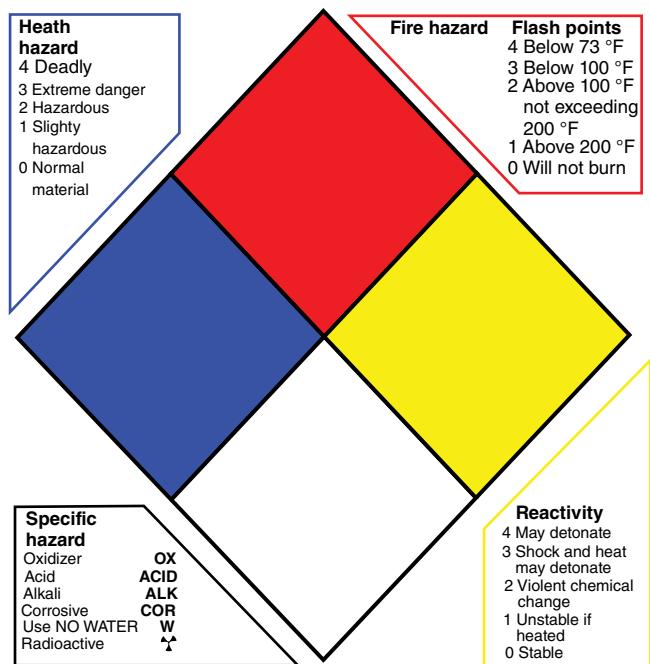


Figure 20.6 Four-color hazard identification symbol (see p. 983 for full caption).

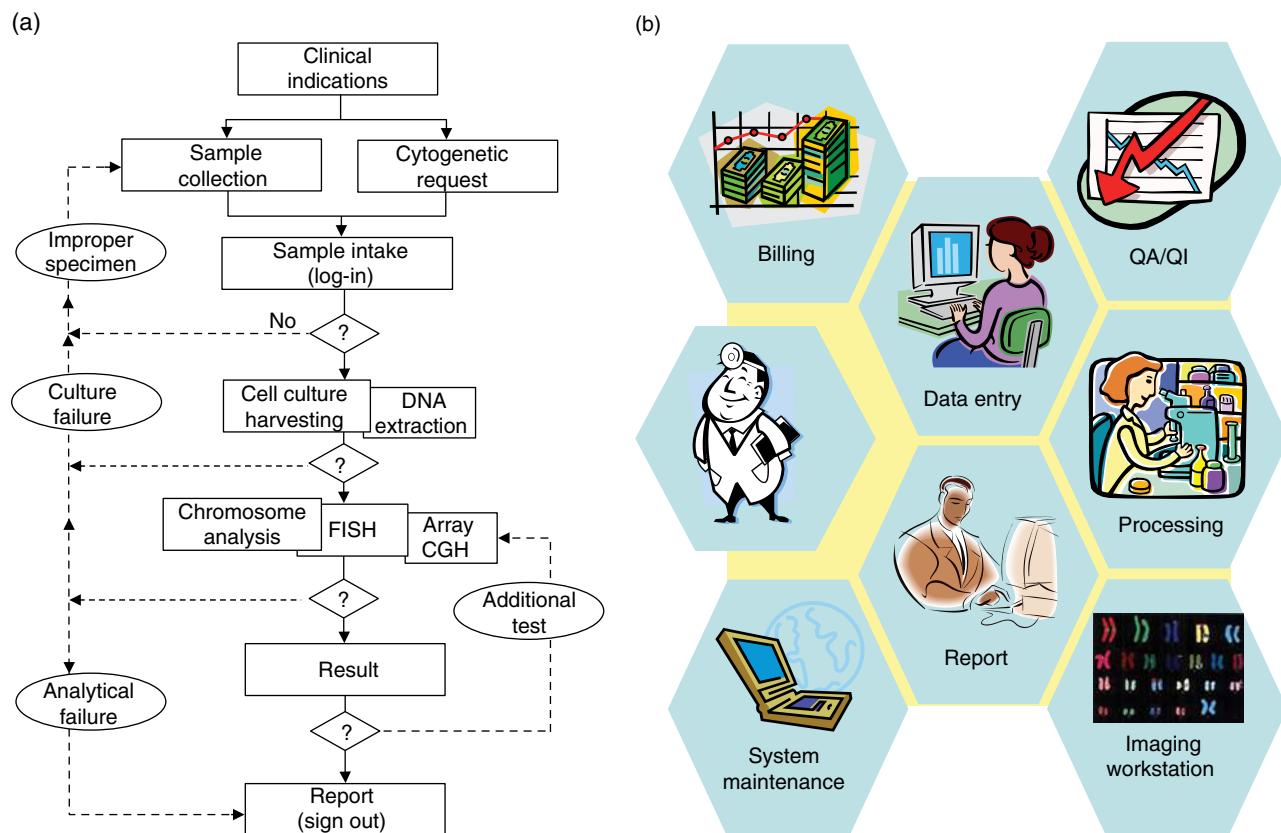


Figure 23.1 Flowchart of a cytogenetic lab (see p. 1046 for full caption).

(b)



(c)



Figure 24.2 Soemmerring's Gazelle (see p. 1058 for full caption).

(b)

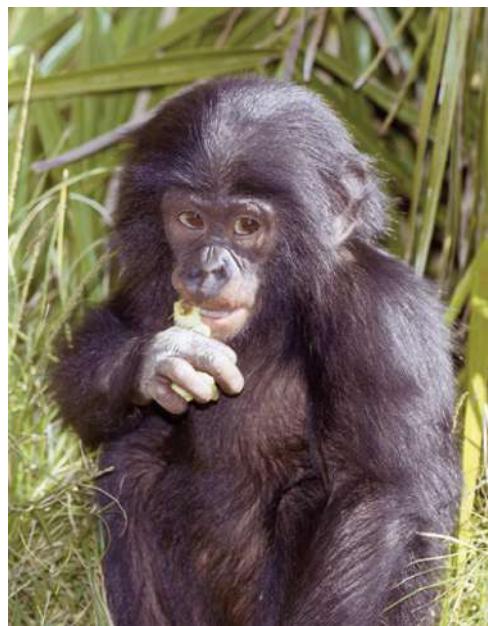


Figure 24.4 (b) Bonobo (see p. 1060 for full caption).



Figure 24.6 (b) Parma wallaby (see p. 1065 for full caption).



Figure 24.9 Photographs are Suni antelope, *Neotragus moschatus* (see p. 1068 for full caption).

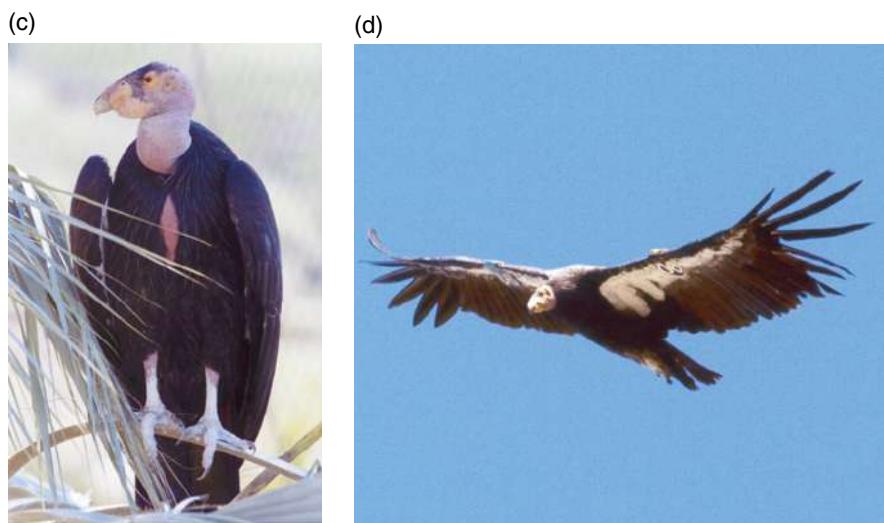


Figure 24.10 California condor (see p. 1069 for full caption).

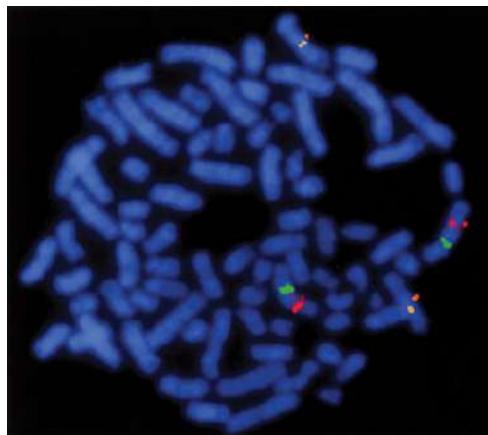


Figure 24.12 BACs from three horse genes. (see p. 1071 for full caption).



Figure 24.13 Blood feathers (see p. 1079 for full caption).