

VeraCode®

Assay Guide

FOR RESEARCH USE ONLY



ILLUMINA PROPRIETARY
Catalog # VC-901-1001
Part # 11312819 Rev. B

illumina®

Notice

This document and its contents are proprietary to Illumina, Inc. and its affiliates ("Illumina"), and are intended solely for the contractual use of its customer in connection with the use of the product(s) described herein and for no other purpose. This document and its contents shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way whatsoever without the prior written consent of Illumina. Illumina does not convey any license under its patent, trademark, copyright, or common-law rights nor similar rights of any third parties by this document.

The instructions in this document must be strictly and explicitly followed by qualified and properly trained personnel in order to ensure the proper and safe use of the product(s) described herein. All of the contents of this document must be fully read and understood prior to using such product(s).

FAILURE TO COMPLETELY READ AND EXPLICITLY FOLLOW ALL OF THE INSTRUCTIONS CONTAINED HEREIN MAY RESULT IN DAMAGE TO THE PRODUCT(S), INJURY TO PERSONS, INCLUDING TO USERS OR OTHERS, AND DAMAGE TO OTHER PROPERTY.

ILLUMINA DOES NOT ASSUME ANY LIABILITY ARISING OUT OF THE IMPROPER USE OF THE PRODUCT(S) DESCRIBED HEREIN (INCLUDING PARTS THEREOF OR SOFTWARE) OR ANY USE OF SUCH PRODUCT(S) OUTSIDE THE SCOPE OF THE EXPRESS WRITTEN LICENSES OR PERMISSIONS GRANTED BY ILLUMINA IN CONNECTION WITH CUSTOMER'S ACQUISITION OF SUCH PRODUCT(S).

FOR RESEARCH USE ONLY

© 2010 Illumina, Inc. All rights reserved.

Illumina, illuminaDx, Solexa, Making Sense Out of Life, Oligator, Sentrix, GoldenGate, GoldenGate Indexing, DASL, BeadArray, Array of Arrays, Infinium, BeadXpress, VeraCode, IntelliHyb, iSelect, CSPro, GenomeStudio, Genetic Energy, HiSeq, and HiScan are registered trademarks or trademarks of Illumina, Inc. All other brands and names contained herein are the property of their respective owners.

Oligonucleotide capture sequences © 2003 Illumina, Inc. All rights reserved.

Illumina customers may reproduce the oligonucleotide capture sequences in the Universal Capture Bead Sets but only in connection with Illumina arrays and assays. All other uses are strictly prohibited. No portion of the oligonucleotide sequences may be published without the prior approval of Illumina.

Revision History

Part Number and Revision	Date
Catalog # VC-901-1001 11312819 Rev. B	March 2010
Catalog # VC-901-1001 11312819 Rev. A	May 2008
Catalog # VC-901-1001 11281021 Rev. A	December 2007
11220990 Rev. A	May 2007
11220990 Beta	December 2006

Table of Contents

Notice	iii
Revision History	v
Table of Contents	vii
List of Figures.....	xi
List of Tables	xv
Chapter 1 Overview	1
Introduction	2
GoldenGate Genotyping Assay for VeraCode.....	3
GoldenGate Methylation Assay for VeraCode.....	3
DASL Gene Expression Assay for VeraCode	3
Multiplex Genotyping with VeraCode Universal Oligo Beads.....	4
Multiplex Protein and Nucleic Acid Assays with VeraCode Carboxyl Beads .	4
BeadXpress Reader System and VeraCode Assays Workflow	5
The BeadXpress Reader.....	5
GenomeStudio Data Analysis Software	6
Technical Assistance	7
.....	8
Chapter 2 Standard Operating Procedures	9
Introduction	10
General Safety Statement	10
Acronyms	10
Prevent PCR Product Contamination.....	14
Standard Equipment, Materials, and Reagents	17
General Lab Setup	21
Perform Automated Protocols with the Tecan 8-Tip Robot.....	23
Chapter 3 GoldenGate Genotyping Assay for VeraCode Manual Protocols	35
Introduction	36
Workflow	38
Tracking Tools	39
Materials and Reagents for this Assay	42
Make DNA Quantitation Plate (Optional)	44

Read QDNA Plate (Optional)	50
Make Single-Use DNA (SUD) Plate	54
Precipitate SUD Plate	56
Resuspend SUD Plate	58
Make Allele-Specific Extension (ASE) Plate	59
Add Master Mix for Extension & Ligation (MEL)	61
Make PCR Plate	64
Inoculate PCR Plate	66
Thermal Cycle PCR Plate	69
Bind PCR Products	70
Make Intermediate Plate for VeraCode Bead Plate	72
Hybridize VeraCode Bead Plate	75
Wash VeraCode Bead Plate	77
Scan VeraCode Bead Plate	78
Troubleshooting	79

Chapter 4 GoldenGate Genotyping Assay for VeraCode Automated Protocols 83

Introduction	84
Workflow	85
Equipment, Materials, and Reagents for This Assay	86
Make Standard DNA Plate (Optional)	89
Make DNA Quantitation Plate (Optional)	92
Read QDNA (Optional)	96
Make Single-Use DNA (SUD) Plate	98
Precipitate SUD Plate	102
Resuspend SUD Plate	106
Make Allele-Specific Extension (ASE) Plate	109
Add Extension and Ligation Reagents (MEL)	112
Make PCR Plate	120
Inoculate PCR Plate	123
Thermal Cycle PCR Plate	127
Bind PCR Products	128
Make VeraCode Bead Plate	131
Hybridize VeraCode Bead Plate	135
Wash VeraCode Bead Plate	136
Scan VeraCode Bead Plate	139

Chapter 5 DASL Gene Expression Assay for VeraCode Protocols . . 141

Introduction	142
Workflow	143
Tracking Tools	144
Materials and Reagents for this Assay	147
Make RNA Quantitation Plate (Optional)	149
Read QRNA Plate (Optional)	154
Make Single-Use RNA (SUR) Plate	158
Make Assay Specific Extension (ASE) Plate	160
Add Master Mix for Extension & Ligation (MEL)	162
Make PCR Plate	165
Inoculate PCR Plate	167

Thermal Cycle PCR Plate	169
Bind PCR Products	170
Make Intermediate Plate for VeraCode Bead Plate	172
Hybridize VeraCode Bead Plate	175
Wash VeraCode Bead Plate	177
Scan VeraCode Bead Plate	178
Troubleshooting	179
Chapter 6 GoldenGate Methylation Assay for VeraCode Protocols . 183	
Introduction	184
Workflow	185
Tracking Tools	186
Sample Sheet	187
Materials and Reagents for this Assay	189
Make DNA Quantitation Plate (Optional)	191
Read QDNA Plate (Optional)	196
Make Bisulfite-Converted DNA (BCD) Plate	200
Make Bisulfite-Converted Single-Use DNA (BCS) Plate	204
Precipitate BCS Plate	206
Resuspend BCS Plate	208
Make Allele-Specific Extension (ASE) Plate	209
Add Master Mix for Extension & Ligation (MEL)	211
Make PCR Plate	214
Inoculate PCR Plate	216
Thermal Cycle PCR Plate	218
Bind PCR Products	219
Make Intermediate Plate for VeraCode Bead Plate	221
Hybridize VeraCode Bead Plate	224
Wash VeraCode Bead Plate	226
Scan VeraCode Bead Plate	227
Chapter 7 Bead Kitting . 229	
Introduction	230
Materials for this Assay	230
Kit VeraCode Beads	231
Store Kitted VeraCode Beads	242
Clean the VeraCode Bead Kitting System	242
Chapter 8 Universal Oligo Beads Example Protocol . 243	
Introduction	244
Materials and Reagents for this Assay	246
Universal Oligo Bead Sets	248
Design PCR and ASPE Primers	256
Match ASPE Primers to VeraCode Capture Sequences	258
Contamination and Controls	260
Two-Plate Protocol for Low-Plex Genotyping	261
Single-Plate Protocol for Low-Plex Genotyping	266
Troubleshooting	271

Chapter 9	Carboxyl Beads Example Protocols	275
Introduction	276	
Equipment, Materials, and Reagents for this Assay	277	
One-Step Carbodiimide Coupling of Amine-Terminated Oligos to Carboxyl VeraCode Beads	280	
Two-Step Protein Immobilization to Carboxyl VeraCode Beads	282	
Quantitation and Manual Bead Kitting	285	
Multiplex Cytokine Reagent Preparation	288	
Multiplex Cytokine Protein Assay	289	
Troubleshooting	292	
Appendix A	GoldenGate Genotyping Assay for VeraCode Controls . . .	295
Introduction	296	
View the Control Graphs	296	
VeraCode Bead Types and IllumiCode Sequence IDs	297	
Allele-Specific Extension Controls	298	
PCR Uniformity	299	
Gender Controls	300	
Extension Gap Control (U3 & U5 Match)	302	
First Hybridization Controls	302	
Second Hybridization Controls	303	
Contamination Detection Controls	304	
Appendix B	DASL Gene Expression Assay for VeraCode Controls . . .	307
Introduction	308	
View the Control Graphs	308	
Control IllumiCode Sequence IDs	309	
Negative Controls	310	
Extension Gap Control	311	
First Hybridization Controls	311	
Second Hybridization Controls	312	
Contamination Detection Controls	313	
Appendix C	GoldenGate Methylation Assay for VeraCode Controls . . .	317
Introduction	318	
View Control Oligos	318	
Control IllumiCode Sequence IDs	319	
Allele-Specific Extension Controls	320	
Bisulfite-Conversion Controls	321	
Gender Controls	322	
Extension Gap Control (U3 and U5 Match)	323	
First Hybridization Controls	324	
Second Hybridization Controls	325	
Negative Controls	326	
Contamination Detection Controls	327	

List of Figures

Chapter 1	Overview	1
Figure 1	Workflow for BeadXpress Reader and VeraCode Assays	5
Chapter 2	Standard Operating Procedures	9
Figure 2	Securing Plates to Vortexer Platform with Velcro Straps.....	21
Figure 3	Robot Control User Interface.....	23
Figure 4	Robot Bleach Wash	27
Figure 5	8-Tip Robot QC Task	29
Chapter 3	GoldenGate Genotyping Assay for VeraCode Manual Protocols	35
Figure 6	Oligo Configuration.....	36
Figure 7	GoldenGate Genotyping Assay for VeraCode Workflow	38
Figure 8	GoldenGate Genotyping Assay for VeraCode Lab Tracking Form	39
Figure 9	VeraCode Sample Sheet	41
Figure 10	MIDI Plate Wells.	45
Figure 11	QDNA Plate with Serial Dilutions of Lambda DNA.....	46
Figure 12	Standard QDNA Plate with PicoGreen	48
Figure 13	Sample QDNA Plate with PicoGreen.	48
Figure 14	Load the PicoGreen Protocol in SoftMax Pro	50
Figure 15	Select Lambda Standards Screen	51
Figure 16	Read Standard QDNA Plate	51
Figure 17	View Standard Curve	52
Figure 18	Read Sample QDNA Plate.....	52
Figure 19	Avoid Tip Contamination.....	62
Figure 20	Apply Label to Filter Plate	70
Figure 21	Assemble Filter Plate	73
Chapter 4	GoldenGate Genotyping Assay for VeraCode Automated Protocols	83
Figure 22	Automated GoldenGate Genotyping Assay for VeraCode Workflow	85
Figure 23	MIDI Plate Wells.	90
Figure 24	DNA Plate Selection Dialog Box (MIDI Selected)	93
Figure 25	GTS Pre-PCR Tasks DNA Prep Make QDNA	94
Figure 26	GTS Fluorometry Analysis Main Screen	96
Figure 27	Number of Sample QDNA Plates	96
Figure 28	Sample QDNA Data	97
Figure 29	GTS Pre-PCR Tasks Single Use DNA Make SUD	99

Figure 30	Robot Bed for Make SUD.....	100
Figure 31	GTS Pre-PCR Tasks Single Use DNA Precip SUD.....	103
Figure 32	Robot Bed for Precip SUD.....	104
Figure 33	GTS Pre-PCR Tasks Single Use DNA Resuspend SUD.....	107
Figure 34	Robot Bed for Resuspend SUD	108
Figure 35	GTS Pre-PCR Tasks Single Use DNA Make SUD ASE	110
Figure 36	Robot Bed for Make ASE	111
Figure 37	GTS Pre-PCR Tasks Biochem Add MEL.....	113
Figure 38	Robot Bed for Add MEL.....	114
Figure 39	First Move ASE Plate Message	115
Figure 40	Bed Map After First Plate Move	115
Figure 41	First Remove and Vortex ASE Plate Message	115
Figure 42	Second Move ASE Plate Message.....	116
Figure 43	Bed Map After Second Plate Move.....	116
Figure 44	Third Move ASE Plate Message.....	117
Figure 45	Bed Map After Third Plate Move.....	117
Figure 46	Second Remove and Vortex ASE Plate Message	117
Figure 47	Fourth Move ASE Plate Message	118
Figure 48	Bed Map After Fourth Plate Move.....	118
Figure 49	Final Vortex and Incubate Message.....	118
Figure 50	GTS Pre-PCR Tasks Biochem Make PCR	121
Figure 51	Robot Bed for Make PCR.....	122
Figure 52	GTS Pre-PCR Tasks Biochem Inoc PCR	124
Figure 53	Robot Bed for Inoc PCR.....	125
Figure 54	Inoc PCR Vortex and Incubate Message	125
Figure 55	VBP Post-PCR Tasks Bind PCR.....	129
Figure 56	Robot Bed for Bind PCR.....	130
Figure 57	VBP Post-PCR Tasks Make HYB VBP	132
Figure 58	Robot Bed for Make VeraCode Bead Plate	133
Figure 59	VBP Post-PCR Tasks Wash VBP	137
Figure 60	Robot Bed for Wash VBP	138

Chapter 5 DASL Gene Expression Assay for VeraCode Protocols . . 141

Figure 61	DASL Gene Expression Assay for VeraCode Laboratory Workflow.....	143
Figure 62	DASL Gene Expression Assay for VeraCode Lab Tracking Form.....	144
Figure 63	VeraCode Sample Sheet	146
Figure 64	Dilution of Ribosomal RNA Standard	150
Figure 65	Ribosomal QRNA Serial Dilutions	151
Figure 66	Standard QRNA Plate with RiboGreen	152
Figure 67	Sample QRNA Plate with RiboGreen.....	153
Figure 68	Load the Illumina QRNA Protocol in SoftMax Pro	154
Figure 69	Select the Standard RNA Screen.....	155
Figure 70	Read the Standard QRNA Plate.....	155
Figure 71	View Standard Curve	156
Figure 72	Read the Sample QRNA Plate	157
Figure 73	Make SUR.....	158
Figure 74	Avoid Tip Contamination.....	163
Figure 75	Apply Label to Filter Plate	170
Figure 76	Assemble Filter Plate	173

Chapter 6	GoldenGate Methylation Assay for VeraCode Protocols .	183
Figure 77	GoldenGate Methylation Assay for VeraCode Workflow.....	185
Figure 78	GoldenGate Methylation Assay for VeraCode Lab Tracking Form	186
Figure 79	VeraCode Sample Sheet	188
Figure 80	MIDI Plate Wells.....	192
Figure 81	QDNA Plate with Serial Dilutions of Lambda DNA.....	193
Figure 82	Standard QDNA Plate with PicoGreen	194
Figure 83	Sample QDNA Plate with PicoGreen.....	195
Figure 84	Load the PicoGreen Protocol in SoftMax Pro	196
Figure 85	Select Lambda Standards Screen	197
Figure 86	Read Standard QDNA Plate	197
Figure 87	View Standard Curve	198
Figure 88	Read Sample QDNA Plate.....	198
Figure 89	Bisulfite Conversion	200
Figure 90	Bisulfite Conversion Workflow	201
Figure 91	Activate Bisulfite-Converted Single-Use DNA.....	204
Figure 92	Avoid Tip Contamination.....	212
Figure 93	Apply Label to Filter Plate	219
Figure 94	Assemble Filter Plate	222
Chapter 7	Bead Kitting.....	229
Figure 95	VeraCode Bead Kitting System	232
Figure 96	VeraCode Bead Kitting System, Deep Reservoir Down	233
Figure 97	Place Rectangular Gasket into Deep Reservoir	233
Figure 98	Add Kitting Buffer	234
Figure 99	Transfer Beads	235
Figure 100	Add Funnel Plate	235
Figure 101	Press Gasket onto Funnel Plate	236
Figure 102	Put Plate on Gasket	236
Figure 103	Close and Latch VeraCode Bead Kitting System	237
Figure 104	Shake VeraCode Bead Kitting System.....	238
Figure 105	Flip VeraCode Bead Kitting System.....	238
Figure 106	Tap VeraCode Bead Kitting System	239
Figure 107	Open VeraCode Bead Kitting System	240
Figure 108	Remove Funnel Plate and Gasket from Deep Reservoir	240
Figure 109	Remove Plate from Deep Reservoir.....	241
Chapter 8	Universal Oligo Beads Example Protocol.....	243
Figure 110	PCR, ASPE Reaction, Hybridization	245
Figure 111	Unwanted PCR Products from Poorly-Designed ASPE Primers.....	257
Figure 112	PCR Gel, ASPE Gel	272
Chapter 9	Carboxyl Beads Example Protocols	275
Figure 113	Multiplex Cytokine Protein Assay.....	290
Appendix A	GoldenGate Genotyping Assay for VeraCode Controls ..	295
Figure 114	ASE Controls	298
Figure 115	PCR Uniformity Controls (U3).....	299

Figure 116	PCR Uniformity Controls (U5)	299
Figure 117	Gender Controls (Set 1)	300
Figure 118	Gender Controls (Set 2)	301
Figure 119	Extension Gap Control (U3 & U5 Match)	302
Figure 120	First Hybridization Controls	302
Figure 121	Second Hybridization Controls	303
Figure 122	Contamination-Free Environment	304
Figure 123	Contaminated Environment without UDG Treatment	305
Figure 124	Contaminated Environment with UDG Treatment.	306
Appendix B	DASL Gene Expression Assay for VeraCode Controls . . .	307
Figure 125	Extension Gap Control	311
Figure 126	First Hybridization Controls (Low T_m)	311
Figure 127	First Hybridization Controls (High T_m)	311
Figure 128	Second Hybridization Controls	312
Figure 129	Contamination-Free Environment	314
Figure 130	Contaminated Environment without UDG Treatment	315
Figure 131	Contaminated Environment with UDG Treatment.	316
Appendix C	GoldenGate Methylation Assay for VeraCode Controls . . .	317
Figure 132	Allele-Specific Extension Controls	320
Figure 133	Bisulfite Conversion Controls.	321
Figure 134	Gender Controls Oligo Configuration (G6PD)	322
Figure 135	Gender Controls Oligo Configuration (ELK1)	322
Figure 136	Extension Gap Control Oligo Configuration	323
Figure 137	First Hybridization (Low T_m) Controls Oligo Configuration	324
Figure 138	First Hybridization (High T_m) Controls Oligo Configuration	324
Figure 139	Second Hybridization Controls Oligo Configuration	325
Figure 140	Contamination-Free Environment	327
Figure 141	Contaminated Environment Without UDG Treatment.	328
Figure 142	Contaminated Environment With UDG Treatment	329

List of Tables

Chapter 1	Overview	1
Table 1	Illumina General Contact Information	7
Table 2	Illumina Customer Support Telephone Numbers	7
Chapter 2	Standard Operating Procedures	9
Table 3	Acronyms	10
Table 4	User-Supplied Equipment	17
Table 5	Illumina-Supplied Equipment	18
Table 6	User-Supplied Materials	19
Table 7	Illumina-Supplied Materials	20
Table 8	User-Supplied Reagents	20
Table 9	Vortexer Calibration Speeds	22
Table 10	Robot Control Software User Interface	24
Chapter 3	GoldenGate Genotyping Assay for VeraCode Manual Protocols	35
Table 11	BeadArray Reader vs. BeadXpress Reader Assays	37
Table 12	Sample Sheet Guidelines	39
Table 13	User-Supplied Reagents	42
Table 14	Illumina-Supplied Materials and Reagents	42
Table 15	Box Contents	43
Table 16	Concentration of Lambda DNA Standards	46
Table 17	QDNA Plate Reagent Volumes	47
Table 18	Thermocycler Program	69
Table 19	Problems Observed During DNA Sample Preparation	79
Table 20	Hyb VBP	79
Table 21	Problems with Signal Intensity	80
Table 22	Problems Observed During Analysis	80
Chapter 4	GoldenGate Genotyping Assay for VeraCode Automated Protocols	83
Table 23	Illumina-Supplied Equipment	86
Table 24	Illumina-Supplied Materials	86
Table 25	VeraCode Box Contents	87
Table 26	User-Supplied Reagents	88
Table 27	Concentration of Lambda DNA Standards	90
Table 28	PicoGreen Reagent Volumes	93
Table 29	Thermocycler Program	127

Chapter 5	DASL Gene Expression Assay for VeraCode Protocols . . . 141	
Table 30	Sample Sheet Guidelines.....	144
Table 31	User-Supplied Reagents.....	147
Table 32	Illumina-Supplied Materials and Reagents.....	147
Table 33	Box Contents	148
Table 34	Concentration of Ribosomal RNA Standards.....	150
Table 35	QRNA Plate Reagent Volumes.....	151
Table 36	Thermocycler Program.....	169
Table 37	Problems Observed During RNA Sample Preparation	179
Table 38	Problems Observed During Hyb VBP	180
Table 39	Problems with Signal Intensity	180
Table 40	Problems Observed During Analysis	181
Chapter 6	GoldenGate Methylation Assay for VeraCode Protocols 183	
Table 41	Sample Sheet Guidelines.....	187
Table 42	User-Supplied Materials and Reagents	189
Table 43	Illumina-Supplied Materials and Reagents.....	189
Table 44	Box Contents	190
Table 45	Concentration of Lambda DNA Standards	192
Table 46	QDNA Plate Reagent Volumes	194
Table 47	Thermocycler Program.....	218
Chapter 7	Bead Kitting 229	
Table 48	Materials for Kitting VeraCode Beads	230
Table 49	Buffers for VeraCode Bead Types	231
Chapter 8	Universal Oligo Beads Example Protocol 243	
Table 50	User-Supplied Materials.....	246
Table 51	User-Supplied Reagents.....	246
Table 52	Illumina-Supplied Reagents	247
Table 53	VeraCode Bead Codes for Individual Universal Oligo Bead Sets	248
Table 54	VeraCode Bead Codes for Pooled Universal Oligo Bead Sets	252
Table 55	Factor V	259
Table 56	Factor II	259
Table 57	MTHFR 667	259
Table 58	MTHFR 1298	259
Table 59	Two-Plate Protocol, PCR Master Mix	262
Table 60	Two-Plate Protocol, SAP/EXO Master Mix.....	262
Table 61	Two-Plate Protocol, ASPE Master Mix	263
Table 62	Single-Plate Protocol, PCR Master Mix	267
Table 63	Single-Plate Protocol, SAP/EXO Master Mix	267
Table 64	Single-Plate Protocol, ASPE Master Mix	268
Table 65	Optimization Reaction	271
Chapter 9	Carboxyl Beads Example Protocols 275	
Table 66	User-Supplied Equipment	277
Table 67	Quantitation and Kitting	277
Table 68	One-Step Carbodiimide Coupling.....	277

Table 69	Two-Step Protein Immobilization.....	278
Table 70	Multiplex Cytokine Protein Assay.....	278
Table 71	VeraCode Carboxyl Bead Sets.....	279
Table 72	Antibody Immobilization, Total Volume	282
Table 73	Dilution of Sulfo-NHS.....	283
Table 74	Dilution of EDC	283
Table 75	High Background	292
Table 76	No Signal	292
Table 77	Too Much Signal	293
Table 78	Low or Flat Standard Curve	293
Table 79	Poor Replicates	293
Table 80	Poor Reproducibility.....	294
Table 81	No Signal in Samples, Standard Curve Fine	294
Table 82	Sample Values too High, Standard Curve Fine	294
Appendix A	GoldenGate Genotyping Assay for VeraCode Controls .	295
Table 83	VeraCode Bead Types and IllumiCode Sequence IDs.....	297
Appendix B	DASL Gene Expression Assay for VeraCode Controls . .	307
Table 84	Control IllumiCode Sequence IDs	309
Table 85	Negative Control IllumiCodes	310
Appendix C	GoldenGate Methylation Assay for VeraCode Controls .	317
Table 86	Used IllumiCode Sequence IDs	319
Table 87	Negative Controls IllumiCodes	326

Chapter 1

Overview

Topics

- 2 Introduction
- 3 GoldenGate Genotyping Assay for VeraCode
- 3 GoldenGate Methylation Assay for VeraCode
- 3 DASL Gene Expression Assay for VeraCode
- 4 Multiplex Genotyping with VeraCode Universal Oligo Beads
- 4 Multiplex Protein and Nucleic Acid Assays with VeraCode Carboxyl Beads
- 5 BeadXpress Reader System and VeraCode Assays Workflow
- 5 The BeadXpress Reader
- 6 GenomeStudio Data Analysis Software
- 7 Technical Assistance

Introduction

The Illumina® BeadXpress® Reader suite is a highly efficient and cost-effective SNP genotyping system that includes:

- ▶ The BeadXpress Reader
- ▶ VeraCode® Bead Plates
- ▶ VeraCode Universal Oligo Beads
- ▶ VeraCode Carboxyl Beads
- ▶ GoldenGate® Assays for Genotyping, Methylation, and DASL® Gene Expression (96- and 384-plex)

Illumina's VeraCode technology and BeadXpress Reader System leverage the power of digital holographic codes to provide a robust detection method for multiplex bioassays requiring high precision, accuracy, and speed. VeraCode is a technology solution that grows with your needs and remains relevant during dynamic changes in your research pursuits. The advantages of this system include:

- ▶ **High Data Quality**—Industry-leading measurement density and sensitivity due to inherent stringency of code detection.
- ▶ **Broad Multiplexing Capability**—Using a patented digital holographic coding technology, the BeadXpress Reader System enables a broad range of multiplexing. Assays ranging from single-plex to 384-plex per sample can be performed from a single well of a standard 96-well VeraCode Bead Plate.
- ▶ **Use of Codes for Increased Quality Metrics**—Bead codes can be used in the assay as identifiers for internal controls, as well as for unique identifiers such as reagent lots, test kits, and sample ID.
- ▶ **Assay Versatility**—A broad range of applications, including genotyping, gene expression, and protein-based assays can be performed on a single platform.
- ▶ **Dual-Color Laser Detection**—The dual-color laser detection of the BeadXpress Reader enables ultimate flexibility in assay design. You can run assays using either two-color detection (e.g., the GoldenGate Assay) or single-color detection (ASPE) on this platform.

To support the broadest range of applications and multiplexing needs, Illumina has developed the following products for the BeadXpress Reader System:

- ▶ The BeadXpress Reader
- ▶ GoldenGate Genotyping Assay for VeraCode, with optional automation
- ▶ GoldenGate Methylation Assay for VeraCode
- ▶ DASL Gene Expression Assay for VeraCode
- ▶ VeraCode Universal Oligo Beads
- ▶ VeraCode Carboxyl Beads
- ▶ The VeraCode Test and Calibration Kit
- ▶ The VeraCode Bead Kitting System
- ▶ BeadXpress Read Buffer

GoldenGate Genotyping Assay for VeraCode

Illumina combines the proven GoldenGate Genotyping Assay with cutting-edge VeraCode technology to deliver one of the most robust systems for SNP genotyping in the industry. Ideally suited for those interested in biomarker validation or creation of custom assay panels, you can now achieve 96 and 384 multiplexing within a single well of a standard microplate. This assay uses an oligo-directed detection method that results in fluorescent products which are hybridized to VeraCode beads, then scanned on the BeadXpress Reader.

Chapter 3, *GoldenGate Genotyping Assay for VeraCode Manual Protocols*, explains how to perform the GoldenGate Assay. Chapter 4, *GoldenGate Genotyping Assay for VeraCode Automated Protocols* shows how to perform the assay using a Tecan 8-tipped robot.

GoldenGate Methylation Assay for VeraCode

The GoldenGate Methylation Assay for VeraCode provides a novel, high-throughput DNA methylation analysis method. Researchers can study how methylation patterns affect the regulation of gene expression in development, differentiation, and diseases such as multiple sclerosis, diabetes, schizophrenia, aging, and cancers. Like the other GoldenGate-based VeraCode assays, the GoldenGate Methylation Assay for VeraCode provides 96- and 384-multiplexing.

Chapter 6, *GoldenGate Methylation Assay for VeraCode Protocols* explains how to perform the Methylation Assay.

DASL Gene Expression Assay for VeraCode

The DASL Gene Expression Assay for VeraCode combines Illumina's proven DASL Gene Expression Assay with VeraCode technology to deliver an extremely robust system for high-throughput, custom 96- and 384-plex gene expression analysis. This assay can be used on both intact and degraded RNA samples, such as those isolated from formalin-fixed, paraffin-embedded (FFPE) samples.

Chapter 5, *DASL Gene Expression Assay for VeraCode Protocols*, explains how to perform the DASL Assay.

Multiplex Genotyping with VeraCode Universal Oligo Beads

Illumina's VeraCode Universal Oligo Bead Sets provide flexibility in the development of multiplex SNP genotyping assays, methylation assays, and DASL gene expression assays. With these highly stable, uniquely coded bead sets that are pre-coupled with capture oligonucleotides, you can develop your own assays based on your desired multiplex and preferred assay methodology.

Chapter 8, *Universal Oligo Beads Example Protocol*, describes one of the many assay design possibilities using VeraCode Universal Oligo Beads.

Multiplex Protein and Nucleic Acid Assays with VeraCode Carboxyl Beads

You can explore a diverse range of bioassay applications using VeraCode Carboxyl Beads. These bead sets enable covalent attachment of proteins, peptides, nucleic acid, and other ligands in a highly multiplexed format that can save time, money, and precious samples. VeraCode Carboxyl Beads are highly stable. Simple immobilization chemistry enables rapid assay design for a variety of analytes, and provides a truly open platform for laboratory-developed tests.

Chapter 9, *Carboxyl Beads Example Protocols*, provides an example of one of the many protocols that can be used with VeraCode Carboxyl Beads.

BeadXpress Reader System and VeraCode Assays Workflow

Figure 1 illustrates the basic workflow of the BeadXpress Reader System and VeraCode Assays, and identifies the user guide that provides guidance for each step.

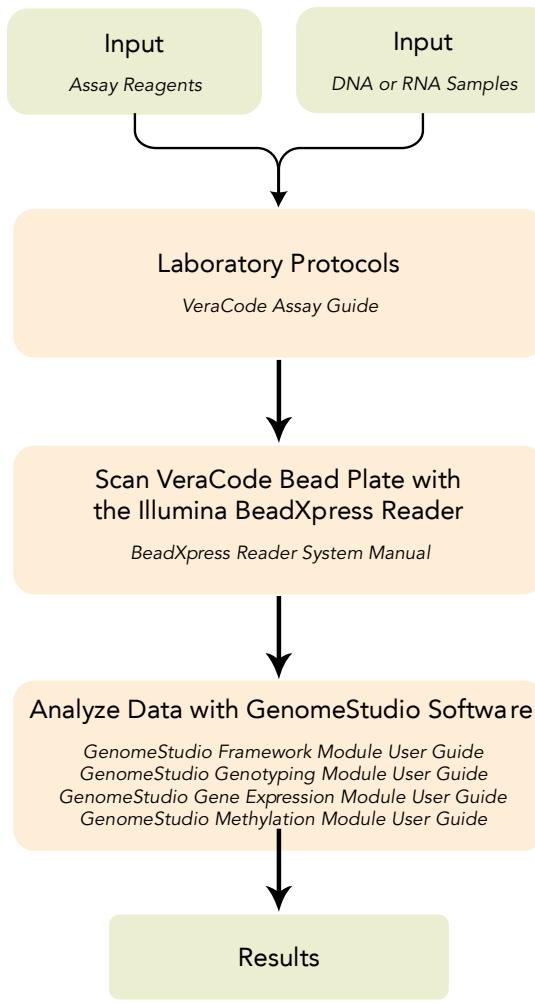


Figure 1 Workflow for BeadXpress Reader and VeraCode Assays

The BeadXpress Reader

Illumina's BeadXpress Reader is a two-channel, 30 µm-resolution non-confocal laser scanner. It scans well plates containing VeraCode beads at two wavelengths and creates intensity files for downstream analysis. As fluorescence data in two colors (corresponding to the two possible alleles present at each SNP locus) are collected, intensity values are determined for each bead type.

For information about scanning well plates with the BeadXpress Reader, see the *BeadXpress Reader System Manual*.

GenomeStudio Data Analysis Software

GenomeStudio software has multiple modules, each one optimized for a particular type of data analysis. Depending on which VeraCode assay you perform, you can use the Genotyping, Gene Expression, or Methylation module.

Genotyping Module

The GenomeStudio Genotyping Module enables you to determine and edit the genotype cluster locations of the AA, AB, and BB clusters for each locus of your custom SNP assay. You can save this information by creating a cluster file (*.egt file) containing the genotype cluster locations for a specific oligo pool. Using the cluster file and your BeadXpress Reader intensity data files (*.idat files), the GenomeStudio Genotyping Module makes genotyping calls and stores them in text file format.

For more information, see the *GenomeStudio Genotyping Module User Guide*.

Gene Expression Module

The GenomeStudio Gene Expression Module enables you to execute two types of data analysis:

- ▶ **Gene Analysis**—Determining gene expression signal levels and whether gene expression is detected.
- ▶ **Differential Analysis**—Determining if gene expression levels have changed between two experimental stages.

You can perform these analyses on individual arrays or on groups of arrays treated as replicates.

For more information, see the *GenomeStudio Gene Expression Module User Guide*.

Methylation Module

The GenomeStudio Methylation Module allows you to perform two types of data analysis:

- ▶ **Methylation Analysis**—Quantifying methylation levels.
- ▶ **Differential Methylation Analysis**—Determining whether methylation levels have changed between a reference group and another experimental group.

You can perform these analyses on individual samples, or on groups of samples treated as replicates.

For more information, see the *GenomeStudio Methylation Module User Guide*.

Framework

GenomeStudio provides numerous tools for exploratory analysis, including:

- ▶ Line plots
- ▶ Bar graphs
- ▶ Scatter plots
- ▶ Histograms
- ▶ Dendograms
- ▶ Box plots
- ▶ Heat maps
- ▶ Control summary reports

Also included are the following powerful data visualization and analysis tools:

- ▶ Illumina Genome Viewer (IGV)
- ▶ Illumina Chromosome Browser (ICB)

For a detailed description of how to use the GenomeStudio Framework (the common elements of the GenomeStudio graphical user interface), see the *GenomeStudio Framework User Guide*.

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 1 Illumina General Contact Information

Illumina Website	http://www.illumina.com
Email	techsupport@illumina.com

Table 2 Illumina Customer Support Telephone Numbers

Region	Contact Number
North America toll-free	1.800.809.ILMN (1.800.809.4566)
United Kingdom toll-free	0800.917.0041
Germany toll-free	0800.180.8994
Netherlands toll-free	0800.0223859
France toll-free	0800.911850

Table 2 Illumina Customer Support Telephone Numbers (Continued)

Other European time zones	+44.1799.534000
Other regions and locations	1.858.202.ILMN (1.858.202.4566)

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at <http://www.illumina.com/msds>.

Product Documentation

If you require additional product documentation, you can obtain PDFs from the Illumina website. Go to <http://www.illumina.com/documentation>. When you click on a link, you will be asked to log in to iCom. After you log in, you can view or save the PDF.

If you do not already have an iCom account, then click **New User** on the iCom login screen and fill in your contact information. Indicate whether you wish to receive the iCommunity newsletter (a quarterly newsletter with articles about, by, and for the Illumina Community), illumiNOTES (a monthly newsletter that provides important product updates), and announcements about upcoming user meetings. After you submit your registration information, an Illumina representative will create your account and email login instructions to you.

Chapter 2

Standard Operating Procedures

Topics

- 10 Introduction
- 10 General Safety Statement
- 10 Acronyms
- 14 Prevent PCR Product Contamination
- 17 Standard Equipment, Materials, and Reagents
- 21 General Lab Setup
- 23 Perform Automated Protocols with the Tecan 8-Tip Robot

Introduction

This chapter describes the standard operating procedures associated with Illumina VeraCode Assays. All other chapters in this guide assume that you have read and are familiar with the contents of this chapter.



CAUTION

Strict regard for the prevention of PCR product contamination is required during all VeraCode assays.

General Safety Statement



CAUTION

Please study and follow all governmental and facility safety standards applicable to your site.

To obtain the latest MSDS for the VeraCode Assays, contact Illumina Technical Support. For contact information, see *Technical Assistance* on page 7.

Acronyms

Table 3 Acronyms

Acronym	Definition	Where Used (Plates and Reagents Only)
AM1	Add MEL 1 (reagent)	Removing excess and non-specifically hybridized oligos in <i>Add Master Mix for Extension and Ligation (MEL)</i> protocol in all VeraCode assays.
ASE	Allele-Specific Extension (plate)	<i>Make Allele-Specific Extension (ASE) Plate, Make PCR Plate, and Inoculate PCR Plate</i> protocols in all VeraCode assays.
ASO	Allele-Specific Oligo	
BCD	Bisulfite-Converted DNA (plate)	<i>Make Bisulfite-Converted DNA (BCD) Plate and Make Bisulfite-Converted Single-Use DNA (BCS) Plate</i> protocols in the GoldenGate Methylation Assay for VeraCode.

Table 3 Acronyms (Continued)

Acronym	Definition	Where Used (Plates and Reagents Only)
BCS	Bisulfite-Converted Single-Use DNA (plate)	<i>Make Bisulfite-Converted Single-Use DNA (BCS) Plate, Precipitate BCS Plate, Resuspend BCS Plate, and Make Allele-Specific Extension (ASE) Plate</i> in the GoldenGate Methylation Assay for VeraCode.
BSA	Bovine Serum Albumin	Assays and bead-kitting procedures using carboxyl beads (Chapter 7, <i>Bead Kitting</i> and Chapter 9, <i>Carboxyl Beads Example Protocols</i>).
DAP	DASL Assay Pool (reagent)	Custom oligo pool used in the <i>Make Allele-Specific Extension (ASE) Plate</i> protocol in the DASL Gene Expression Assay for VeraCode.
DASL	cDNA-Mediated Annealing, Selection, Extension, and Ligation	
EDTA	Ethylenediamine Tetraacetic Acid	<i>Make Single-Use DNA (SUD) Plate</i> protocol in the manual GoldenGate Genotyping Assay for VeraCode. <i>Make Quantitated DNA (QDNA) Plate</i> and <i>Make Single-Use DNA (SUD) Plate</i> protocols in the automated GoldenGate Genotyping Assay for VeraCode. <i>Single-Plate Protocol ASPE</i> and <i>Two-Plate Protocol ASPE</i> in the Universal Oligo Beads Example Protocol.
gDNA	Genomic DNA	
GTS	Genotyping System	
GT	Genotyping	
Hyb	Hybridization	
INT	Intermediate Plate	<i>Make Intermediate Plate for VeraCode Bead Plate</i> and <i>Hybridize VeraCode Bead Plate</i> protocols in all manual VeraCode assays. <i>Make VeraCode Bead Plate</i> protocol in the automated GoldenGate Genotyping Assay for VeraCode.
IP1	Inoc PCR 1 (reagent)	Eluting extended and ligated products in the <i>Inoculate PCR Plate</i> protocol for all VeraCode assays.
LSO	Locus-Specific Oligo	
MCS	Master Mix cDNA Synthesis for Single Use (reagent)	Activating DNA in the <i>Make Single-Use RNA (SUR) Plate</i> protocol for the DASL Gene Expression Assay for VeraCode.
MEL	Master Mix for Extension/Ligation (reagent)	Add <i>Master Mix for Extension and Ligation (MEL)</i> protocol in all VeraCode assays.

Table 3 Acronyms (Continued)

Acronym	Definition	Where Used (Plates and Reagents Only)
MH2	Make HYB 2 (reagent)	Making the VeraCode Bead Plate in the <i>Make Intermediate Plate for VeraCode Bead Plate and Hybridize VeraCode Bead Plate</i> protocols in all manual VeraCode assays. Used to make the VeraCode Bead Plate in the <i>Make VeraCode Bead Plate</i> protocol in the automated GoldenGate Genotyping Assay for VeraCode.
MMP	Master Mix for PCR (reagent)	Providing biotinylated and fluorophore-labeled primers for the <i>Make PCR Plate</i> protocol in all VeraCode assays.
MPB	Paramagnetic Particles B (reagent)	Binding double-stranded PCR products in the <i>Bind PCR Products</i> protocol in all VeraCode assays.
MS1	Make SUD 1 (reagent)	Activating DNA in the <i>Make Single-Use DNA (SUD) Plate</i> protocol in the manual and automated GoldenGate Genotyping Assay for VeraCode, and in the <i>Make Bisulfite-Converted Single-Use DNA (BCS) Plate</i> protocol in the GoldenGate Methylation Assay for VeraCode.
OB1	Oligo Hybridization and DNA Binding Buffer 1 (reagent)	Hybridizing oligos and binding cDNA and gDNA in the <i>Make Allele-Specific Extension (ASE) Plate</i> protocol in all VeraCode assays.
OMA	Oligo Methylation Pool All (reagent)	Custom oligo pool used in the GoldenGate Methylation Assay for VeraCode.
OPA	Oligo Pool All (reagent)	Custom oligo pool used in the manual and automated GoldenGate Genotyping Assay for VeraCode.
PBS	Phosphate Buffered Saline	Assays and bead-kitting procedures using carboxyl beads (Chapter 7, <i>Bead Kitting</i> and Chapter 9, <i>Carboxyl Beads Example Protocols</i>).
PBS-BSA	Phosphate Buffered Saline + Bovine Serum Albumin	
PBST	Phosphate Buffered Saline + Tween 20	
PCR	Polymerase Chain Reaction (plate)	<i>Make PCR Plate, Inoculate PCR Plate, Thermal Cycle PCR Plate, and Bind PCR Products</i> protocols in all VeraCode assays.
PMPs	Paramagnetic Particles	
PS1	Precipitation Solution 1 (reagent)	Precipitating DNA in the manual and automated GoldenGate Genotyping Assay for VeraCode and the GoldenGate Methylation Assay for VeraCode.

Table 3 Acronyms (Continued)

Acronym	Definition	Where Used (Plates and Reagents Only)
QDNA	Quantitated DNA (plate)	<i>Make DNA Quantitation Plate, Read QDNA Plate, and Make Single-Use DNA (SUD) Plate</i> protocols in the manual and automated GoldenGate Genotyping Assay for VeraCode. <i>Make DNA Quantitation Plate, Read QDNA Plate, and Make Bisulfite-Converted DNA (BCD) Plate</i> protocols the GoldenGate Methylation Assay for VeraCode.
QRNA	Quantitated RNA (plate)	<i>Make RNA Quantitation Plate and Read QRNA Plate</i> protocols in the DASL Gene Expression Assay for VeraCode.
RFU	Relative Fluorescent Levels	
RS1	Resuspension Solution 1 (reagent)	Resuspending precipitated DNA in the manual and automated GoldenGate Genotyping Assay for VeraCode and the GoldenGate Methylation Assay for VeraCode.
SUD	Single-Use DNA (plate)	<i>Make Single-Use DNA (SUD) Plate, Precipitate SUD Plate, Resuspend SUD Plate, and Make Allele-Specific Extension (ASE) Plate</i> protocols in the manual and automated GoldenGate Genotyping Assay for VeraCode.
SUR	Single-Use RNA (plate)	<i>Make Single-Use RNA (SUR) Plate and Make Allele-Specific Extension (ASE) Plate</i> protocols in the DASL Gene Expression Assay for VeraCode.
UB1	Universal Buffer 1 (reagent)	Washing paramagnetic beads in the Add Master Mix for Extension and Ligation (MEL) and Inoculate PCR Plate protocols in all VeraCode assays.
UB2	Universal Buffer 2 (reagent)	Washing magnetic particles in the <i>Make Intermediate Plate for VeraCode Bead Plate</i> protocol in all manual VeraCode assays, and in the <i>Make VeraCode Bead Plate</i> protocol in the automated GoldenGate Genotyping Assay for VeraCode.
UDG	Uracil DNA Glycosylase (reagent)	Optional reagent for degrading post-PCR products contaminating the PCR process in the <i>Make PCR Plate</i> protocol in all VeraCode assays.
VBP	VeraCode Bead Plate	<i>Hybridize VeraCode Bead Plate, Wash VeraCode Bead Plate, and Scan VeraCode Bead Plate</i> protocols in all VeraCode assays. Also used in the <i>Make VeraCode Bead Plate</i> protocol in the automated GoldenGate Genotyping Assay for VeraCode.
VR1	VeraCode Read Buffer (reagent)	Used for the BeadXpress Reader. Not used in any VeraCode assays.
VW1	VeraCode Wash Buffer (reagent)	Washing VeraCode beads in the <i>Wash VeraCode Bead Plate</i> protocol in all VeraCode assays.
xg	Multiple of gravitational acceleration	

Prevent PCR Product Contamination

The PCR (polymerase chain reaction) process is commonly used in the laboratory to amplify specific DNA sequences.

Unless you exercise sufficient caution, PCR products may contaminate reagents, instrumentation, and samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.



You must establish procedures for preventing PCR product contamination before you begin work in the lab.

The following sections outline practices that help reduce the risk of PCR product contamination.

Physically Separate Pre- and Post-PCR Areas

The laboratory space where pre-PCR processes (DNA extraction, quantitation, and normalization) are performed should be physically separate from the laboratory space where PCR products are made and processed (post-PCR processes).

Ideally, pre-PCR processes should be performed in a separate, dedicated laboratory space.

General rules:

- Never use the same sink to wash pre- and post-PCR reservoirs
- Never share the same water purification system for pre- and post-PCR processes
- Store all supplies used in the protocols in the pre-PCR area, and transfer to the post-PCR area as needed

Provide Dedicated Equipment and Supplies

Dedicate separate, full sets of instruments (pipettes, centrifuges, ovens, heat blocks, etc.) to the pre- and post-PCR lab processes, and never share the instruments between processes.

Perform Daily and Weekly Bleaching

Use the following guidelines for daily and weekly bleaching of the Post-PCR and Pre-PCR areas. Train personnel responsible for this activity about how to prevent PCR product contamination.

Daily Pre-PCR Bleaching

Establish a regular daily and weekly bleaching schedule for the pre-PCR area. This helps eliminate PCR product that may have entered the pre-PCR area.



To prevent sample or reagent degradation, ensure that all bleach vapors that remain after cleaning have fully dissipated before starting any processes.

Identify high-risk pre-PCR items such as the ones listed below. Clean these items with a 10% bleach solution each morning before beginning any pre-PCR processes:

- ▶ Bench tops
- ▶ Door handles
- ▶ Refrigerator and freezer door handles
- ▶ Computer mouse and keyboard

Daily Post-PCR Bleaching

Daily and weekly bleaching help reduce the risk of PCR contamination by controlling the amount of PCR product in the post-PCR area.



To prevent sample or reagent degradation, ensure that all bleach vapors that remain after cleaning have fully dissipated before starting any processes.

Identify post-PCR area “hot spots” that pose the highest risk of contamination, and clean these items daily with a 10% bleach solution. These hot spots include, but may not be limited to:

- ▶ Thermocyclers
- ▶ Bench space used to process amplified DNA
- ▶ Door handles
- ▶ Refrigerator and freezer door handles
- ▶ Computer mouse and keyboard

Weekly Bleaching for All Lab Areas

Once a week, perform a thorough bleaching of the pre-PCR and post-PCR areas. Mop floors with a 10% bleach solution, and clean all bench tops and instruments that are not cleaned daily.

Correctly Handle Items That Fall to the Floor

The pre-PCR floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-PCR area; therefore, anything that has fallen to the floor should be treated as contaminated.

Throw away any disposable items that fall to the floor, such as empty tubes, pipette tips, gloves, lab coat hangers, etc.

Non-disposable items that fall to the floor (such as a pipette, an important sample container, etc.) should be immediately and thoroughly cleaned with a 10% bleach solution to remove PCR product contamination.



Be sure to clean any lab surface with which a contaminated item has come into contact.

Individuals handling anything that has fallen to the floor, disposable or not, must throw away their lab gloves and put on a new pair.

Uracil DNA Glycosylase (Optional)

You may choose to add UDG (Uracil DNA Glycosylase) to the PCR master mix to help prevent PCR product contamination.

The PCR master mix delivered with the VeraCode kits contains a balanced mixture of the following items:

- ▶ Universal PCR primers
- ▶ PCR buffer
- ▶ dUTP
- ▶ dATP
- ▶ dGTP
- ▶ dCTP

The dUTP is incorporated into the post-PCR products. UDG targets dUTP for specific degradation in subsequent PCR reactions, thus reducing the chance for PCR products to contaminate the Pre-PCR products.

Detect PCR Product Contamination

The oligo pools include internal controls to help determine whether contamination has occurred. PCR contamination detection controls are divided into four types, and only a single type is added to each oligo pool tube. When a single oligo pool is run, only one contamination control type will have high signal. Should two or more contamination control types have high signal, significant contamination may have occurred. For more information about contamination controls, see:

- ▶ Appendix A, *GoldenGate Genotyping Assay for VeraCode Controls*
- ▶ Appendix B, *DASL Gene Expression Assay for VeraCode Controls*
- ▶ Appendix C, *GoldenGate Methylation Assay for VeraCode Controls*

Do Not Reuse Reagents

Never reuse excess reagents. Discard excess reagents according to your facility requirements.

Standard Equipment, Materials, and Reagents

All assays run on a BeadXpress Reader system require the following equipment, materials, and reagents. Remember to maintain separate stocks for pre- and post-PCR areas.

Individual assays usually have additional requirements. These requirements are identified at the start of each chapter.

Equipment User-Supplied

Table 4 User-Supplied Equipment

Item	Source
Lab coats	General lab supplier
Protective gloves	General lab supplier
Safety glasses	General lab supplier
Tube vortexer	General lab supplier
Stopwatch/timer	General lab supplier
[Optional] Single-channel precision pipettes (10 µl and 200 µl)	General lab supplier
8-channel precision pipettes (5 µl to 200 µl)	General lab supplier
[Optional] 12-channel precision pipettes (5 µl to 200 µl)	General lab supplier
1000 µl pipette	General lab supplier
Cap mat applicator	Corning #3081
[Optional] Foil Stripper	ABgene catalog # AB-0592
Microtiter plate centrifuge with g-force range of 8–3000 xg	General lab supplier
[Optional] Fluorometer	Gemini XS or XPS (Molecular Devices)
Vacuum flask assembly (flask, stopper, tubing, and vacuum source)	General lab supplier
Vacuum regulator	Qiagen catalog # 19530
96-well thermocycler with heated lid	Available as part of the optional GoldenGate Accessory Kit for BeadXpress

Illumina-Supplied

Table 5 Illumina-Supplied Equipment

Item	Catalog #
BeadXpress Reader System <ul style="list-style-type: none"> • BeadXpress Reader (110V or 220V) • Reagent carrier • Reagent and waste bottles • USB cable, Type A-B, 1.0 meter • Detachable AC line cord, 2.0.1 • PC workstation with monitor • <i>BeadXpress Reader System Manual</i> • <i>VeraCode Assay Guide, Experienced User Cards (EUCs), and Lab Tracking Forms (LTFs)</i> • BeadXpress Reader System CD • BeadXpress Starter Kit (110V or 220V) 	VC-101-1000 (110V) VC-101-1001 (220V)
BeadXpress Reader Starter Kit <ul style="list-style-type: none"> • VeraCode Bead Kitting System • VeraCode Vortex Incubator (110V or 220V) • 8-pin vacuum manifold 	VC-120-1000 (110V, included with VC-101-1000) VC-120-1001 (220V, included with VC-101-1001)
GoldenGate Satellite Kit for BeadXpress Reader <ul style="list-style-type: none"> • Microplate shaker (110V or 220V) • High-temperature loop fastener • Nylon hook • Raised-bar magnet • Heat block with microtubes block • Digital optical stroboscope • Combi heat sealer (110V or 220V) • 96-well base adapter 	VC-120-1200 (110V) VC-120-1201 (220V)
[Optional] GoldenGate Accessory Kit for BeadXpress <ul style="list-style-type: none"> • Refrigerated benchtop centrifuge (110V or 220V) • Horizontal M4 rotor • Microplate carrier for M4 rotor • Conical inserts 9x15 ml, set of 4 • 750 ml bucket, set of 4 • 96-well thermocycler with heated lid (110V or 220V) • Alpha unit module, 96V-well 	VC-120-1300 (110V) VC-120-1301 (220V)

Materials User-Supplied

Table 6 User-Supplied Materials

Item	Source
96-well, black, flat-bottom Fluotrac 200 plates	Greiner, catalog # 655076
MultiScreen filter plates, 0.45 µM, clear, Styrene	Millipore, catalog # MAHV-N45 10/50
Corning Costar* brand Polypropylene 96-well, V-bottom plates, cap mats not included	Fisher Scientific, catalog # 07-200-695 (Corning # 3363)
Microseal 96-well skirted polypropylene microplates, 8x12 well array or Thermo-Fast 96 skirted microplates	MJ Research, catalog # MSP-9601 ABgene, catalog # AB-0800
96-well cap mats	Corning, Fisher Scientific, catalog # 07-200-744
Heat sealing foil sheets (thermoseal)	ABgene, catalog # AB-0559
Aluminum adhesive seals	VWR, catalog # BK538619
2mil Sealplate clear adhesive film, non-sterile	Phenix Research Products, catalog # LMT-SEAL-EX
Microseal "A" film (PCR plate sealing film) (Check your thermocycler to find out what type of sealing film it requires, and use that type.)	MJ Research, catalog # MSA-5001
Centrifuge tubes (50 ml and 15 ml)	Corning catalog # 430828 and # 430055
Conical tubes (15 ml)	VWR, catalog # 21008-103
Non-sterile solution basins, 55 ml	Labcor Products, Inc., catalog # 730-001 VWR, catalog # 21007-970
Sterile plastic container, minimum 100 ml capacity	General lab supplier
Serological pipettes (10, 25, and 50 ml)	General lab supplier
Aerosol filter pipette tips, (5 µl to 200 µl)	General lab supplier
Aluminum foil	General lab supplier
Absorbent pads	General lab supplier

Illumina-Supplied

This section lists Illumina-supplied materials that you must have in order to perform VeraCode assays. You must have one of the VeraCode Assay kits (96- or 384-plex), the BeadXpress System Buffer kit, and the VeraCode Test and Calibration kit.

Table 7 Illumina-Supplied Materials

Item	Catalog #
BeadXpress Read Buffer (500 ml VR1 buffer, 10X, used in the BeadXpress Reader)	VC-400-1001
VeraCode Test and Calibration Kit (performed monthly—12-month supply)	VC-321-1000

Reagents User-Supplied

Table 8 User-Supplied Reagents

Item	Source
10 mM Tris-HCL pH 8.0, 1 mM EDTA	General lab supplier
1X TE	General lab supplier
2-propanol	General lab supplier
Titanium Taq DNA polymerase	Clontech, catalog # 639220
0.1N NaOH (sodium hydroxide)	Sigma-Aldrich, catalog # S0899
Reagent alcohol (90% ethanol, 5% methanol, 5% isopropanol)	General lab supplier
Deionized H ₂ O	General lab supplier
10% bleach (plain, unscented household bleach)	General lab supplier

General Lab Setup

Calibrate the Vortexer

Follow the instructions below to calibrate the microplate shaker.



The displayed speed of the vortexer may vary from the actual speed. BeadXpress Reader recommends using a digital stroboscope to determine the actual vortex speed. Once you have determined the actual vortex speed, record it along with the displayed speed and use these measurements for reference throughout the assay. Check the vortexer speed periodically.

1. Replace top tray of the vortexer (used to secure the plate) with three Velcro straps for securing 96-well plates.
2. Cut six two-inch lengths of adhesive-backed Velcro hooks. Attach these hooks to the underside of the bottom tray of the shaker platform.
3. Cut three 20-inch lengths of Velcro loops. Use these as straps to secure the plates onto the vortexer platform (Figure 2).



Figure 2 Securing Plates to Vortexer Platform with Velcro Straps

4. Set the digital stroboscope display speed to 1600 rpm.
5. Turn the vortexer on and set it to 1600 rpm. Adjust the vortexer speed until the actual vortex speed as indicated on the digital stroboscope reaches 1600 rpm.
6. Record the vortexer display speed.
7. Use the method described above to determine the displayed speed for the actual vortex speed of 1800 rpm, 2000 rpm, and 2300 rpm. These four vortex speeds are used in the VeraCode Assays.
8. Place an Illumina-provided label on the vortexer with the calibration information. Table 9 lists the vortexer display speeds and actual speeds reflected on the Illumina-provided label.

Table 9 Vortexer Calibration Speeds

Display Speed	Actual Vortex Speed
1450 rpm	1600 rpm
1625 rpm	1800 rpm
1800 rpm	2000 rpm
1975 rpm	2300 rpm

**NOTE**

The protocols always refer to the actual vortex speed, not the displayed value.

Balance the Centrifuge

Whenever you centrifuge plates, place a balance plate opposite each plate being centrifuged.

Prepare Multichannel Pipettes

Ensure that multichannel (8-channel) pipettes are properly calibrated, clean, and decontaminated. Use close-fitting pipette tips to control the dispensing volume.

Apply Barcode Labels to Plates

As a convention, apply barcode labels to the right side of the plate (column #12 end of the plate).

Prepare for Sample Tracking

You are responsible for tracking:

- ▶ The position of samples in the plates. Lab tracking forms are provided for this purpose on your Documentation CD.
- ▶ Appropriate information on your Sample Sheet. Sample sheet templates are included on the Documentation CD.

Prepare Fewer than 96 Samples

Each reagent tube supplied with your Illumina BeadXpress Reader System and assay kit contains sufficient volume to process 96 samples at once, using an 8-channel pipette and a reservoir.

When processing smaller sample batches (fewer than 96 samples) using a reagent reservoir, dead volume and pipetting error losses can increase. To ensure accurate reagent volume for all samples, single-pipette reagent into each well.

To store remaining reagent, Illumina recommends freezing aliquots, rather than repeatedly freezing and thawing the supplied reagent tube.

Perform Automated Protocols with the Tecan 8-Tip Robot

Follow the instructions in this section to use and maintain the Tecan 8-Tip Robot:

- ▶ *Robot Control Software* on page 23
- ▶ *Prepare the Robot for the First Use of the Day* on page 26
- ▶ *Prepare the Robot Before Each Use* on page 28
- ▶ *Perform Quality Control* on page 28
- ▶ *Clean the Robot* on page 30
- ▶ *Liquid-Handling Automation Notes* on page 32

Note that not all assays have optional automation.

Robot Control Software

This section explains how to use the GTS robot control software. Figure 3 shows the main screen.

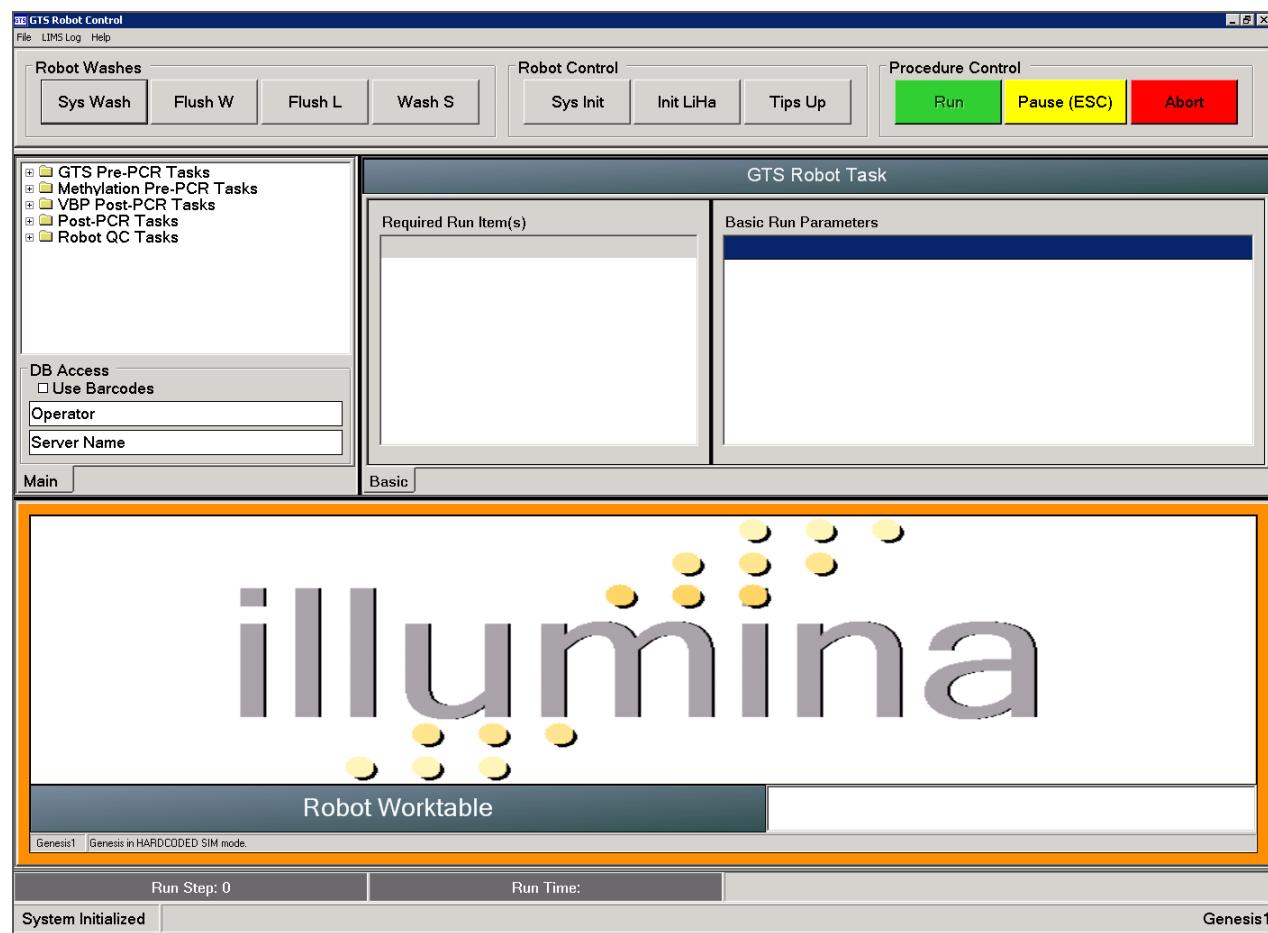


Figure 3 Robot Control User Interface

Table 10 lists each part of the GTS robot control software main screen and explains how to use it.

Table 10 Robot Control Software User Interface

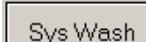
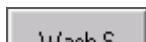
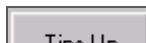
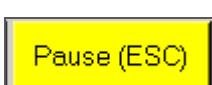
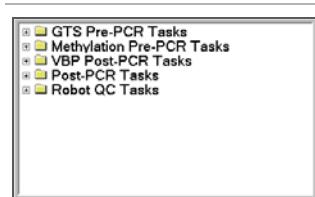
Screen Element	Function
Robot Washes Pane	
	<p>Click to start a complete flush of system fluids:</p> <ul style="list-style-type: none"> • Daily, per robot maintenance schedule (please refer to the documentation that came with the robot) • When changing system fluid • If bubbles are present in the lines • Per instructions in individual laboratory protocols
	<p>Click to flush a large amount of water through the robot tips over the robot waste station:</p> <ul style="list-style-type: none"> • If a robot process was stopped unexpectedly, and you want to flush the tips completely before proceeding • To validate the robot fast-wash pump operation
	<p>Click to flush the robot tips in the “long wash” station (tips are deeply immersed):</p> <ul style="list-style-type: none"> • If the robot tips were immersed in a deep receptacle (deep-well plate, tube, etc.) during a robot process that was stopped unexpectedly, and you want to flush the tips completely before proceeding
	<p>Click to move the robot diluters up and down and wash the robot tips in the short-wash station (tips are immersed to a shallow depth):</p> <ul style="list-style-type: none"> • To remove air bubbles that still remain following a flush wash • To validate the robot diluters’ operation
Robot Control Pane	
	Click to initialize the system at the beginning of each day.
	<p>Click to initialize the liquid handling system (moves to its home position):</p> <ul style="list-style-type: none"> • If a robot process was stopped unexpectedly, and you want to re-initialize the LiHa robot arm
	<p>Click to move the robot tips to their “up” position:</p> <ul style="list-style-type: none"> • When you need to lift the robot tips to the maximum height without re-initializing the LiHa robot arm • During selected robot maintenance tasks
Procedure Control Pane	
	After selecting a robot procedure from the expandable list of options at the left, entering basic run parameters, and setting up the robot bed per the robot bed map, click Run to start the selected robot process. If you are <i>not</i> using LIMS, clear the Use Barcodes checkbox first.
	Should it become necessary to interrupt the robot run for any reason, click Pause on the robot PC (or press the Esc keyboard key). The button changes to Continue . When ready to resume the run, click Continue .
	

Table 10 Robot Control Software User Interface (Continued)

Screen Element	Function
	Should it become necessary to abort the robot run for any reason, click Abort on the robot PC. The button changes to Restart . When you are ready to restart the run, click Restart .
	

Procedures Pane

To select a robot procedure, expand the appropriate task area in the pane on the left, and then select the procedure.

DB Access pane

Clear the **Use Barcodes** checkbox before starting any procedure. The barcodes feature only applies to systems running LIMS, which is not available for VeraCode.

Robot Task Pane

The Required Run Items pane displays the items needed to run the selected procedure. The fields in the Basic Run Parameters pane are automatically filled with the default values for the selected process. You may change these values per individual laboratory protocols.

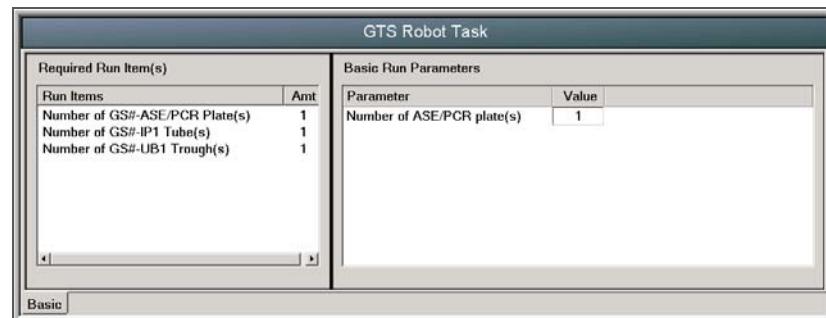


Table 10 Robot Control Software User Interface (Continued)

Screen Element	Function
Robot Bed Map	
<p>After you have accepted or changed the basic run parameters for the selected procedure, the robot bed map shows where to place the various plates, tubes, and reservoirs on the robot bed.</p> <ul style="list-style-type: none"> • Reagent Tubes appear as color-coded dots at the left, corresponding to their positions in the tube rack. The reagent associated with each color is listed in the lower right corner of the pane. • Reagent Reservoirs (quarter, half, or full) appear as lettered boxes to the right of the reagent tubes. The reagent for each reservoir is also listed in the lower right corner of the pane. • Plates are located to the right of the reservoirs, and are color-coded to distinguish them from each other. Each plate shows the plate type (e.g., 96 TCY for a 96-well TCY plate, and 96 FBLK for a 96-well black Fluotrac plate) and the plate name (ASE, PCR, etc.). • Numbers along the bottom of the bed map show the positions of the items. For example, the green ASE plates in the image below are in position 14 and the grey PCR plates are in position 20. 	

Prepare the Robot for the First Use of the Day

Before using the robot for the first time each day, perform the procedures described in this section.



Do not place your hands on or near the robot bed while the robot is running.

1. Reboot the robot PC.
2. From the robot PC desktop, open the Robot Programs folder.
3. Launch the GTS Robot Control program.
The robot takes a few moments to initialize.
4. Check the system fluid level and add fluid if necessary.



If adding fluid, do so *before* the Bleach Wash step.

5. Select **QC Tasks | Robot Bleach Wash** (Figure 4).

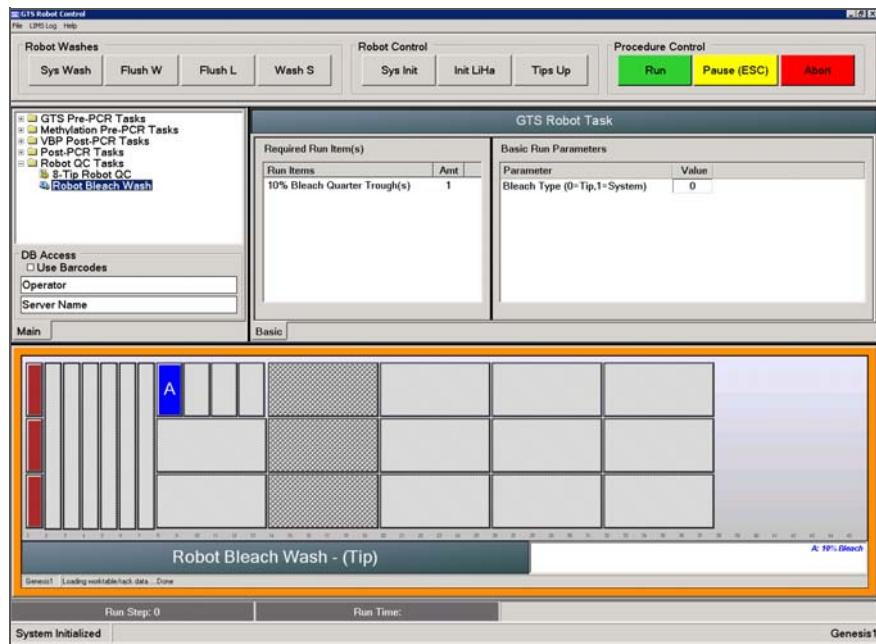


Figure 4 Robot Bleach Wash

6. In the Basic Run Parameters pane, enter "0" for a tip wash or "1" for a system wash.
7. Click **Run**.
8. If you selected a system wash ("1"), follow these steps:
 - a. When prompted, swap out the System Liquid carboy for a 10% commercial bleach solution. When you have done this, click **OK**. The robot initiates a bleach soak procedure.
 - b. When prompted, swap out the bleach solution for the System Liquid carboy. Click **OK**. The robot continues the system flush.
 - c. Observe the lines for air bubbles.
 - d. Repeat the system flush process until the lines are completely free of air bubbles.



CAUTION

To prevent contamination, ensure all bleach vapors have fully dissipated before starting any process involving samples.

9. If you selected a tip wash ("0"), place a quarter reservoir with 5 ml of 10% commercial bleach solution in position A of the reservoir frame. Click **OK**. The robot initiates the tip wash. When the bleach procedure is complete, the robot returns to the main robot task screen.

Prepare the Robot Before Each Use

After initializing the robot at the start of the day, follow this procedure every time you are about to use the robot. However, if this is the first time the robot is being used today, follow the steps described under *Prepare the Robot for the First Use of the Day* on page 26 instead.



Do not place your hands on or near the robot bed while the robot is running.

1. If the robot has already been used today, you only need to perform a system flush. Click **Sys Wash**.
2. Observe the lines for air bubbles.
3. Repeat the system wash process until the lines are completely free of air bubbles.
4. Observe the robot tips for any dripping.
5. During the fast wash cycle, observe robot tips as they dispense system liquid. Liquid should dispense in a straight stream. All tips should dispense liquid in equal volumes and at equal velocities.



If you observe any irregularities, do not proceed. Contact the lab manager.

Perform Quality Control

Perform regular robot QC procedures ensure that the liquid handling system causes minimal variation in the assay.

You should perform robot QC tasks:

- As part of a regular robot maintenance schedule
- After any robot or robot tips mechanical problem has been resolved

There are two robot QC tests:

► **The individual-column dispense test** (Ind-Col Dispense Plate)

In this test, eight robot tips dispense reagent to the plate one column at a time. Ind-Col Dispense Plate tests for three volumes. The tips are washed after reagent has been dispensed to all columns.

► **The multi-column dispense test** (Multi-Col Dispense Plate)

In this test, a large volume of reagent is dispensed multiple times over the entire plate (i.e., multiple times per six columns). Multi-Col Dispense Plate tests for two volumes. The tips are washed after reagent has been dispensed to six columns.

Select both tests for a thorough robot QC.

Test Volume Accuracy of the Robot Tips

1. Label a new Fluotrac 200 plate "Ind-Col Dispense."
2. Label another new Fluotrac 200 plate "Multi-Col Dispense."

3. Select Robot QC Tasks | 8-Tip Robot QC (Figure 5).

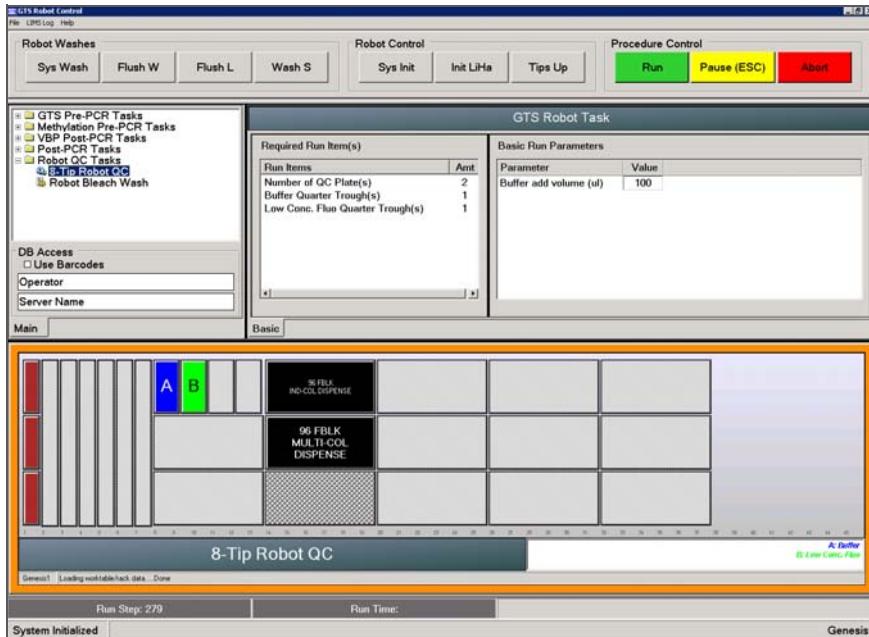


Figure 5 8-Tip Robot QC Task

4. Add 35 ml fluorescein dilution buffer to a quarter reservoir. Place the reservoir in position A of the reservoir frame, as shown on the robot bed map (Figure 5).
5. Add 6 ml low concentration fluorescein (0.025 mg/ml) to a quarter module reservoir and place in position B, as shown on the robot bed map.



Different reagent volumes will apply if you select fewer than three tests.

6. Place the Fluotrac 200 plates on the robot bed according to the robot bed map.
7. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
8. Click **Run**. The robot conducts an internal QC process. A message in the lower status bar indicates when this is complete.
9. Immediately cover the Fluotrac 200 plates with aluminum foil.
10. If you plan to analyze the 8-Tip Robot QC data in the post-PCR area, transfer the Fluotrac 200 plates to the post-PCR area or to the area where the fluorometer resides.
11. Once the robot QC tests have all been successfully completed, dispose of any remaining reagents in accordance with your facility requirements.

12. Select QC Tasks | Robot Bleach Wash.**CAUTION**

To prevent fluorescein from contaminating sample solutions, you must perform this bleach wash step after every robot QC testing.

13. Place a quarter reservoir with 5 ml of 10% bleach in position A of the reservoir frame.**14. Click Run.**

When the bleach procedure is complete, the robot returns to the main robot task screen.

15. When the tip bleach process is complete, click **Sys Flush.****16. Observe the lines for air bubbles.****17. Repeat the system flush process at least **three times**, until the lines are completely free of air bubbles.****Clean the Robot****Clean the Outer Surface of the Robot Tips (Weekly)**

Perform this activity once a week.

Required Materials:

- Kimwipes
- Ethanol

1. At the beginning of each day, check for any leaks.
2. In the robot software, click **Sys Init**.
3. Fold a Kimwipe in half lengthwise.
4. Using a squeeze bottle, soak the folded Kimwipe with 70% ethanol.
5. In the robot software, click **Tips Up**.
6. Fold the Kimwipe around the robot tip.
7. Starting from one end of the tip row, wipe each tip gently along the entire lower half of the tip.
8. Reverse the Kimwipe to use the other, fresh side.
9. Starting from the other end of the tip row, wipe each tip a second time along the entire lower half of the tip.
10. Clean the waste station with the Kimwipe.
11. If needed, clean the waste station a second time.

Bleach-Bathe the Robot Carriers (Daily)

Perform this activity daily.

Required Materials:

- Bleach

- DiH₂O
 - Two soaking trays or standard dishwashing tubs (24" L x 18" W x 6" D)
 - Absorbent bench underpad
1. Prepare a 10% bleach bath (~500 ml concentrated bleach in 4500 ml DiH₂O) in one of the soaking trays.
 2. Fill the other soaking tray with DiH₂O.
 3. Lay out at least two rows of absorbent bench underpad on a benchtop.
 4. Remove the robot carriers from the robot bed. Note the carriers' original positions, so that you can replace them correctly.
 5. Submerge the carriers in the prepared bleach solution for about 1 minute.
 6. Remove the carriers from the bleach solution and submerge them in the DiH₂O soaking tray for about 1 minute.
 7. Remove the carriers from the DiH₂O soaking tray and rinse them under running water in the sink.
 8. Dry the carriers on the absorbent bench underpad.
 9. Allow carriers to dry on the underpad overnight before returning them to their proper positions on the robot bed.

**CAUTION**

To prevent contamination, ensure all bleach vapors have fully dissipated before starting any process involving samples.

Liquid-Handling Automation Notes

This section provides general notes for automating the liquid handling processes.

Design an Automated Process Flow

Clearly design a process flow and name each component of that process. The process flow charts at the start of each protocol chapter may be helpful as a guide.

Prepare Reagents

If your liquid-handling system cannot aspirate from a tube, pour reagents into reservoirs or microtiter plates.

Set Robot Tip Height

For accurate reagent dispensing, set your robot tip height so that the tips touch the reagent after it is dispensed. As the tips withdraw from the reagent, the surface tension of the solution helps prevent solution from adhering to the exterior shaft of the tip, and allows it to remain in the well. Slow down retraction speed to keep carryover to a minimum.



This is particularly important when dispensing volumes of 30 µl or less.

Omit Some Wash Steps

To speed up a robot process, reagents can be consecutively dispensed ("multi-dispensed") to multiple wells of an empty destination plate without washing tips between dispenses.

Dispense Reagents

Robot tips can vary in their absolute positions with regard to X-Y-Z coordinates. All pipetting must allow for a worst-case error in any critical dimension to avoid crashing the tips.

When using robots that pipette using system liquid, it is important to keep this liquid from diluting the reagents. It may be necessary to use liquid-separating air gaps and throw-away reagent volumes.

Never draw liquid into the robot diluter syringe. Cleaning the syringe of residual reagents is very difficult.

A reagent may be multi-dispensed to wells containing unique samples *only if* the tip height is set so that the tips do not come in contact with the sample-containing solutions. This is only recommended for dispensing large volumes (greater than 30 µl). Take special care to ensure that any solution splashed during reagent dispensing does not come into contact with the tips.

When dispensing small volumes (less than 10 µl), a conditioning dispense back into the source container can help ensure an accurate first-dispensed volume into the destination container.

To avoid cross-contamination when pipetting unique samples, always wash or change tips between samples. Wash volume required can vary widely depending on the robot and the type of reagent being washed out of the system.

Work with Viscous Solutions

For viscous solutions, aspirate and dispense slowly to ensure volume accuracy.

Fluorescence-Testing the Robot Procedure

While developing the robotic procedure, use a fluorescent solution to check the volume of liquid dispensed into a well. The degree of fluorescence in the well can be compared to a standard curve generated manually.

Chapter 3

GoldenGate Genotyping Assay for VeraCode Manual Protocols

Topics

- 36 Introduction
- 38 Workflow
- 39 Tracking Tools
- 42 Materials and Reagents for this Assay
- 44 Make DNA Quantitation Plate (Optional)
- 50 Read QDNA Plate (Optional)
- 54 Make Single-Use DNA (SUD) Plate
- 56 Precipitate SUD Plate
- 58 Resuspend SUD Plate
- 59 Make Allele-Specific Extension (ASE) Plate
- 61 Add Master Mix for Extension & Ligation (MEL)
- 64 Make PCR Plate
- 66 Inoculate PCR Plate
- 69 Thermal Cycle PCR Plate
- 70 Bind PCR Products
- 72 Make Intermediate Plate for VeraCode Bead Plate
- 75 Hybridize VeraCode Bead Plate
- 77 Wash VeraCode Bead Plate
- 78 Scan VeraCode Bead Plate
- 79 Troubleshooting

Introduction

This chapter describes in detail the pre- and post-PCR laboratory protocols associated with the GoldenGate Genotyping Assay for VeraCode. Perform each protocol in the order shown.

The instructions assume that you are preparing 96 DNA samples. If you are preparing fewer than 96 samples, scale down the protocols accordingly. For more information, see *Prepare Fewer than 96 Samples* on page 22.

The instructions in this chapter assume that you have already familiarized yourself with Chapter 2, *Standard Operating Procedures* and have set up the lab area appropriately.



CAUTION

It is very important to prevent PCR product contamination during this assay. To learn about safe lab practices for Illumina assays, see Chapter 2, *Standard Operating Procedures*. In addition, follow all of the safety procedures described in this chapter.

GoldenGate Genotyping Assay for VeraCode

Illumina's GoldenGate Genotyping Assay for VeraCode targets specific SNPs in genomic DNA samples. The genotyping application is based on sequence-specific extension and ligation of correctly hybridized query oligos, which are distinguished by their shared primer landing sites (Figure 6).

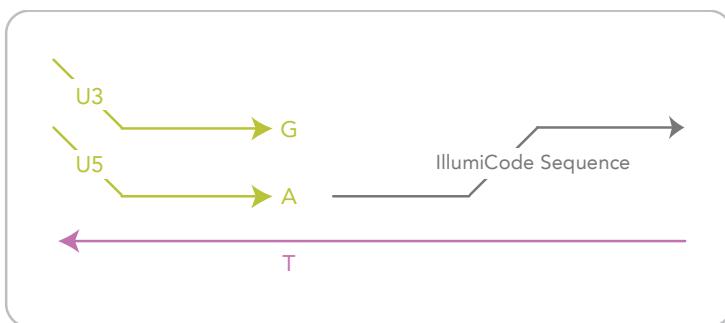


Figure 6 Oligo Configuration

In the GoldenGate Genotyping Assay for VeraCode, DNA is first activated through a chemical reaction with biotin. The biotinylated DNA is then purified from excess biotin. Assay oligonucleotides (oligos) are added and hybridized to the DNA, and the mixture is bound to streptavidin-conjugated paramagnetic particles (SA-PMPs). After the oligo hybridization, mis- and non-hybridized oligos are washed away, and allele-specific extension and ligation of the hybridized oligos is performed. The extended and ligated products form a synthetic template that is transferred to a PCR reaction and amplified. The strand containing the fluorescent signal in the PCR products is isolated and hybridized to the VeraCode beads via the address sequence. After the hybridization, the VeraCode beads are washed and scanned on the BeadXpress Reader.

The GoldenGate Assay for VeraCode and the BeadXpress Reader is nearly identical to the GoldenGate Assay for the BeadArray Reader, but differs in the following ways:

Table 11 BeadArray Reader vs. BeadXpress Reader Assays

	GoldenGate Assay for the BeadArray Reader	GoldenGate Assay for VeraCode and the BeadXpress Reader
Reagent Used to Make HYB/VBP Plate	MH1	MH2
Hyb Temperature	60°C, then 45°C	45°C
Hyb Time	Overnight	3 hours
Final Hyb Volume	50 µl	100 µl

Workflow

Figure 7 graphically represents the GoldenGate Genotyping Assay for VeraCode process flow.

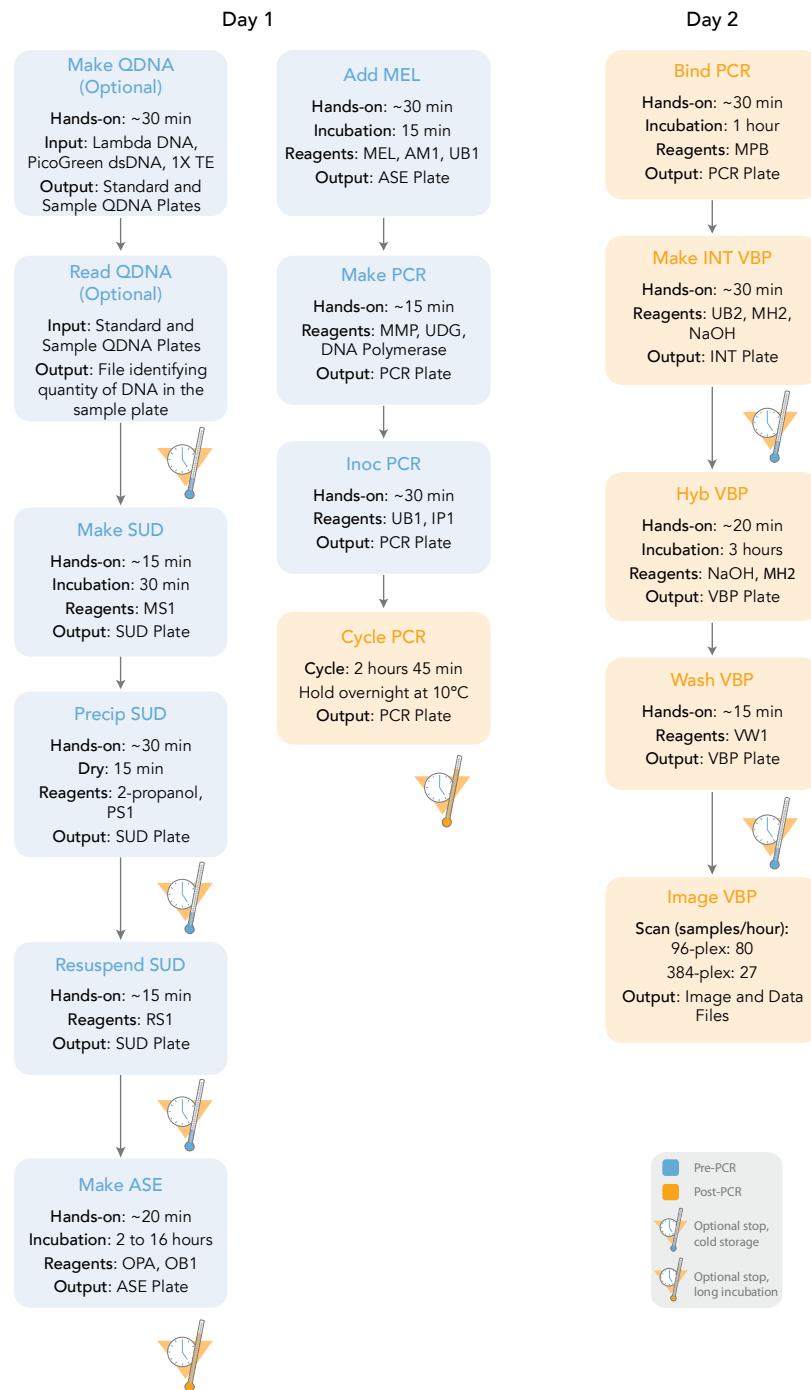


Figure 7 GoldenGate Genotyping Assay for VeraCode Workflow

Tracking Tools

Lab Tracking Forms

Create a copy of the two-page lab tracking form for each run (Figure 8). Use it to track information such as operator ID and reagent barcodes, and to record the sample location in each plate.



GoldenGate® Genotyping Assay for VeraCode® Lab Tracking Form	
<p>1 Activate DNA (Make, Precipitate, Resuspend)</p> <p>Date/Time: _____ SUD Plate: _____ Operator: _____ Robot: _____ M1 Reagent: _____ Plate Positions on Robot: _____ P1 Reagent: _____ (Make) _____ (Precip) _____ (Resus) _____ R1 Reagent: _____ Heat block (95°C, 30 m): Start: _____ Stop: _____ Centrifuge (2000 xg, 20 m): Start: _____ Stop: _____ Air dry (22°C, 15 m): Start: _____ Stop: _____</p> <p>2 Make ASE</p> <p>Date/Time: _____ SUD Plate: _____ Operator: _____ Robot: _____ ASE Plate: _____ Plate Positions on Robot: _____ O1 Reagent: _____ Heat block (2 °C): _____ OPA Reagent: _____ Start (70°C): _____ Stop (30°C): _____</p> <p>3 Add MEL</p> <p>Date/Time: _____ ASE Plate: _____ Operator: _____ Robot: _____ AM1 Reagent: _____ Plate Position on Robot: _____ U1 Reagent: _____ Heat block (45°C, 5 m): Start: _____ Stop: _____ MEL Reagent: _____</p> <p>4 Make PCR</p> <p>Date/Time: _____ PCR Plate: _____ Operator: _____ Robot: _____ MM1 Reagent: _____ Plate Position on Robot: _____ <input type="checkbox"/> Add Recommended DNA Polymerase <input type="checkbox"/> Add Uracil DNA Glycosylase (UDG)</p> <p>5 Inoc PCR</p> <p>Date/Time: _____ ASE Plate: _____ Operator: _____ Robot: _____ PCR Plate: _____ Plate Positions on Robot: _____ IP1 Reagent: _____ <input type="checkbox"/> Incubate in Heat Block (95°C, 1 m) U81 Reagent: _____</p>	
<i>Pre-PCR</i> <i>Post-PCR</i>	<i>Post-PCR</i> <i>Pre-PCR</i>
<small>Catalog # VC-R01-1001 Part # 11312721 Rev A</small>	

GoldenGate® Genotyping Assay for VeraCode® Lab Tracking Form	
<p>6 Cycle PCR</p> <p>Date/Time: _____ PCR Plate: _____ Operator: _____ Thermal Cycler: _____ Thermal Cycle Program Name: _____ Start: _____ Stop: _____</p> <p>7 Bind PCR</p> <p>Date/Time: _____ MPB Reagent: _____ Operator: _____ Robot: _____ Plate Position on Robot: _____ Incubate in light-protected drawer (22°C, 1 hour): _____ Start: _____ Stop: _____</p> <p>8 Make INT</p> <p>Date/Time: _____ INT Plate: _____ Operator: _____ Robot: _____ UB2 Reagent: _____ Plate Positions on Robot: _____ M1H Reagent: _____ Centrifuge (1000 xg, 25°C, 5 m): _____ NaOH Lot #: _____ <input type="checkbox"/> Start _____ Stop: _____</p> <p>9 Hyb VBP</p> <p>Date/Time: _____ INT Plate: _____ Operator: _____ VBP Plate: _____ Vortex Incubator ID: _____ M1H Reagent: _____ (850 rpm, 45°C, 3 h): Start: _____ Stop: _____ NaOH Lot #: _____</p> <p>10 Wash VBP</p> <p>Date/Time: _____ VBP Plate: _____ Operator: _____ VW1 Reagent: _____ <input type="checkbox"/> Vacuum-Aspirate Supernatant (50 mbar)</p> <p>11 Scan VBP</p> <p>Date/Time: _____ VBP Plate: _____ Operator: _____ BeadXpress® Reader ID: _____</p>	
<i>Post-PCR</i>	<i>Post-PCR</i>
<small>Catalog # VC-R01-1001 Part # 11312721 Rev A</small>	

Figure 8 GoldenGate Genotyping Assay for VeraCode Lab Tracking Form

Sample Sheet

To effectively track your samples and assay, Illumina recommends that you create a Sample Sheet. You will use the Sample Sheet with GenomeStudio to analyze your data. For more information about data analysis, see the *GenomeStudio Genotyping Module User Guide*.

Create your Sample Sheet according to the guidelines provided in Table 12.

Table 12 Sample Sheet Guidelines

Section	Description	Required (R) or Optional (O)
Sample_Name	Example: S12345 If not user-specified, the GenomeStudio application will assign a default sample name, concatenating the sample plate and sample well names.	O

Table 12 Sample Sheet Guidelines

Section	Description	Required (R) or Optional (O)
Sample_Plate	Example: GS0005623-DNA User-specified name for the plate containing DNA samples.	O
Sample_Well	Example: A01 The well containing the specific sample in the 96-well DNA plates.	O
Sentrix_ID	Example: CK1234567-VBP The VeraCode Bead Plate.	R
Sentrix_Position	Example: R001_C001 The VeraCode Bead Plate well position to which the sample is hybridized.	R
NOTES	Figure 9 shows an example sample sheet. Your sample sheet header may contain any number of columns, and whatever additional information you choose. Your sample sheet must be in a comma-delimited (*.csv) file format	

Save the sample sheet under any name you wish (for example, your user-defined experiment name).

Figure 9 provides an example of the Sample Sheet format. The VeraCode Assay Documentation CD includes a Sample Sheet template file that you can copy and use.

The screenshot shows a Microsoft Excel window titled "Microsoft Excel - 060607_Sample_Sheet_Methyl-384_VeraCode.csv". The window displays a table with data. The columns are labeled A through G. Row 1 contains "[Header]". Rows 2 through 5 contain project details: Investigator Name (Steffen), Project Name (VeraCode), Experiment Name, and Date (6/6/2007). Row 7 contains "[Data]". Rows 8 through 30 show sample information for 30 samples, each with a unique Sentrix_ID (60507) and position (R001_C001 to R002_C010). The table has a header row and 29 data rows.

	A	B	C	D	E	F	G
1	[Header]						
2	Investigator Name	Steffen					
3	Project Name	VeraCode					
4	Experiment Name						
5	Date	6/6/2007					
6							
7	[Data]						
8	Sample_Name	Sample_Well	Sample_Plate	Sample_Group	Pool_ID	Sentrix_ID	Sentrix_Position
9						60507	R001_C001
10						60507	R001_C002
11						60507	R001_C003
12						60507	R001_C004
13						60507	R001_C005
14						60507	R001_C006
15						60507	R001_C007
16						60507	R001_C008
17						60507	R001_C009
18						60507	R001_C010
19						60507	R001_C011
20						60507	R001_C012
21						60507	R002_C001
22						60507	R002_C002
23						60507	R002_C003
24						60507	R002_C004
25						60507	R002_C005
26						60507	R002_C006
27						60507	R002_C007
28						60507	R002_C008
29						60507	R002_C009
30						60507	R002_C010

Figure 9 VeraCode Sample Sheet

Materials and Reagents for this Assay

These items are specifically required for the GoldenGate Genotyping Assay for VeraCode. For a list of equipment, materials, and reagents required for all assays in a BeadXpress Reader lab, see *Standard Equipment, Materials, and Reagents* on page 17.

User-Supplied

Table 13 User-Supplied Reagents

Item	Source
Quant-iT PicoGreen DNA quantification reagent	Molecular Probes Invitrogen, catalog # P7581
Lambda DNA	Invitrogen, catalog # 25250-028

Illumina-Supplied

Table 14 Illumina-Supplied Materials and Reagents

Item	Catalog #
VeraCode 96-Plex GoldenGate Kit, 480 samples <ul style="list-style-type: none"> • BOX A VeraCode DNA Activation Kit • BOX B VeraCode GoldenGate Pre-PCR #1 • BOX C VeraCode GoldenGate Pre-PCR #2 • BOX D VeraCode GoldenGate Post-PCR • BOX E 96-Plex VeraCode Bead Plates • OPA GoldenGate Assay custom oligo pool, shipped separately <i>Description of box contents appears below.</i>	VC-201-0096
VeraCode 384-Plex GoldenGate Kit, 480 samples <ul style="list-style-type: none"> • BOX A VeraCode DNA Activation Kit • BOX B VeraCode GoldenGate Pre-PCR #1 • BOX C VeraCode GoldenGate Pre-PCR #2 • BOX D VeraCode GoldenGate Post-PCR • BOX E 384-Plex VeraCode Bead Plates • OPA GoldenGate Assay custom oligo pool, shipped separately <i>Description of box contents appears below.</i>	VC-201-0384

VeraCode GoldenGate Kit Box Contents

Table 15 Box Contents

Box	Contents
BOX A VeraCode DNA Activation Kit	MS1 —Reagent used to activate sufficient DNA PS1 —Precipitation solution for DNA activation RS1 —Resuspension solution for DNA activation
BOX B VeraCode GoldenGate Pre-PCR #1	OB1 —Oligo hybridization, and cDNA and gDNA binding buffer MMP —Master mix for PCR reagent IP1 —Reagent used to elute extended and ligated products UB1 —Universal buffer used to wash paramagnetic beads
BOX C VeraCode GoldenGate Pre-PCR #2	MEL —Reagent used for extension and ligation AM1 —Reagent used to wash away non-specifically hybridized and excess oligos from the gDNA
BOX D VeraCode GoldenGate Post-PCR	MPB —Magnetic particle reagent used to bind double-stranded PCR products MH2 —Reagent used to make the VBP plate UB2 —Universal buffer used to wash magnetic particles VW1 —Reagent used to wash the VeraCode beads
BOX E 96-Plex VeraCode Bead Plates	96-plex VeraCode Bead Plate (5)
BOX E 384-Plex VeraCode Bead Plates	384-plex VeraCode Bead Plate (5)

Other Materials

- ▶ QDNA barcode labels
- ▶ GS#-DNA barcode labels
- ▶ SUD barcode labels
- ▶ ASE barcode labels
- ▶ PCR barcode labels
- ▶ Filter plate: GS_____ -PCR labels
- ▶ INT barcode labels
- ▶ Filter plate adapter
- ▶ Vortexer calibration label

Make DNA Quantitation Plate (Optional)

This process uses the Quant-iT PicoGreen dsDNA quantitation reagent to quantitate double-stranded DNA samples for the GoldenGate Genotyping Assay for VeraCode.

Illumina recommends the Molecular Probes PicoGreen assay for quantitating dsDNA samples in the Illumina GoldenGate Genotyping Assay for VeraCode for the BeadXpress System and VeraCode technology. The PicoGreen assay can quantitate small DNA volumes, and measures DNA directly. Other techniques may pick up contamination such as RNA and protein. Illumina recommends using a fluorometer, as fluorometry provides DNA-specific quantitation, whereas spectrophotometry may also measure RNA, and may yield values that are too high.

Estimated Time Hands-on: 20 minutes per plate, plus 10 minutes to prepare the PicoGreen

Consumables

Item	Quantity	Storage	Supplied By
PicoGreen dsDNA quantitation reagent	See instructions	-20°C	User
1X TE	See instructions	Room temperature	User
Lambda DNA	See instructions	-20°C	User
96-well 0.65 ml microplate (MIDI)	1 per 96 samples		User
Fluotrac 200 (96-well black flat-bottom) plate	2 per 96 samples		User

Preparation

- ▶ Remove PicoGreen reagent from freezer and thaw at room temperature for 60 minutes in a light-impermeable container.
- ▶ Label a 96-well MIDI plate "Standard QDNA."
- ▶ Label a 96-well black flat-bottom plate "Standard QDNA."
- ▶ Label a 96-well black flat-bottom plate "Sample QDNA."

Steps **Make Standard QDNA MIDI Plate**

In this process, you create a Standard QDNA plate with serial dilutions of stock Lambda DNA in the wells of column 1.

1. Place stock Lambda DNA in well A1 of the Standard QDNA MIDI plate and dilute it to 75 ng/ μ l in a final volume of 233.3 μ l.
 - a. Use the following formula to calculate the amount of stock Lambda DNA to add to A1:

$$\frac{(233.3 \mu\text{l}) \times (75 \text{ ng}/\mu\text{l})}{(\text{stock Lambda DNA concentration})} = \mu\text{l of stock Lambda DNA to add to A1}$$

- b. Dilute the stock DNA in well A1 using the following formula:

$$\mu\text{l of 1X TE to add to A1} = 233.3 \mu\text{l} - \mu\text{l of stock Lambda DNA in well A1}$$

2. Add 66.7 μ l 1X TE to well B1 of the same plate.
3. Add 100 μ l 1X TE to wells C, D, E, F, G, and H of column 1 of the same plate.

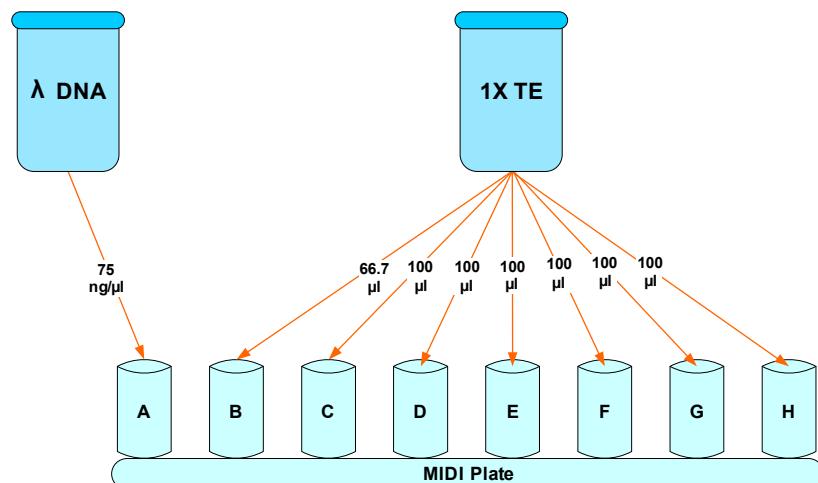


Figure 10 MIDI Plate Wells

4. Pipette the contents of A1 up and down 10 times to mix.
5. Transfer 133.3 μ l of Lambda DNA from well A1 into well B1, and then pipette the contents of well B1 up and down 10 times.
6. Change pipette tips. Transfer 100 μ l from well B1 into well C1, and then pipette the contents of well C1 up and down 10 times.
7. Change pipette tips. Transfer 100 μ l from well C1 into well D1, and then pipette the contents of well D1 up and down 10 times.
8. Change pipette tips. Transfer 100 μ l from well D1 into well E1, and then pipette the contents of well E1 up and down 10 times.
9. Change pipette tips. Transfer 100 μ l from well E1 into well F1, and then pipette mix the contents of well F1 up and down 10 times.

10. Change pipette tips. Transfer 100 µl from well F1 into well G1, and then pipette the contents of well G1 up and down 10 times.
11. **Do not transfer solution from well G1 to well H1.** Well H1 serves as the blank 0 ng/µl Lambda DNA.

Table 16 Concentration of Lambda DNA Standards

Row-Column	Conc. (ng/µl)	Final Volume in Well (µl)
A1	75	100
B1	50	100
C1	25	100
D1	12.5	100
E1	6.25	100
F1	3.125	100
G1	1.5625	200
H1	0	100

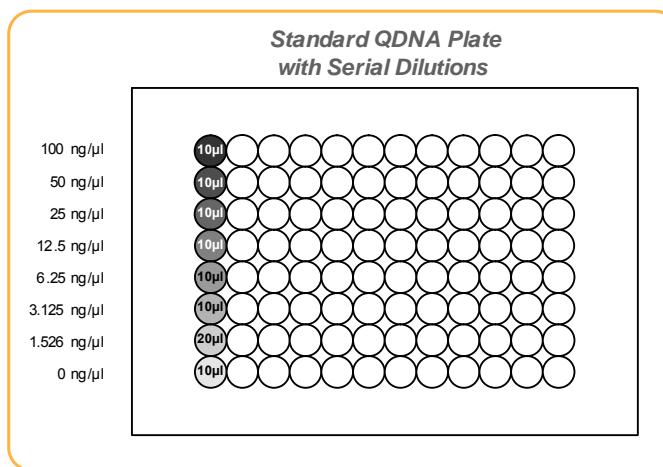


Figure 11 QDNA Plate with Serial Dilutions of Lambda DNA

12. Cover the plate with a cap mat.
13. Do one of the following:
 - Proceed to *Prepare Standard QDNA Fluotrac Plate with PicoGreen Dilution*.
 - Store the plate at 4°C for future use.

Prepare Standard QDNA Fluotrac Plate with PicoGreen Dilution

In this process you create a new Standard QDNA Fluotrac plate by transferring the serial dilutions of the Standard QDNA MIDI plate into the new plate and adding PicoGreen.



CAUTION

PicoGreen reagent degrades quickly in the presence of light. Do not use glass containers for PicoGreen reagent.

1. Prepare a 1:200 dilution of PicoGreen to 1X TE, using the kit supplies and a sterile 100 ml plastic container wrapped in aluminum foil. Refer to Table 17 to identify the volumes needed to produce diluted reagent for multiple 96-well QDNA plates. For fewer than 96 samples, scale down the volumes.

Table 17 QDNA Plate Reagent Volumes

# QDNA Plates	PicoGreen Volume (μ l)	1X TE Volume (ml)
1	115	23
2	215	43
3	315	63

2. Cap the sterile plastic container and vortex to mix.
3. Pour the PicoGreen/1X TE dilution into a sterile reservoir.
4. Using an 8-channel pipette, transfer 195 μ l PicoGreen/1X TE dilution into each well of columns 1 and 2 of the Standard QDNA Fluotrac plate (Figure 12).
5. Add 2 μ l of each stock Lambda DNA dilution from column 1 of the original Standard QDNA MIDI plate into the corresponding wells of columns 1 and 2 in the Standard QDNA Fluotrac plate.

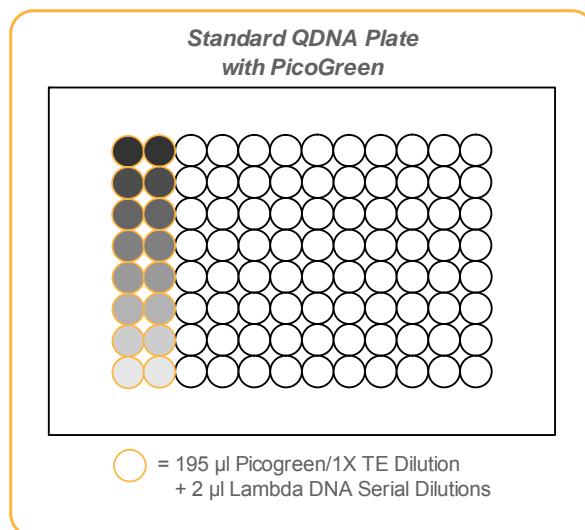


Figure 12 Standard QDNA Plate with PicoGreen

6. Pipette mix the contents of the new Standard QDNA plate.
7. Immediately cover the plate with an aluminum adhesive seal.

Prepare Sample QDNA Fluotrac plate with PicoGreen and DNA

In this process, you create a new Sample QDNA Fluotrac plate that contains DNA sample and PicoGreen.

1. Transfer 195 µl of the PicoGreen/1X TE dilution that you made earlier into each well of the new black flat-bottom plate labelled "Sample QDNA" (Figure 13).
2. Add 2 µl sample DNA to each well of the Sample QDNA plate.

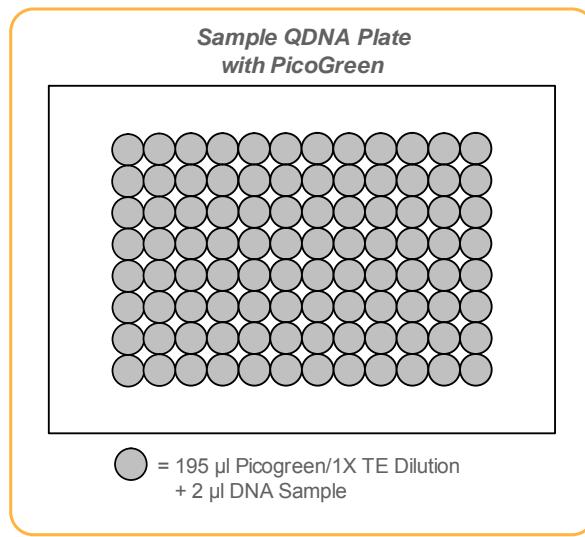


Figure 13 Sample QDNA Plate with PicoGreen

3. Pipette mix the contents of the Sample QDNA plate.
4. Immediately cover the new plate with an aluminum adhesive seal.
5. Proceed to *Read QDNA Plate (Optional)* on page 50.

Read QDNA Plate (Optional)

This process uses the Gemini XS or XPS Fluorometer to provide DNA-specific quantitation. Illumina recommends using a fluorometer, because fluorometry provides DNA-specific quantitation, whereas spectrophotometry may also measure RNA and yield values that are too high.

Estimated Time Fluorometer: 5 minutes per plate

- Steps**
1. Turn on the fluorometer.
 2. At the PC, open the SoftMax Pro program.
 3. Load the Illumina QDNA.ppr file (available on the installation CD that came with your system).
 4. Select **Assays | Nucleic Acids | Illumina QDNA** (Figure 14).

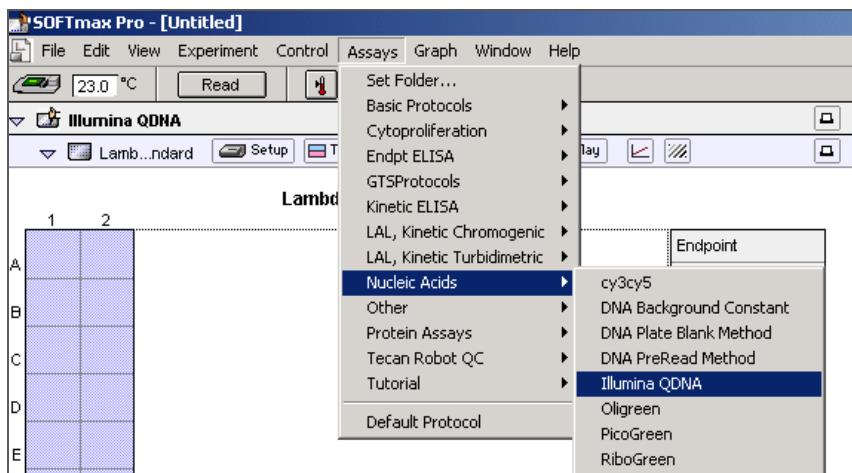


Figure 14 Load the PicoGreen Protocol in SoftMax Pro

5. Place the Standard QDNA Plate into the fluorometer loading rack with well A1 in the upper-left corner.
6. Click the blue arrow next to **Lambda Standard** (Figure 15).

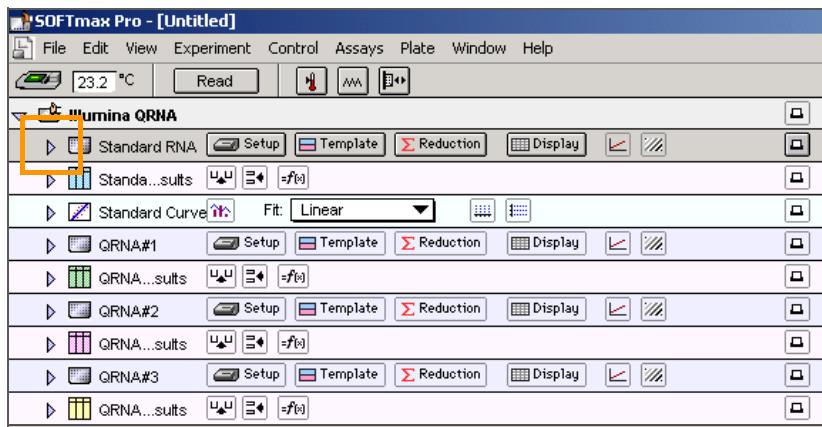


Figure 15 Select Lambda Standards Screen

7. Click **Read** in the SoftMax Pro interface (Figure 16) to begin reading the Standard QDNA Plate.

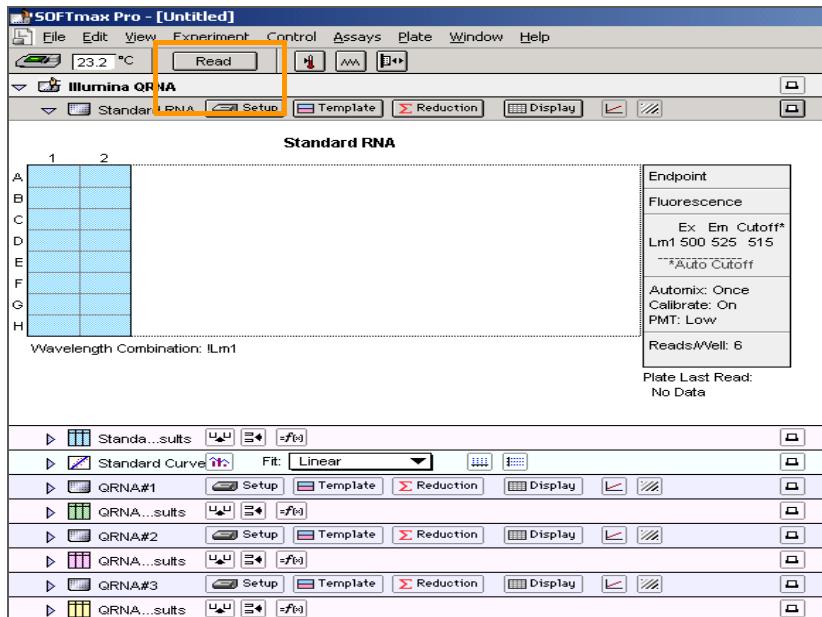


Figure 16 Read Standard QDNA Plate

8. When the software finishes reading the plate, the plate drawer opens. Remove the Standard QDNA Plate from the drawer.
9. Click the blue arrow next to **Standard Curve** to view the standard curve graph (Figure 17).
10. If the standard curve is acceptable, continue with the sample plate. Otherwise, click **Standard Curve** again.

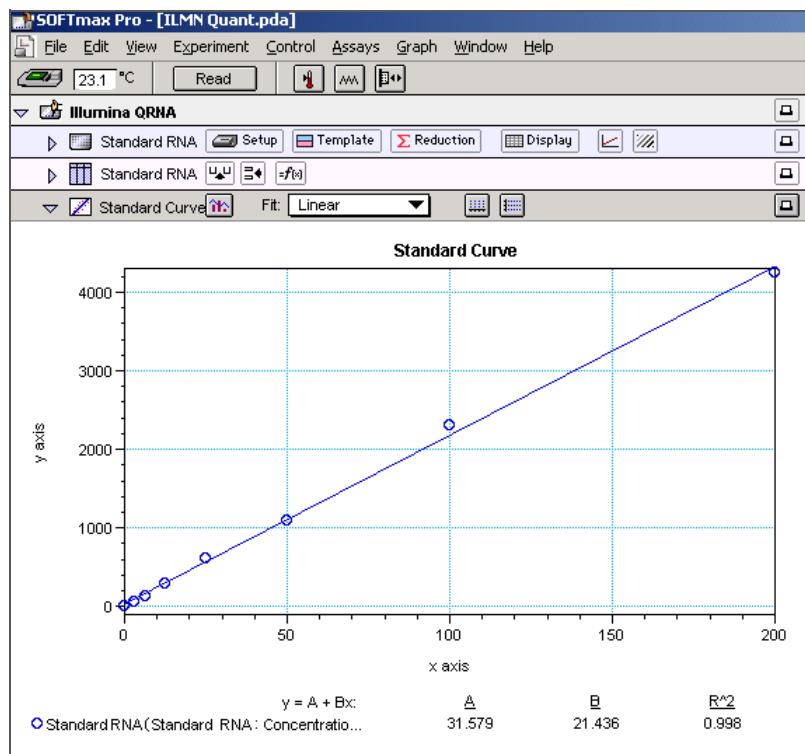


Figure 17 View Standard Curve

11. Place the Sample QDNA plate in the reader with well A1 in the upper left corner.
12. Click the blue arrow next to **QDNA#1** and click **Read**.

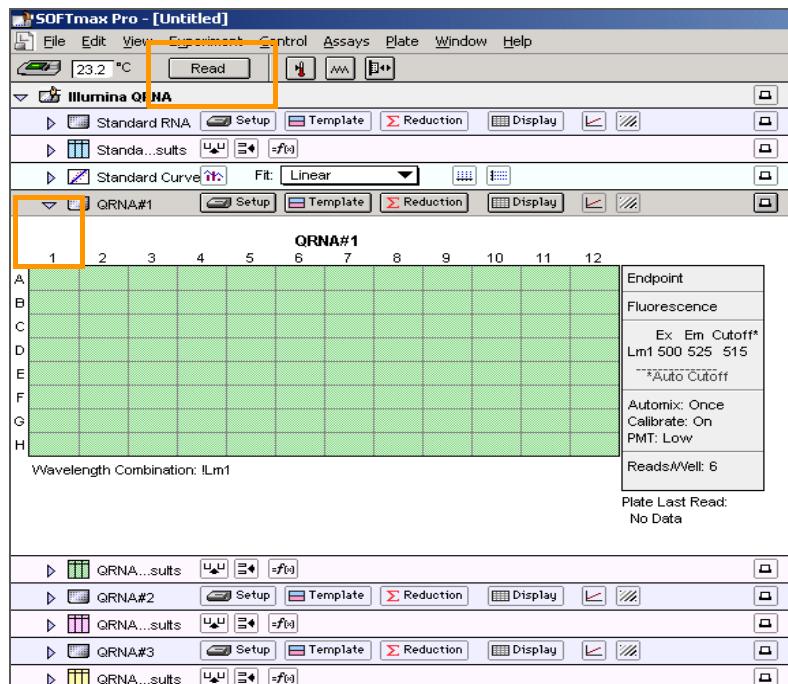


Figure 18 Read Sample QDNA Plate

13. When the software finishes reading the plate, the plate drawer opens.
Remove the plate from the drawer.
14. Repeat steps 11 through 13 for all sample plates that you want to quantitate.
15. Once all plates have been read, click **File | Save** to save the output data file (*.pda).
16. Once you save the *.pda file, click **File | Import/Export | Export** and export the file as a *.txt file. You can open the *.txt file in Microsoft Excel for data analysis.
17. Proceed to *Make Single-Use DNA (SUD) Plate* on page 54.

Make Single-Use DNA (SUD) Plate

This process activates sufficient DNA of each individual sample to be used once in the GoldenGate Genotyping Assay for VeraCode.

Estimated Time	Hands-on: ~15 minutes Incubation: 30 minutes
-----------------------	---

Consumables

Item	Quantity	Storage	Supplied By
10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE)	See instructions	Room temperature	User
MS1 reagent	1 tube per SUD plate	-20°C	Illumina
DNA samples and controls	96 or 384	-20°C	User
96-well 0.2 ml skirted microplate	1 per sample plate		User

Preparation

- ▶ In the appropriate columns of the Sample Sheet, enter the Sample_Name and Sample_Plate for each Sample_Well defined in the Sample Sheet. See *Sample Sheet* on page 39.
- ▶ Preheat the heat block to 95°C and allow the temperature to stabilize.
- ▶ Turn on the heat sealer to preheat it. Allow 15 minutes.
- ▶ Thaw the MS1 reagent tube to room temperature. Vortex to mix the contents, and pour the entire tube into a new, non-sterile reservoir.
- ▶ Thaw the DNA samples and controls to room temperature and vortex to mix the contents.
- ▶ Apply a SUD barcode label to a new 96-well microplate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - SUD plate barcode
 - MS1 reagent barcode



You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

- Steps**
1. Normalize DNA samples to 50 ng/ μ l with 10 mM Tris-HCl pH 8.0, 1 mM EDTA.
 2. Add 5 μ l MS1 reagent to each well of the SUD plate.

3. Using an 8-channel pipette, transfer 5 µl normalized DNA sample to each well of the SUD plate. Change tips between column dispenses.
4. Apply a microplate foil heat seal to the SUD plate and seal it with the heat sealer (3 seconds). Ensure that all wells are completely sealed.
5. Pulse centrifuge the SUD plate to 250 xg.
6. Vortex at 2300 rpm for 20 seconds, making sure the plate is firmly strapped to the vortexer platform to prevent plate movement.
7. Pulse centrifuge to 250 xg.

**NOTE**

It is important to centrifuge the SUD plate to 250 xg before the 95°C incubation to prevent the wells from drying out during incubation.

8. Place the SUD plate in the preheated heat block and close the lid.
9. Incubate the SUD plate at 95°C for exactly 30 minutes.

**CAUTION**

Do not allow the 95°C incubation period to exceed 30 minutes.

10. On the lab tracking form, record the start and stop times.
11. Pulse centrifuge the plate to 250 xg.
12. If you plan to perform the Make ASE protocol today, then immediately set the heat block to 70°C.
13. Proceed to *Precipitate SUD Plate* on page 56.

Precipitate SUD Plate

In this process, PS1 and 2-propanol are added to the SUD plate to precipitate the DNA and remove excess DNA activation reagent MS1.

Estimated Time	Hands-on: ~30 minutes
	Drying: 15 minutes

Consumables

Item	Quantity	Storage	Supplied By
PS1 reagent	Bottle	4°C	Illumina
2-propanol	Bottle	Room temperature	User

Preparation

- ▶ Pour 1 ml PS1 into a reagent reservoir.
- ▶ Pour 2 ml 2-propanol into a second reservoir.
- ▶ On the lab tracking form, record the PS1 reagent barcode.



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Remove the heat seal from the heated SUD plate, taking care to avoid splashing from the wells.

2. Add 5 µl PS1 reagent to each well of the SUD plate.



CAUTION

To avoid contaminating the pipette tips, place the tips against the top edge of the well. If you suspect the tips are contaminated with the contents of the well, discard them and use new tips.

3. Seal the SUD plate with clear adhesive film.
4. Pulse centrifuge the plate to 250 xg.
5. Vortex at 2300 rpm for 20 seconds or until the solution is uniformly blue.
6. Remove the film and add 15 µl 2-propanol to each well of the SUD plate.
7. Seal the SUD plate with clear adhesive film.
8. Vortex at 1600 rpm for 20 seconds or until the solution is uniformly blue.
9. Centrifuge the sealed SUD plate to 3000 xg for 20 minutes.

**NOTE**

If you do not see a faint blue pellet at the bottom of each well, the DNA has not precipitated. In some cases, depending on DNA quality, the blue pellet may appear diffuse.

10. On the lab tracking form, record the start and stop times.

Perform the next step immediately to avoid dislodging the activated DNA pellets. If any delay occurs, re-centrifuge to 3000 xg for 10 minutes before proceeding.

11. Remove the SUD plate seal and decant the supernatant by inverting the SUD plate and smacking it down onto an absorbent pad.**CAUTION**

Do not tilt the plate, as this can cause cross-contamination between wells. Tap the plate firmly enough to decant all the supernatant; tapping lightly will not work as well.

12. Tap the inverted plate onto the pad to blot excess supernatant.**13.** Place the inverted SUD plate on an absorbent pad and centrifuge to 8 xg for 1 minute.**WARNING**

Do not spin the inverted plate to more than 8 xg, or the sample will be lost!

14. Remove the SUD plate from the centrifuge.**15.** Set the plate upright and allow it to dry at room temperature for 15 minutes.**16.** On the lab tracking form, record the start and stop times.**17.** Do one of the following:

- Proceed to *Resuspend SUD Plate* on page 58.
- Seal the plate with adhesive film and store at -20°C for up to 24 hours.

Resuspend SUD Plate

In this process, RS1 is added to the SUD plate to resuspend the DNA.

Estimated Time Hands-on: ~15 minutes

Consumables

Item	Quantity	Storage	Supplied By
RS1 reagent	Bottle	Room temperature	Illumina

Preparation

- ▶ Pour 1.2 ml RS1 into a reagent reservoir.
- ▶ On the lab tracking form, record the RS1 reagent barcode.



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Add 10 µl RS1 reagent to each well of the SUD plate.
2. Seal the SUD plate with microplate clear adhesive film.
3. Pulse centrifuge to 250 xg.
4. Vortex at 2300 rpm for 1 minute or until the blue pellet is completely dissolved. Ensure that the plate is firmly strapped to the vortexer platform to prevent plate movement.
SUD sample plate activation is complete.
5. Do one of the following:
 - Proceed immediately to *Make Allele-Specific Extension (ASE) Plate* on page 59.
 - Store the SUD plate at 4°C overnight.

Make Allele-Specific Extension (ASE) Plate

This process combines the biotinylated gDNAs from the SUD plate with query oligos, hybridization reagents, and paramagnetic particles in an Allele Specific Extension (ASE) plate. The ASE plate is placed in a heat block and the query oligos for each sequence target of interest are allowed to anneal to the biotinylated gDNA samples. The gDNA is simultaneously captured by paramagnetic particles. The resulting ASE plate is ready for the extension and ligation of the hybridized oligos on the bound gDNAs.

This process is designed for one plate, using the SUD plate as input.

Estimated Time

Hands-on: ~20 minutes

Incubation: 2–16 hours

Consumables

Item	Quantity	Storage	Supplied By
OB1 reagent	1 tube per plate	-20°C	Illumina
OPA reagent	1 tube per plate	-20°C	Illumina
96-well 0.2 ml skirted microplate	1 per SUD plate		User

Preparation

- ▶ In the Pool_ID column of the Sample Sheet, enter the OPA for each Sample_Well.
- ▶ Preheat the heat block to 70°C and allow the temperature to stabilize.
- ▶ Turn on the heat sealer to preheat it. Allow 15 minutes.
- ▶ Thaw the OPA reagent tube to room temperature. Vortex the tube, and then pulse centrifuge to 250 xg. Pour the contents of the OPA tube into a reagent reservoir.
- ▶ Thaw the OB1 reagent tube to room temperature. Vortex the tube to completely resuspend the beads. Invert tube to verify that all the paramagnetic particles are evenly suspended in solution. Pour the contents of the OB1 tube into a second reagent reservoir.



Do not centrifuge the OB1 tube.

- ▶ Apply an ASE barcode label to a new 96-well microplate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - SUD plate barcode
 - ASE plate barcode

- OB1 reagent barcode
- OPA reagent barcode

**NOTE**

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Pulse centrifuge the SUD plate to 250 xg.
2. Add 10 µl OPA reagent to each well of the ASE plate.
3. Add 30 µl OB1 reagent to each well of the ASE plate.
4. Carefully remove the heat seal from the SUD plate.
5. Transfer 10 µl of biotinylated sample from each well of the SUD plate (where 10 µl is the entire volume) to the corresponding well of the ASE plate.
6. Using a microplate heat seal, heat-seal the ASE plate (3 seconds). Ensure that all wells are completely sealed.
7. Pulse centrifuge the ASE plate to 250 xg.
8. Vortex the ASE plate at 1600 rpm for 1 minute or until all beads are completely resuspended.
9. Place the sealed ASE plate on the 70°C heat block and close the lid.
10. Immediately reset the temperature to 30°C.
11. Allow the ASE plate to cool to 30°C for about 2 hours. The ASE plate may remain in the heat block for up to 16 hours.
12. On the lab tracking form, record the start and stop times.
13. Proceed to *Add Master Mix for Extension & Ligation (MEL)* on page 61.

Add Master Mix for Extension & Ligation (MEL)

In this process, AM1 and UB1 reagents are added to the ASE plate to wash away non-specifically hybridized and excess oligos. An enzymatic extension and ligation master mix (MEL) is added to each DNA sample. The extension and ligation reaction occurs at 45°C.

Estimated Time	Hands-on: ~30 minutes Incubation: 15 minutes
-----------------------	---

Consumables

Item	Quantity	Storage	Supplied By
AM1 reagent	Bottle	4°C	Illumina
UB1 reagent	Bottle	4°C	Illumina
MEL reagent	1 tube per plate	-20°C	Illumina

Preparation

- ▶ Remove the ASE plate from the heat block.
- ▶ Preheat the heat block to 45°C for about one hour.
- ▶ Thaw the MEL tube to room temperature.
- ▶ Pour 11 ml AM1 into a reagent reservoir. Add 10 ml for each additional plate.
- ▶ Pour 11 ml UB1 into a second reagent reservoir. Add 10 ml for each additional plate.
- ▶ Pour the thawed MEL tube contents into a third reagent reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - ASE plate barcode
 - AM1 reagent barcode
 - UB1 reagent barcode
 - MEL reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps



CAUTION

In this procedure, you will remove all the liquid from the wells several times, leaving only the beads. Work quickly so that the beads do not dry out.

1. Centrifuge the ASE plate to 250 xg.
2. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.

If you are using the raised-bar magnetic plate from Illumina, the beads in odd-numbered columns will be pulled to the right wall of the well, and the beads in even-numbered columns will be pulled to the left wall of the well.

**NOTE**

To avoid aspirating the beads during this procedure, slant the pipette tips so that they draw liquid from the side of the well opposite the beads. Aspirate all of the odd columns first, and then rotate the plate and aspirate the even columns (or vice-versa). This enables you to keep the pipettor at the same angle throughout.

3. Carefully remove the heat seal from the ASE plate.
4. Using an 8-channel pipette with new tips, remove and discard all the liquid (50 µl) from the wells. Leave the beads in the wells.
It is not necessary to change pipette tips until you have removed the liquid from all 12 columns.
5. Visually inspect pipette tips after removing solution from each column to ensure no beads have been removed. If beads are visible in pipette tips, return the solution to the same wells, allow magnet to re-collect beads, and change the pipette tips.
6. With the ASE plate on the raised-bar magnetic plate, use an 8-channel pipette with new tips to add 50 µl AM1 to each well of the ASE plate.

**CAUTION**

To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (Figure 19, page 62). If you suspect the tips are contaminated with the contents of the well, use new tips.



Figure 19 Avoid Tip Contamination

7. Seal the ASE plate with microplate clear adhesive film.

8. Vortex the ASE plate at 1600 rpm for 20 seconds or until all beads are resuspended.
9. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
10. Remove the seal from the ASE plate, taking care to avoid splashing from the wells.
11. Using the same 8-channel pipette with the same tips, remove all AM1 reagent from each well. Leave the beads in the wells.
It is not necessary to change pipette tips until liquid has been removed from all 12 columns.
12. Repeat steps 6 through 11 once.
13. Remove the ASE plate from the raised-bar magnetic plate.
14. Using an 8-channel pipette with new tips, add 50 μ l UB1 to each well of the ASE plate.
15. Place the ASE plate onto the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
16. Using the same 8-channel pipette with the same tips, remove all UB1 reagent from each well. Leave the beads in the wells.
It is not necessary to change pipette tips until liquid has been removed from all 12 columns.
17. Repeat steps 13 through 16 once.
18. Using an 8-channel pipette with new tips, add 37 μ l MEL to each well of the ASE plate.
19. Seal the plate with microplate clear adhesive film.
20. Vortex the plate at 1600–1700 rpm for 1 minute or until the beads are resuspended.
21. Incubate the ASE plate on the preheated 45°C heat block for exactly 15 minutes.

**CAUTION**

Do not allow the ASE plate to incubate at 45°C longer than 15 minutes.

22. During the incubation, perform the *Make PCR Plate* procedure.
23. On the lab tracking form, record the start and stop times.
24. Proceed to *Inoculate PCR Plate* on page 66. Leave the ASE plate at room temperature if you proceed immediately, or store it at 4°C for up to 1 hour.

Make PCR Plate

This process adds the Illumina-recommended DNA Polymerase and optional Uracil DNA Glycosylase (UDG) to the master mix for PCR (MMP reagent) and creates a 96-well plate for the Inoc PCR process.

Estimated Time Hands-on: ~15 minutes

Consumables

Item	Quantity	Storage	Supplied By
MMP reagent	1 tube per plate	-20°C	Illumina
Titanium Taq DNA Polymerase	64 µl	-20°C	User
Uracil DNA Glycosylase (UDG, Optional)	50 µl	-20°C	User
96-well 0.2 ml skirted microplate	1 per ASE plate		User

Preparation

- ▶ Thaw the MMP tube to room temperature.
- ▶ Apply a PCR barcode label to a new 96-well microplate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - PCR plate barcode
 - MMP reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Add 64 µl DNA Polymerase to the MMP tube. Check off this action in the lab tracking form.
2. [Optional] Add 50 µl Uracil DNA Glycosylase to the MMP tube. Check off this action in the lab tracking form.
3. Invert the tube several times to mix the contents, and then pour the contents of the tube into a reagent reservoir.
4. Using an 8-channel pipette, add 30 µl of the mixture into each well of the PCR plate.
5. Seal the PCR plate with microplate clear adhesive film.

6. Pulse centrifuge to 250 xg, and then place the PCR plate in a light-protected location.
7. Proceed to *Inoculate PCR Plate* on page 66.

Inoculate PCR Plate

This process uses the template formed in the extension and ligation process in a PCR reaction. This PCR reaction uses three universal primers (MMP reagent): two are labeled with fluorescent dyes and the third is biotinylated. The biotinylated primer allows capture of the PCR product and elution of the strand containing the fluorescent signal. The eluted samples are transferred from the ASE plate to the PCR plate.

Estimated Time	Hands-on: ~30 minutes
-----------------------	-----------------------

Consumables

Item	Quantity	Storage	Supplied By
UB1 reagent	Bottle	4°C	Illumina
IP1 reagent	1 tube per plate	-20°C	Illumina

Preparation

- ▶ Remove the ASE plate from the heat block.
- ▶ Reset the heat block to 95°C.
- ▶ Pour 6 ml UB1 into a reagent reservoir.
- ▶ Thaw the IP1 reagent to room temperature. Pour the contents of the tube into a reagent reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - ASE plate barcode
 - PCR plate barcode
 - IP1 reagent barcode
 - UB1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
2. Remove the microplate clear adhesive film from the ASE plate.
3. Using an 8-channel precision pipette, remove and discard the supernatant (~50 µl) from all wells of the ASE plate. Leave the beads in the wells.

It is not necessary to change pipette tips until liquid has been removed from all 12 columns.

4. Visually inspect pipette tips after removing solution from each column to ensure no beads have been removed. If beads are visible in pipette tips, return the solution to the same wells, allow magnet to re-collect beads, and change the pipette tips.
5. Leaving the plate on the magnet and using an 8-channel precision pipette with new tips, add 50 µl UB1 to each well of the ASE plate.

**CAUTION**

To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (Figure 19, page 62). If you suspect the tips are contaminated with the contents of the well, discard the tips and use new tips.

6. Leave the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
7. Remove and discard the supernatant (~50 µl) from all wells of the ASE plate. Leave the beads in the wells.
It is not necessary to change pipette tips until liquid has been removed from all 12 columns.
8. Remove the plate from the magnet.
9. Using an 8-channel precision pipette with new tips, add 35 µl IP1 to each column of the ASE plate.
10. Seal the plate with microplate clear adhesive film.
11. Vortex at 1800 rpm for 1 minute, or until all the beads are resuspended.
12. Place the plate on the 95°C heat block for 1 minute.
13. Place the ASE plate back onto the raised-bar magnetic plate for 2 minutes or until the beads have been completely captured.
14. Using an 8-channel pipette with new tips, transfer 30 µl supernatant from each well in the first column of the ASE plate to the first column of the PCR plate.
15. Repeat for each column of the ASE plate. Change tips between column dispenses.

**CAUTION**

Take special care not to disturb or transfer the beads when aspirating eluted product.

16. Discard the ASE plate.
17. Seal the PCR plate with the appropriate PCR plate-sealing film for your thermocycler.
18. Immediately transfer the PCR plate to the thermocycler.
19. Proceed to *Thermal Cycle PCR Plate* on page 69.

This concludes the Pre-PCR processes for the manual GoldenGate Genotyping Assay for VeraCode. If you remove materials such as experienced user cards from the Pre-PCR lab, do not return with them into the Pre-PCR lab at any time.

Thermal Cycle PCR Plate

This process thermal cycles the PCR plate to fluorescently label and amplify the templates generated in the pre-PCR process.

Estimated Time Thermal Cycle: ~2 hours 45 minutes

Preparation

- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - PCR plate barcode
 - Thermocycler ID
 - Thermocycler program

Steps

1. Place the sealed plate into the thermocycler and run the thermocycler program shown in this table.

Table 18 Thermocycler Program

	Temperature	Time
	37°C	10 minutes
	95°C	3 minutes
X 34 {	95°C	35 seconds
	56°C	35 seconds
	72°C	2 minutes
	72°C	10 minutes
	4°C	5 minutes

2. Do one of the following:

- Proceed immediately to *Bind PCR Products* on page 70. Store the PCR plate at room temperature (22°C) in a light-protected drawer.
- Seal and store the PCR plate at -20°C overnight.

Bind PCR Products

In this process, MPB reagent is added to the PCR plate and the solution is transferred to a filter plate. The filter plate is incubated at room temperature to bind the biotinylated strand to paramagnetic particles, thus immobilizing the double-stranded PCR products.

Estimated Time	Hands-on: ~30 minutes Incubation: 1 hour
-----------------------	---

Consumables

Item	Quantity	Storage	Supplied By
MPB reagent	1 tube per PCR plate	4°C	Illumina
0.45 µM clear Styrene filter plate with lid	1 per PCR plate		User

Preparation

- ▶ Vortex the MPB tube several times or until the beads are well resuspended. Pour the contents of the tube into a non-sterile reagent reservoir.
- ▶ Write the PCR plate barcode number in the space provided on a “Filter Plate: GS _____ -PCR” label. Apply the label to the top surface of the filter plate, adjacent to column 12 (Figure 20).



This ensures that the filter plate is associated with the correct PCR plate. Do not attach the label to the lid of the filter plate, as the lid could get separated from the plate.

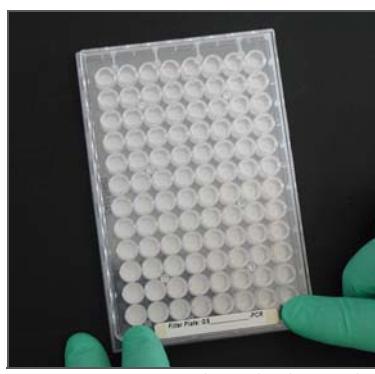


Figure 20 Apply Label to Filter Plate

- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - MPB reagent barcode
 - Filter plate barcode

**NOTE**

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Pulse centrifuge the PCR plate to 250 xg.
 2. Place new tips onto an 8-channel pipette.
 3. Add 20 µl resuspended MPB into each well of the PCR plate.
It is not necessary to change pipette tips until liquid has been transferred to all 12 columns.
-
-
- CAUTION**
- To avoid contaminating the tips, place the tips against the top edge of the wells (Figure 19, page 62). If you suspect the tips are contaminated with the contents of the well, use new tips.
-
4. Set an 8-channel pipette to 85 µl to allow space for bubbles, and attach new tips.
 5. Pipette the solution in the PCR plate up and down several times to mix the beads with the PCR product.
 6. Transfer the mixed solution into the first column of the filter plate. There should be about 70 µl fluid in each well.
 7. Repeat step 6 for each column of the PCR plate. Change tips between column dispenses.
 8. Discard the empty PCR plate.
 9. Cover the filter plate with its lid and store it at room temperature, protected from light, for 60 minutes.
 10. On the lab tracking form, record the start and stop times.
 11. Proceed to *Make Intermediate Plate for VeraCode Bead Plate* on page 72.

Make Intermediate Plate for VeraCode Bead Plate

In this process, the PCR product is washed in the filter plate with UB2 and NaOH. The single-stranded, fluor-labeled material is then eluted into an INT plate containing MH2 reagent.

Estimated Time Hands-on: ~30 minutes

Consumables

Item	Quantity	Storage	Supplied By
MH2 reagent	1 tube per PCR plate	Room temperature	Illumina
UB2 reagent	Bottle	Room temperature	Illumina
0.1N NaOH	Bottle	4°C	User
96-well V-bottom plate	2 per PCR plate		User

Preparation

- ▶ Apply a INT barcode label to a new 96-well V-bottom plate.
- ▶ Using a serological pipette, transfer 6 ml UB2 into a sterile reservoir.
- ▶ Pour 4 ml 0.1N NaOH into a second sterile reservoir.
- ▶ Pour the contents of the MH2 tube into a third sterile reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - INT plate barcode
 - UB2 reagent barcode
 - MH2 reagent barcode
 - NaOH lot number



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Place the filter plate adapter on an empty, unlabeled 96-well V-bottom plate (waste plate) (Figure 21).
2. Place the filter plate containing the bound PCR products onto the filter-plate adapter (Figure 21).



Figure 21 Assemble Filter Plate

3. Centrifuge to 1000 xg for 5 minutes at 25°C.
4. Remove the filter plate lid.
5. Using an 8-channel pipette with new tips, add 50 µl UB2 to each well of the filter plate. Dispense slowly to avoid disturbing the beads.



CAUTION

To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (see Figure 19, page 62).

6. Replace the filter plate lid.
7. Centrifuge to 1000 xg for 5 minutes at 25°C.
8. Using an 8-channel pipette with new tips, add 30 µl MH2 to each well of the INT plate.
9. Replace the waste plate with the INT plate. Orient the INT plate so that well A1 of the filter plate matches well A1 of the INT plate.



WARNING

Be sure to replace the waste plate with the INT plate. Failure to replace the waste plate will result in loss of samples.

10. Discard the waste plate.
11. Place new tips onto the 8-channel pipette.
12. Dispense 30 µl 0.1N NaOH to all wells of the filter plate.
13. Replace the filter plate lid.



NOTE

Due to the sensitivity of the dyes to 0.1N NaOH, proceed quickly. Prolonged incubation with NaOH is unnecessary; less than 5 minutes is sufficient. The DNA is denatured almost instantly.

14. Centrifuge immediately at 1000 xg for 5 minutes at 25°C. At the end, no beads should be visible in the wells of the INT plate.
15. Discard the filter plate. Save the adapter for later use in other protocols.
16. Gently mix the contents of the INT plate by moving it from side to side without splashing.
17. Cover the INT plate with clear adhesive seal.
18. Do one of the following:
 - Proceed to *Hybridize VeraCode Bead Plate* on page 75.
 - Seal the INT plate with a cap mat and store it at -20°C.

Hybridize VeraCode Bead Plate

This process uses the VeraCode Vortex Incubator, an incubating microplate shaker, to hybridize the VeraCode Bead Plate (VBP). Once the samples are transferred to the VBP, they are ready for hybridization at 45°C.

Estimated Time	Hands-on: ~20 minutes Incubation: 3 hours
-----------------------	--

Consumables

Item	Quantity	Storage	Supplied By
MH2 reagent	1 tube per plate	Room temperature	Illumina
0.1N NaOH	Bottle	4°C	User
VeraCode Bead Plate	1 per INT plate		Illumina

Preparation

- ▶ If the INT plate has been frozen, thaw to room temperature in a light-protected drawer, then pulse centrifuge to 250 xg.
- ▶ Preheat the VeraCode Vortex Incubator to 45°C and allow it to equilibrate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - INT plate barcode
 - VBP plate barcode
 - MH2 reagent barcode
 - NaOH lot number



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps Add Neutralized MH2 to INT VBP

1. Using a serological pipette, transfer 3 ml MH2 into a 15 ml conical tube.
2. Using a different serological pipette, transfer 3 ml 0.1 N NaOH to the 15 ml tube.
3. Vortex the tube until the contents are mixed.
4. Pour the mixture into a sterile reservoir.

5. Using an 8-channel pipette, add 50 µl of neutralized MH2 to each of the INT plate wells that contain sample. Be careful not to let the pipette tips touch the samples.

Hybridize VBP

1. Remove the VBP from the 4°C refrigerator and pulse centrifuge to 250 xg. If the beads are not at the bottoms of the wells, pulse centrifuge again.
2. Remove the cap mat from the VeraCode Bead Plate. Save the cap mat for subsequent use in hybridization.
3. Using an 8-channel pipette with new tips, pipette each column of sample in the INT plate up and down 4–5 times.
4. Using the same tips, transfer 100 µl of each assay product from the INT plate into the corresponding well of the VeraCode Bead Plate.
5. Place the cap mat back on the VeraCode Bead Plate.
6. Place the VeraCode Bead Plate Bead Plate, which now contains samples, into the VeraCode Vortex Incubator. You can load up to 2 VBP plates in the vortexer.



NOTE

If you load only one plate, load an empty 96-well plate in the opposite position as a balance.

7. Close the lid and make the following settings:
 - Push the **Encoder** knob until **RPM** is highlighted. Rotate the knob to 85 (850 rpm).
 - Push the **Encoder** knob until **Time** is highlighted. Rotate the knob below 0.30 or above 99.5 until HLD appears. This sets the Vortex Incubator to run continuously.
 - Push the **Encoder** knob until **Temperature** is highlighted. Rotate the knob to 45 (45°C).
8. Press **Start/Stop** and incubate for 3 hours.
9. Proceed to *Wash VeraCode Bead Plate* on page 77.

Wash VeraCode Bead Plate

In this process, the VeraCode Bead Plate is removed from the VeraCode Vortex Incubator and washed two times with the VW1 reagent.

Estimated Time Hands-on: ~15 minutes

Consumables

Item	Quantity	Storage	Supplied By
VW1 reagent	Bottle	Room temperature	Illumina

Preparation

- ▶ Pour 45 ml of VW1 into a nonsterile reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - VW1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Stop the VeraCode Vortex Incubator. When the speed indicator reaches 0, open the lid and remove the VeraCode Bead Plate.
2. Pulse centrifuge the plate to 250 xg.
3. Remove the cap mat.
4. Using an 8-channel pipette, add 200 μ l VW1 buffer to each well. Make sure to agitate the bead pellet.
5. Gently swirl the plate in a circular motion on the benchtop.
6. Wait 2 minutes for the beads to collect in the bottom of the well.
7. Aspirate the supernatant with the vacuum manifold at a pressure of 50 mbar.
8. Repeat steps 4 through 7 once.
9. Do one of the following:
 - a. Proceed to *Scan VeraCode Bead Plate* on page 78.
 - b. Seal the VBP plate with an adhesive seal and store it in the dark at room temperature for up to 24 hours.

Scan VeraCode Bead Plate

The BeadXpress Reader uses lasers to excite the Cy3 and Cy5 fluors of the single-stranded PCR products bound to the VeraCode beads. Light emissions from these fluors are then recorded in a data file. Fluorescence data are analyzed to derive genotyping results using Illumina's GenomeStudio software package.

Estimated Time	80 samples/hour at 96-plex 27 samples/hour at 384-plex
-----------------------	---

Preparation	<ul style="list-style-type: none">▶ Prepare a scan settings file containing information about your samples, the BeadXpress Reader settings, and VeraCode beads. If you intend to analyze GoldenGate genotyping data in GenomeStudio, you should enter the VeraCode Bead Plate serial number (CK#-VBP) in the Plate_ID field.
Steps	For instructions on scanning VeraCode Bead Plate, see the <i>BeadXpress Reader System Guide</i> .

Troubleshooting

Use the information in this section to troubleshoot the GoldenGate Genotyping Assay for VeraCode.

DNA Sample Preparation

Table 19 Problems Observed During DNA Sample Preparation

Symptom	Probable Cause	Resolution	Comments
Some or all of the contents of the wells in the SUD plate evaporated during the 95°C incubation.	The heat seal was not completely sealed to the plate, allowing evaporation.	Check the heat sealer to ensure that it is functioning properly.	
	The incorrect seal was used to seal the plate.	Use ABgene (catalog # AB-0559) foil seals for this step.	
Excessive condensation was observed on the bottom side of the heat seal after the Make ASE incubation.	The heated lid was not used.	Centrifuge the plate to remove condensation from the seal and proceed to the Add MEL step.	Some condensation is normal. To minimize condensation, use an ABgene (catalog # AB-0559) foil seal.
The heat block was left at 70°C overnight for the Make ASE incubation.	The heat block temperature was not set to 30°C after loading the plate into the heat block.	Repeat the experiment. The samples have been ruined.	Set the temperature to 30°C immediately after loading the ASE plate into the heat block.
Beads are difficult to resuspend during Add MEL and Inoc PCR.	The high-speed shaker may be out of calibration.	Recalibrate the high-speed shaker (see <i>Calibrate the Vortexer</i> on page 21).	Particularly difficult samples can be resuspended manually using a pipettor.

Hybridize VeraCode Bead Plate

Table 20 Hyb VBP

Symptom	Probable Cause	Resolution	Comments
The cap mat came up in parts of the VBP during the 45°C incubation.	The cap mat was not completely sealed.	Use the cap mat applicator to ensure that the cap mat is completely sealed to the plate.	

Signal Intensity

Table 21 Problems with Signal Intensity

Symptom	Probable Cause	Resolution	Comments
Low intensity was observed in all the bead types of the VBP while scanning.	Incorrect wash buffer.	Repeat the experiment.	Use VW1 to wash the sample plate.
	Incorrect PMT setting.	Stop the current scan and rescan the VBP using a higher PMT setting.	This can only be done for the current column and those following it.
	DNA concentration was too low.	Recheck the DNA concentration with PicoGreen.	

Analysis

Table 22 Problems Observed During Analysis

Symptom	Probable Cause	Resolution	Comments
Low correlation between sample replicates.	The incorrect cycler program was used or there were problems with the cycler temperature control.	Recheck the cycler program or measure the time for completion of PCR plate cycling. Expected times range from 2 hours and 45 minutes to 3 hours and 5 minutes, depending on the cycler.	Always record times and compare them to historical norms.
	The post-hyb wash was not done after the 3-hour 45°C incubation.	Repeat the experiment.	
Strong signal from multiple contamination controls observed in the control panel.	The OPA tubes were pooled during the experiment.	For the contamination controls to be informative, do not pool the contents of multiple OPA tubes.	OPA tubs with the same barcode may still have different contamination controls.
	Cross-contamination may have occurred.	Take care to avoid PCR amplicon contamination. For example, use the GoldenGate kit with UDG to control amplicon contamination. Treat lab work surfaces with 10% bleach and allow them to air-dry.	

Table 22 Problems Observed During Analysis (Continued)

Symptom	Probable Cause	Resolution	Comments
Cy3/Cy5 ratio was higher than usual in the second hybridization controls.	The PCR plate with MPB beads in the Bind PCR process was subjected to excessive light.	Protect the PCR plate from light. Fluorescent lighting is permissible, but keep the plates in the dark when they are not in use.	
	Bleach or bleach fumes may have been present.	Remove the bleach container during the procedure. Allow bleach fumes to dissipate after cleaning lab surfaces.	In sufficient concentration, bleach affects Cy3.
	The washed VBP plate was subjected to excessive light before scanning.	Protect the washed VBP plate from light while waiting for the scanner.	It is normal to see a slightly increased Cy3/Cy5 ratio with a washed VBP plate that has been staged for longer than 1 day.
The samples appeared to have intensity but did not get called.	There were fewer than three beads for that bead type.	You can make the correct call by looking at the raw intensities.	
The genotyping results did not correlate with the samples.	The plate orientations were reversed.	Re-sort the data in inverse order, H12 to A1, and reanalyze them.	Illumina recommends adding a positive known control sample in a standardized, non-symmetric well position.
	The wrong scan settings file was loaded.	Load the correct scan settings file.	
	The wrong OPA manifest was loaded into GenomeStudio or associated with a sample sheet.	Load the correct OPA manifest.	
	The samples were copied directly from the scan settings file to the sample sheet, or vice versa.	Rearrange the sample names according to the templates for a row-major or column-major file.	

Chapter 4

GoldenGate Genotyping Assay for VeraCode Automated Protocols

Topics

- 84 Introduction
- 85 Workflow
- 86 Equipment, Materials, and Reagents for This Assay
- 89 Make Standard DNA Plate (Optional)
- 92 Make DNA Quantitation Plate (Optional)
- 96 Read QDNA (Optional)
- 98 Make Single-Use DNA (SUD) Plate
- 102 Precipitate SUD Plate
- 106 Resuspend SUD Plate
- 109 Make Allele-Specific Extension (ASE) Plate
- 112 Add Extension and Ligation Reagents (MEL)
- 120 Make PCR Plate
- 123 Inoculate PCR Plate
- 127 Thermal Cycle PCR Plate
- 128 Bind PCR Products
- 131 Make VeraCode Bead Plate
- 135 Hybridize VeraCode Bead Plate
- 136 Wash VeraCode Bead Plate
- 139 Scan VeraCode Bead Plate

Introduction

The instructions in this chapter explain how to perform the GoldenGate Genotyping Assay for VeraCode using a Tecan eight-tip robot.

The instructions assume that you are preparing 96 DNA samples. If you are preparing fewer than 96 samples, scale down the protocols accordingly.

The instructions in this chapter assume that you have already familiarized yourself with Chapter 2, *Standard Operating Procedures* and have set up the lab area appropriately.



CAUTION

It is very important to prevent PCR product contamination during this assay. Follow all of the safety procedures described in this chapter.

Workflow

Figure 22 graphically represents the workflow for the automated GoldenGate Genotyping Assay for VeraCode.

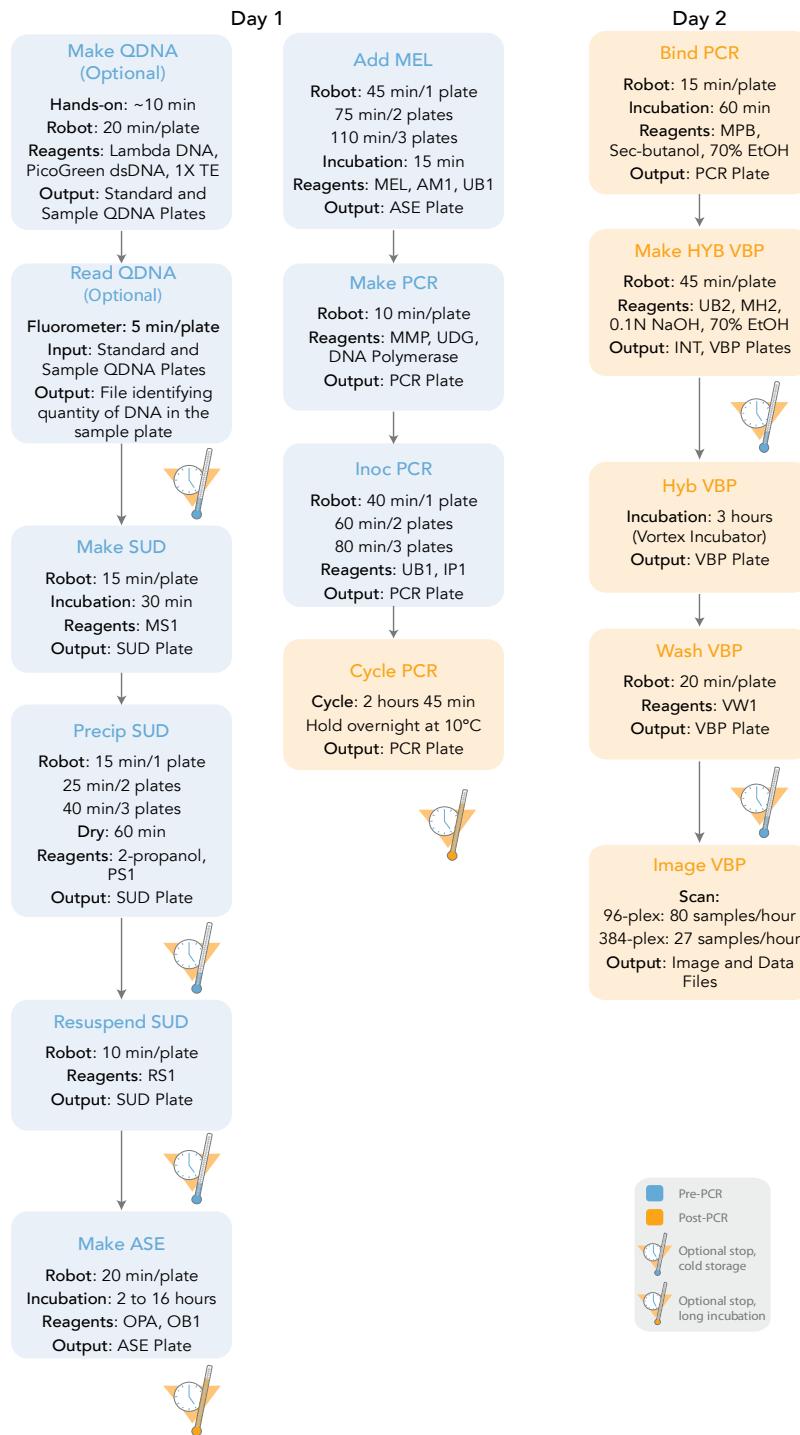


Figure 22 Automated GoldenGate Genotyping Assay for VeraCode Workflow

Equipment, Materials, and Reagents for This Assay

These materials are specific to the automated GoldenGate Genotyping Assay for VeraCode. For a list of other equipment, materials, and reagents required in a BeadXpress Reader lab, see *Standard Equipment, Materials, and Reagents* on page 17.

For information about tracking tools for this assay, see *Tracking Tools* on page 39.

Equipment Illumina-Supplied

Table 23 Illumina-Supplied Equipment

Item	Catalog #
Two Tecan eight-tip robots One for pre- and one for post-PCR processes	Non-LIMS customers <ul style="list-style-type: none"> • SC-16-401 (110V)—North America • SC-16-402 (220V)—EU and Asia Pacific (Except Japan) LIMS customers <ul style="list-style-type: none"> • SC-16-403 (110V)—North America • SC-16-404 (220V)—EU and Asia Pacific (Except Japan)
Reservoir, full, 150 ml	Beckman Coulter, catalog # 372784
Reservoir, half, 75 ml	Beckman Coulter, catalog # 372786
Reservoir, quarter, 40 ml	Beckman Coulter, catalog # 372790
Reservoir frame	Beckman Coulter, catalog # 372795

Materials and Illumina-Supplied Reagents

Table 24 Illumina-Supplied Materials

Item	Catalog #
Filter plate adapter	
VeraCode 96-Plex GoldenGate Kit, 480 samples <ul style="list-style-type: none"> • BOX A VeraCode DNA Activation Kit • BOX B VeraCode GoldenGate Pre-PCR #1 • BOX C VeraCode GoldenGate Pre-PCR #2 • BOX D VeraCode GoldenGate Post-PCR • BOX E 96-Plex VeraCode Bead Plates • OPA GoldenGate Assay custom oligo pool, shipped separately <i>Description of box contents appears below.</i>	VC-201-0096

Table 24 Illumina-Supplied Materials (Continued)

Item	Catalog #
VeraCode 384-Plex GoldenGate Kit, 480 samples • BOX A VeraCode DNA Activation Kit • BOX B VeraCode GoldenGate Pre-PCR #1 • BOX C VeraCode GoldenGate Pre-PCR #2 • BOX D VeraCode GoldenGate Post-PCR • BOX E 384-Plex VeraCode Bead Plates • OPA GoldenGate Assay custom oligo pool, shipped separately <i>Description of box contents appears below.</i>	VC-201-0384

VeraCode GoldenGate Kit Box Contents

Table 25 VeraCode Box Contents

Box	Contents
BOX A VeraCode DNA Activation Kit	MS1 —Reagent used to activate sufficient DNA PS1 —Precipitation solution for DNA activation RS1 —Resuspension solution for DNA activation
BOX B VeraCode GoldenGate Pre-PCR #1	OB1 —Oligo hybridization and cDNA and gDNA binding buffer MMP —Master mix for PCR reagent IP1 —Reagent used to elute extended and ligated products UB1 —Universal buffer used to wash paramagnetic beads
BOX C VeraCode GoldenGate Pre-PCR #2	MEL —Reagent used for extension and ligation AM1 —Reagent used to wash away non-specifically hybridized and excess oligos from the gDNA
BOX D VeraCode GoldenGate Post-PCR	MPB —Magnetic particle reagent used to bind double-stranded PCR products MH2 —Reagent used to make the VBP plate UB2 —Universal buffer used to wash magnetic particles and the SAM VW1 —Reagent used to wash the VeraCode beads
BOX E 96-Plex VeraCode Bead Plates	96-plex VeraCode Bead Plate (5)
BOX E 384-Plex VeraCode Bead Plates	384-plex VeraCode Bead Plate (5)

Labels

- ▶ QDNA barcode labels
- ▶ GS#-DNA barcode labels
- ▶ SUD barcode labels
- ▶ ASE barcode labels
- ▶ PCR barcode labels
- ▶ Filter plate: GS_____ -PCR labels
- ▶ INT barcode labels

- ▶ Vortexer calibration label

Reagents User-Supplied

Table 26 User-Supplied Reagents

Item	Source
Quant-iT PicoGreen DNA quantification reagent	Molecular Probes Invitrogen, catalog # P7581
Lambda DNA	Invitrogen, catalog # 25250-028

Make Standard DNA Plate (Optional)

This process creates a Standard DNA plate with specific concentrations of DNA in the wells. Use this plate as input into the Make QDNA process.

Estimated Time Hands-on: ~10 minutes

Consumables

Item	Quantity	Storage	Supplied By
1X TE (10 mM Tris-HCl pH8.0, 1 mM EDTA)	See instructions	Room temperature	User
Lambda DNA	See instructions	-20°C	User
96-well 0.65 ml microtiter plate (MIDI)	1 per 96 samples		User

Preparation

- ▶ Remove PicoGreen reagent from freezer and thaw at room temperature for 60 minutes in a light-impermeable container.
- ▶ Label a 96-well MIDI plate "Standard DNA."

Steps **Make Standard DNA MIDI Plate**

In this process, you create a Standard DNA plate with serial dilutions of stock Lambda DNA in the wells of column 1.

1. Place stock Lambda DNA in well A1 of the Standard DNA MIDI plate and dilute it to 75 ng/µl in a final volume of 233.3 µl.
 - a. Use the following formula to calculate the amount of stock Lambda DNA to add to A1:

$$\frac{(233.3 \mu\text{l}) \times (75 \text{ ng}/\mu\text{l})}{(\text{stock Lambda DNA concentration})} = \mu\text{l of stock Lambda DNA to add to A1}$$

- b. Dilute the stock DNA in well A1 using the following formula:

$$\mu\text{l of 1X TE to add to A1} = 233.3 \mu\text{l} - \mu\text{l of stock Lambda DNA in well A1}$$

2. Add 66.7 µl 1X TE to well B of column 1 of the same plate.
3. Add 100 µl 1X TE to wells C, D, E, F, G, and H of column 1 of the same plate.

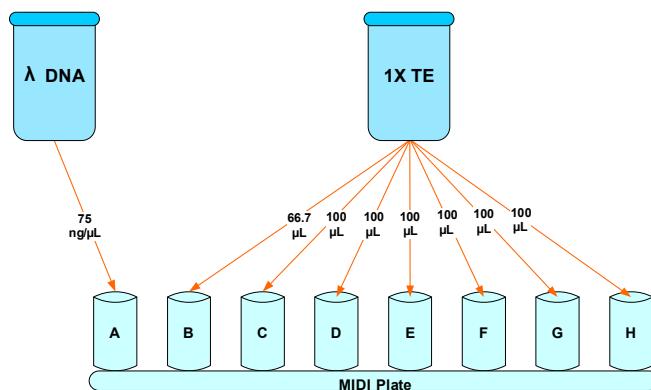


Figure 23 MIDI Plate Wells

4. Pipette the contents of A1 up and down 10 times to mix.
5. Transfer 133.3 μ L of Lambda DNA from well A1 into well B1, and then pipette the contents of well B1 up and down 10 times.
6. Change pipette tips. Transfer 100 μ L from well B1 into well C1, and then pipette the contents of well C1 up and down 10 times.
7. Change pipette tips. Transfer 100 μ L from well C1 into well D1, and then pipette the contents of well D1 up and down 10 times.
8. Change pipette tips. Transfer 100 μ L from well D1 into well E1, and then pipette the contents of well E1 up and down 10 times.
9. Change pipette tips. Transfer 100 μ L from well E1 into well F1, and then pipette mix the contents of well F1 up and down 10 times.
10. Change pipette tips. Transfer 100 μ L from well F1 into well G1, and then pipette the contents of well G1 up and down 10 times.
- 11. Do not transfer solution from well G1 to well H1.** Well H1 serves as the blank 0 ng/ μ L Lambda DNA.

Table 27 Concentration of Lambda DNA Standards

Row-Column	Conc. (ng/ μ L)	Final Volume in Well (μ L)
A1	75	100
B1	50	100
C1	25	100
D1	12.5	100
E1	6.25	100
F1	3.125	100
G1	1.5625	200
H1	0	100

12. Cover the Standard DNA plate with a cap mat.

13. Do one of the following:

- Proceed to *Make DNA Quantitation Plate (Optional)* on page 92.
- Store the plate at 4°C for future use.

Make DNA Quantitation Plate (Optional)

In this process, you create one to three QDNA plates for use in the Molecular Dynamics Fluorometer (if available). Quantitation ensures that there is enough sample DNA to generate good data.

Estimated Time	Hands-on: ~10 minutes Robot: 20 minutes per plate
-----------------------	--

Consumables

Item	Quantity	Storage	Supplied By
1X TE	10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE)	Room temperature	User
PicoGreen dsDNA	See instructions	-20°C	User
Black Fluotrac plate	2 per 96 samples		Illumina
Standard DNA plate	1 per 96 samples	-20°C	Illumina
GS#-DNA plate with DNA samples, normalized to 50 ng/ μ l	8, 16, 24, 32, 48, or 96 DNA samples in 1 to 3 plates	-20°C	User



NOTE

PicoGreen is susceptible to differential contaminants. False positives may occur for whole-genome amplification. Therefore, it is important to quantitate the input into the whole-genome amplification reaction.

Preparation

- ▶ Thaw the PicoGreen reagent for 60 minutes in a light-protected environment.
- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ Thaw the GS#-DNA plate to room temperature (22°C).
- ▶ For each GS#-DNA plate, apply a QDNA barcode label to a new black Fluotrac plate.
- ▶ Label the remaining black Fluotrac plate "Standard QDNA."
- ▶ In the Sample Sheet, enter the Sample_Name (optional) and Sample_Plate for each Sample_Well.

Prepare PicoGreen Dilution



CAUTION

PicoGreen reagent degrades quickly in the presence of light. Do not use glass containers for PicoGreen reagent.

1. Wrap aluminum foil around a sterile plastic container to prevent light penetration.
2. Make a 1:200 dilution of PicoGreen to 1X TE in the sterile plastic container.

Table 28 PicoGreen Reagent Volumes

# QDNA Plates	PicoGreen Volume (μ l)	1X TE Volume (ml)
1	125	25
2	230	45
3	325	65

You can prepare dilutions for up to three sample plates at a time.

3. Mix dilution thoroughly.

Steps Make QDNA

1. At the robot PC, select **GTS Pre-PCR Tasks | DNA Prep | Make QDNA**.
2. In the DNA Plate Selection dialog box, select the plate type of the Standard DNA and Sample DNA plates. They should all be MIDI plates or all be TCY plates. Roll the mouse pointer over each picture to see a description of the plate.

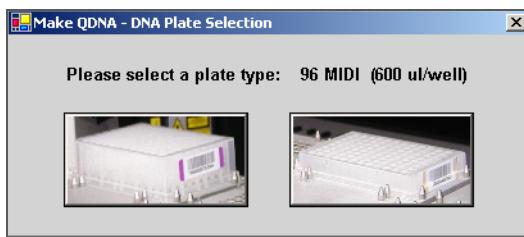


Figure 24 DNA Plate Selection Dialog Box (MIDI Selected)

3. In the Basic Run Parameters pane, enter the **Number of DNA/QDNA plates** (1, 2, or 3) and the number of **Total Samples in DNA**.
The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

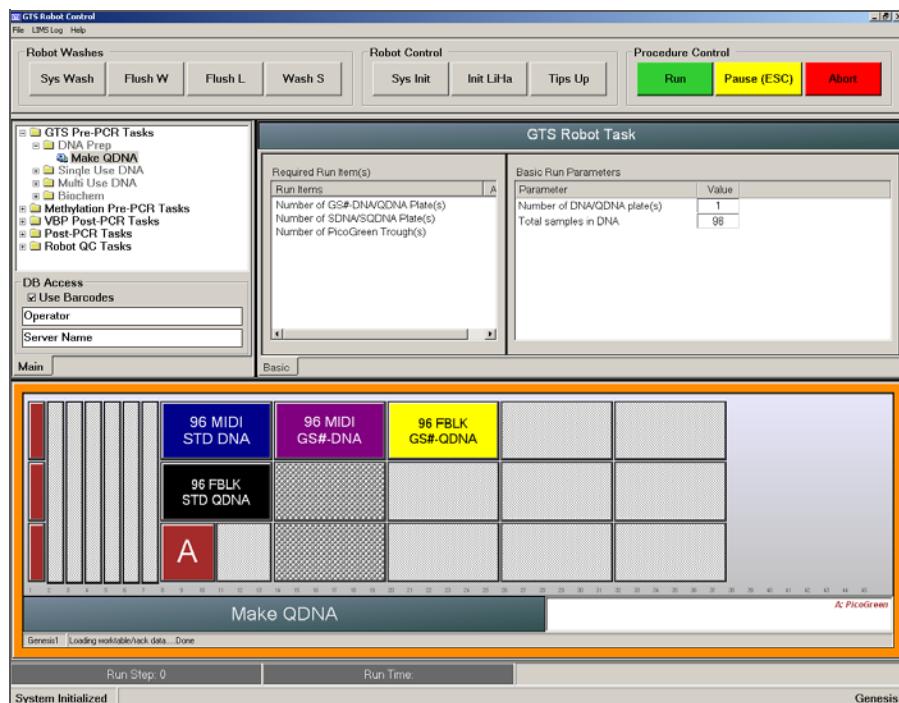


Figure 25 GTS Pre-PCR Tasks / DNA Prep / Make QDNA

4. Do one of the following:
 - Vortex MIDI plates at 1450 rpm (actual vortex speed) for 1 minute
 - Vortex TCY plates at 1250 rpm for 1 minute
5. Centrifuge the GS#-DNA plate to 280 xg for 1 minute.
6. Vortex the Standard DNA plate at 1450 rpm for 1 minute.
7. Centrifuge the Standard DNA plate to 280 xg for 1 minute.
8. Place the GS#-DNA, Standard DNA, Standard QDNA, and QDNA plates on the robot bed according to the robot bed map. Place well A1 at the top-left corner of its robot bed carrier. Remove any plate seals.
9. Pour the PicoGreen dilution into half reservoir A and place it on the robot bed.
The robot transfers stock Lambda DNA dilution into the Standard QDNA plate, and then to the Standard QDNA plate. Sample DNA from the GS#-DNA plate will be transferred into the GS#-QDNA Fluotrac plate.
10. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
11. On the lab tracking form, record the position of the plate(s) on the robot bed.
12. Clear the **Use Barcodes** checkbox and click **Run**. Observe the robot start to run to ensure there are no problems.
The robot PC sounds an alert and displays a message when the process is complete.

13. Click **OK** in the message box.
14. On the lab tracking form, record:
 - Date/Time
 - Operator
 - Robot
 - The QDNA barcode that corresponds to each GS#-DNA barcode
 - The Standard QDNA plate that corresponds to each Standard DNA plate
15. After the robot finishes, immediately seal all plates:
 - a. Place foil adhesive seals over GS#-QDNA and Standard QDNA plates.
 - b. Place cap mats on GS#-DNA and Standard DNA plates.
16. Discard unused reagents in accordance with facility requirements.
17. Centrifuge the QDNA and Standard QDNA plates to 280 xg for 1 minute.
18. Proceed to *Read QDNA (Optional)* on page 96.

Read QDNA (Optional)

In this process, you use a fluorometer along with the GTS Fluorometry Analysis software to interpret the quantitated DNA in the QDNA plate(s) and obtain the exact concentration of DNA in the sample. Illumina recommends using a fluorometer because fluorometry provides DNA-specific quantitation. Spectrophotometry may also measure RNA, yielding values that are too high.

Estimated Time Fluorometer: 5 minutes per plate

- Steps**
1. Turn on the fluorometer.
 2. Open the GTS Fluorometry Analysis software.
 3. Select **Reader Tasks | Read QDNA**.

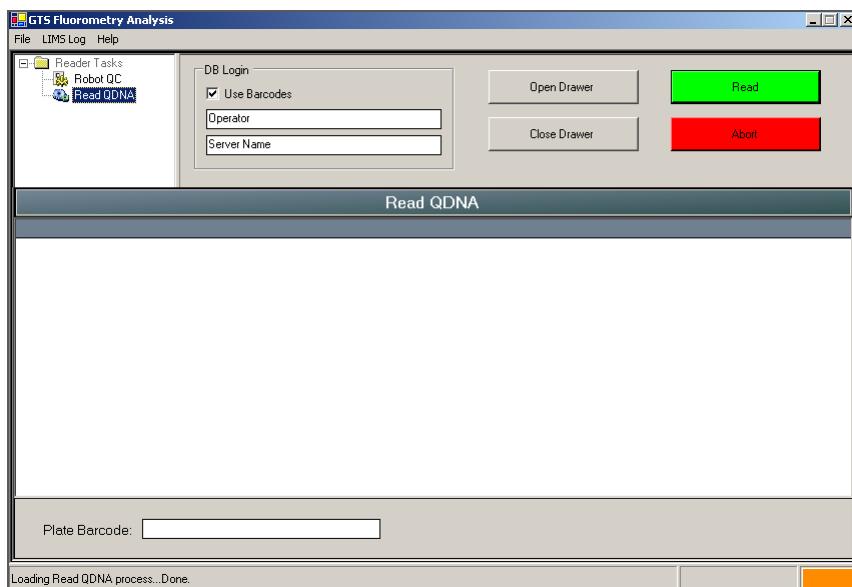


Figure 26 GTS Fluorometry Analysis Main Screen

4. Clear the **Use Barcodes** checkbox.
5. Click **Read**.
6. When prompted, enter the number of Sample QDNA plates you want to read (1, 2, or 3). Do not include the Standard QDNA plate in this number. Click **OK**.

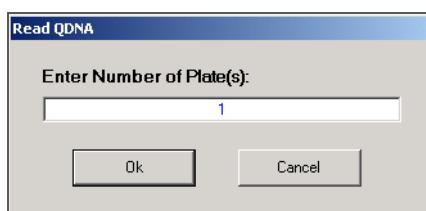


Figure 27 Number of Sample QDNA Plates

7. Remove the seal from the Standard QDNA plate, place the plate in the fluorometer tray, and click **OK**. The fluorometer reads the plate data.
8. Review the data from the Standard QDNA plate. Either accept it and go on to the next step, or reject it and read another plate.
9. Remove the Standard QDNA plate from the fluorometer tray.
10. When prompted, hand-scan the Sample QDNA plate barcode. Click **OK**.
11. When prompted, remove the plate seal from the Sample QDNA plate and load it into the fluorometer tray, with well A1 at the upper left corner. Click **OK**.
The fluorometer reads the Sample QDNA plate.
12. When prompted, click **Yes** to review the raw Sample QDNA plate data. Microsoft Excel opens automatically at the same time and displays the quantitation data for the Sample QDNA plate. There are three tabs in the file:
 - **QDNA_STD**—Plots the RF values against the concentration (ng/µl).
 - **QDNA**—Plots the concentration (ng/µl) for each well.
 - **Data**—Compares the data from the Standard QDNA plate to the Sample QDNA plate you just read.

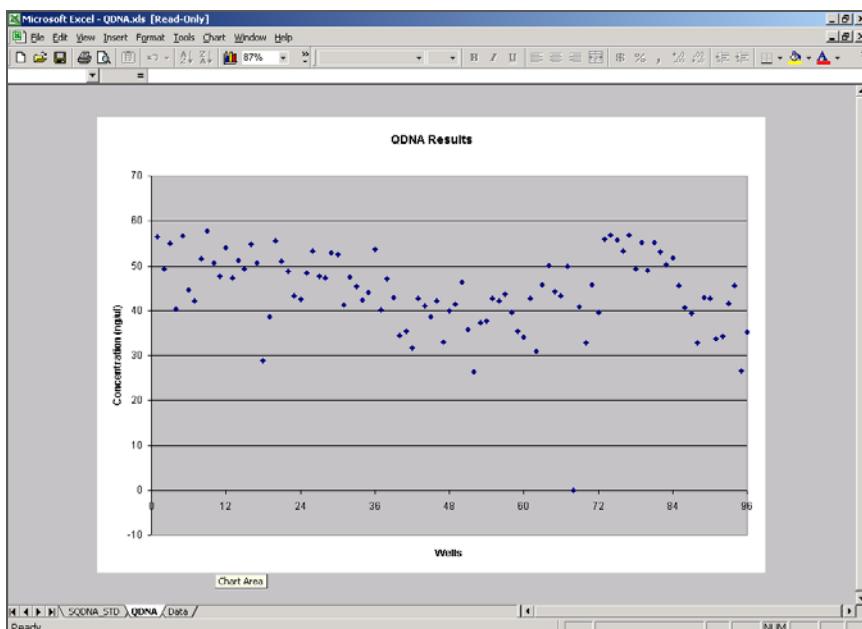


Figure 28 Sample QDNA Data

The GTS Fluorometer Analysis software prompts you to indicate whether you wish to save the QDNA data shown in the Excel file.

13. If you entered more than one Sample QDNA plate to read, repeat steps 10 to 12 for each additional plate.
14. Discard the QDNA plates and reagents in accordance with facility requirements.
15. Proceed to *Make Single-Use DNA (SUD) Plate* on page 98.

Make Single-Use DNA (SUD) Plate

In this process, the robot transfers nucleic acid activator reagent (MS1) to the SUD plate, followed by sample. After the robot procedure is compete, the plate is sealed and incubated on a heat block at 95°C for 30 minutes to activate the gDNA.

Estimated Time

Robot:

- 1 plate: 15 minutes
- 2 plates: 30 minutes
- 3 plates: 45 minutes

Incubation: 30 minutes

Consumables

Item	Quantity	Storage	Supplied By
MS1 reagent	1 tube per SUD plate	-20°C	Illumina
10 mM Tris pH 8.0/1 mM EDTA	Bottle	Room temperature	User
gDNA samples and controls	96 or 384 At least 12 µl per sample well	-20°C	User
96-well 0.2 ml skirted microplate (TCY)	1 per 96 samples		User

Preparation

- ▶ Preheat the heat block to 95°C and allow it to equilibrate.
- ▶ Turn on the heat sealer to preheat it.
- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ Thaw the DNA samples and controls to room temperature and vortex to mix the contents.
- ▶ Normalize all samples to a concentration of 50 ng/µl.
- ▶ Thaw the MS1 tube to room temperature in a light-protected location. Vortex briefly to mix.



MS1 reagent is photosensitive. Store it and thaw it away from light.

- ▶ Apply a SUD barcode label to a new 96-well microplate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator

- Robot
- SUD plate barcode
- MS1 reagent barcode

**NOTE**

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. From the robot PC, select **GTS Pre-PCR Tasks | Single Use DNA | Make SUD**.
2. Select the plate type (MIDI or TCY).

**CAUTION**

Do not mix plate types on the robot bed.

3. In the Basic Run Parameters pane, enter the **Number of DNA/SUD Plates** (1, 2, 3) and the **Total samples in DNA plate** (16, 24, 32, 48, 96).

The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

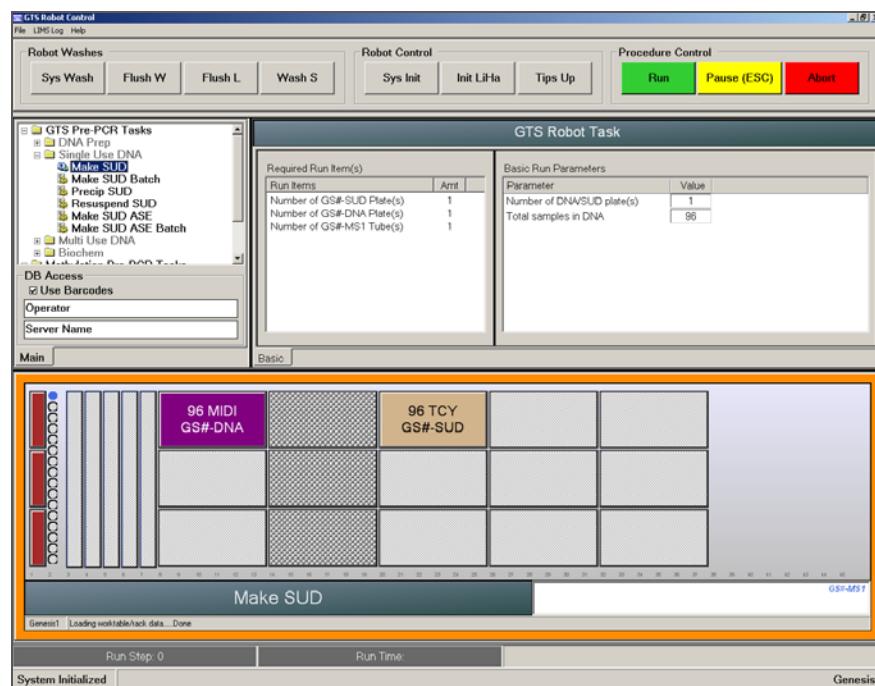


Figure 29 GTS Pre-PCR Tasks | Single Use DNA | Make SUD

4. Pulse centrifuge the MS1 tube to 280 xg for 1 minute.

5. Place the MS1 tube in the robot tube rack according to the robot bed map. Remove the cap.
6. Pulse centrifuge the GS#-DNA plate to 280 xg for 1 minute.
7. Place the GS#-DNA and SUD plates on the robot bed according to the robot bed map. Remove any plate seals.

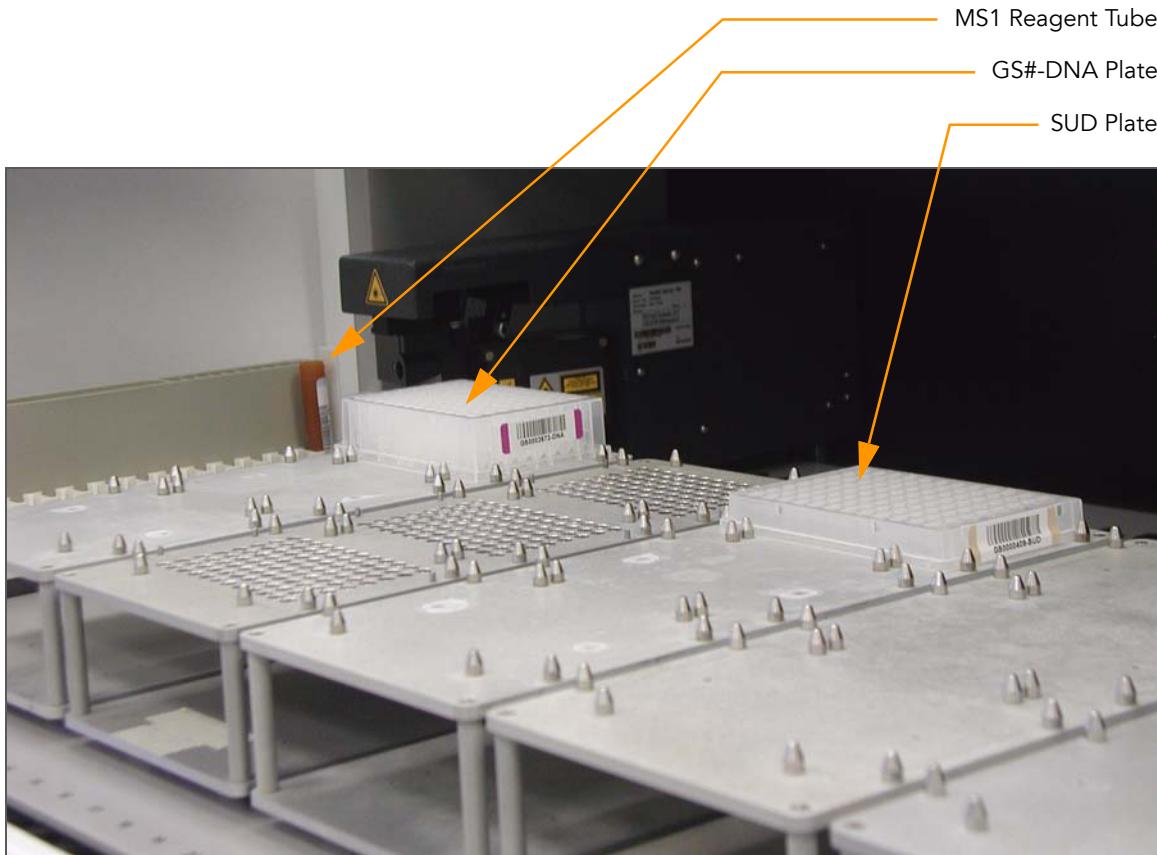


Figure 30 Robot Bed for Make SUD

8. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
9. On the lab tracking form, record the position of the plate(s) on the robot bed.
10. Clear the **Use Barcodes** checkbox and click **Run**. Observe the robot start to run to ensure there are no problems.
The robot PC sounds an alert and displays a message when the process is complete.
11. When the robot finishes, heat-seal the SUD plate with a foil heat seal.
12. Pulse centrifuge the SUD plate to 280 xg for 1 minute.
13. Vortex the plate at 2300 rpm for 20 seconds, or until all wells are completely mixed.
14. Pulse centrifuge the SUD plate to 280 xg for 1 minute.

15. Place the sealed SUD plate in the preheated 95°C heat block and close the lid. Incubate the plate for 30 minutes.
16. [Optional] After incubation, place a weight on the plate and allow it to cool for 3 minutes.
17. Pulse centrifuge the SUD plate to 3000 xg for 1 minutes.
18. If you plan to perform the Make ASE protocol today, preheat the heat block to 70°C.
19. Do one of the following:
 - Proceed to *Precipitate SUD Plate* on page 102.
 - Store the sealed SUD plate at 4°C for up to 12 hours.

Precipitate SUD Plate

In this process, the robot adds PS1 reagent and 2-propanol to the SUD plate to precipitate the DNA.

Estimated Time

Robot:

- 1 plate: 15 minutes
- 2 plates: 25 minutes
- 3 plates: 40 minutes

Hands-on: 30 minutes

Drying: 1 hour

Consumables

Item	Quantity	Storage	Supplied By
PS1 reagent	Bottle	Room temperature	Illumina
2-propanol	Bottle	Room temperature	User

Preparation

- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - Robot
 - PS1 reagent barcode
 - 2-propanol lot number



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. From the robot PC, select **GTS Pre-PCR Tasks | Single Use DNA | Precip SUD**.
2. Select the plate type (MIDI or TCY). All plates on the robot bed must be the same type.
3. In the Basic Run Parameters pane, enter the **Number of SUD Plates** (1, 2, 3) and the **Total samples in DNA plate** (16, 24, 32, 48, 96).
The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

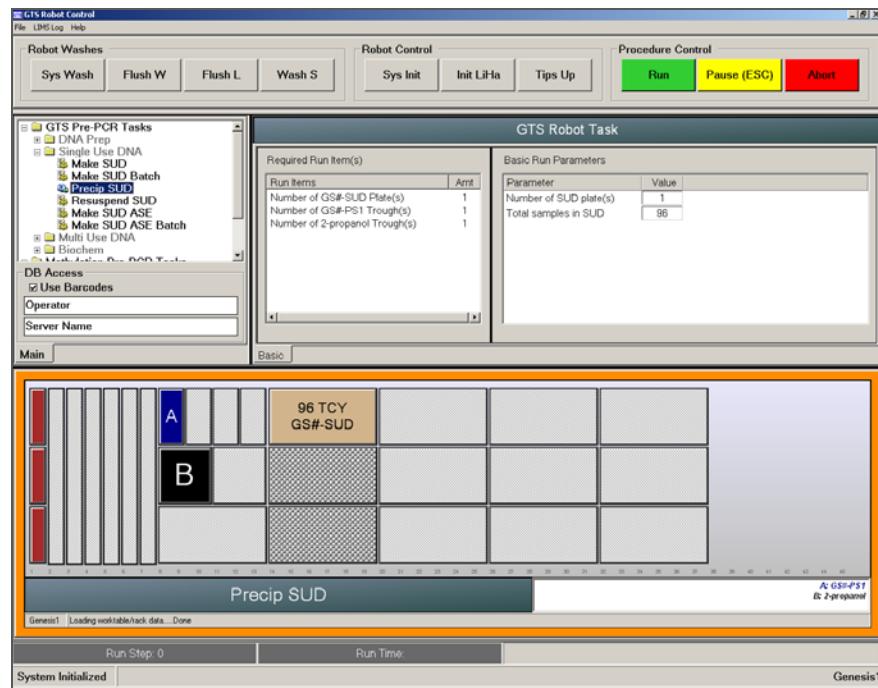


Figure 31 GTS Pre-PCR Tasks / Single Use DNA / Precip SUD

4. Place quarter reservoir A onto the robot bed according to the robot bed map. Dispense PS1 as follows:
 - 1 plate: 2 ml
 - 2 plates: 2.5 ml
 - 3 plates: 3 ml
5. Place half reservoir B onto the robot bed according to the robot bed map. Dispense 2-propanol as follows:
 - 1 plate: 9 ml
 - 2 plates: 17 ml
 - 3 plates: 25 ml
6. Place the SUD plate on the robot bed according to the robot bed map. Remove any plate seals.

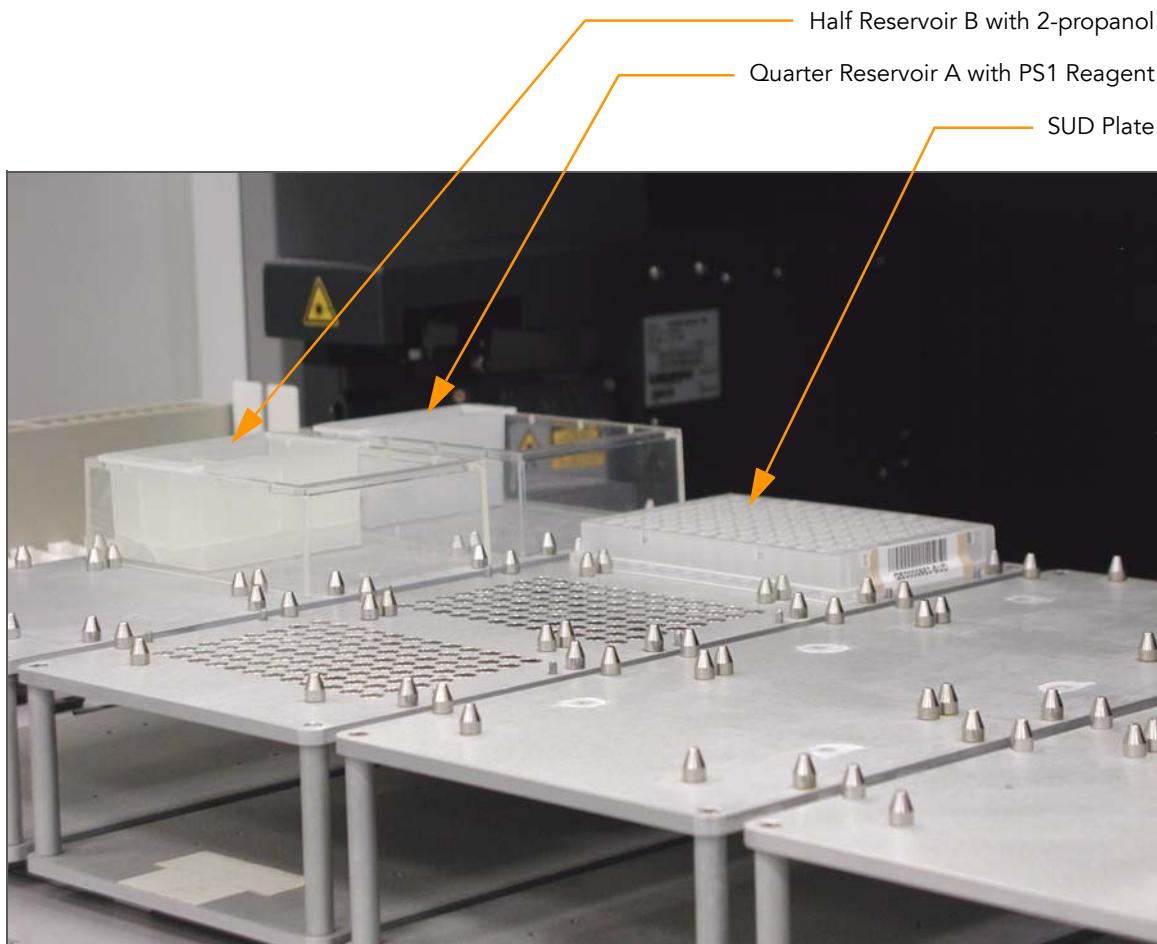


Figure 32 Robot Bed for Precip SUD

7. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
8. On the lab tracking form, record the position of the plate(s) on the robot bed.
9. Clear the **Use Barcodes** checkbox and click **Run**. Observe the robot start to run to ensure there are no problems.
The robot PC sounds an alert and displays a message when the process is complete.
10. When the robot finishes, seal the SUD plate with adhesive film.
11. Vortex the plate at 1600 rpm for 20 seconds, or until all wells are uniformly blue.
12. Centrifuge the SUD plate to 3000 xg for 20 minutes. A faint blue pellet should be at the bottom of each well.

**NOTE**

If you do not see a faint blue pellet at the bottom of each well, the DNA has not precipitated. In some cases, depending on DNA quality, the blue pellet may appear diffuse.

13. On the lab tracking form, record the start and stop times.

Perform the next step immediately to avoid dislodging the activated DNA pellets. If any delay occurs, recentrifuge to 3000 xg for 10 minutes before proceeding.

14. Remove the SUD plate seal and decant the supernatant by inverting the plate and smacking it down onto an absorbent pad.**CAUTION**

Do not tilt the plate, as this can cause cross-contamination between wells. Tap the plate firmly enough to decant all the supernatant; tapping lightly will not work as well.

15. Tap the inverted plate onto the pad to blot excess supernatant.**16.** Place the inverted SUD plate on an absorbent pad and centrifuge to 8 xg for 1 minute.**WARNING**

Do not spin the inverted plate to more than 8 xg, or the sample will be lost!

17. Remove the SUD plate from the centrifuge.**18.** Set the plate upright and allow it to dry at room temperature for 1 hour.**19.** On the lab tracking form, record the start and stop times.**20.** Do one of the following:

- Proceed to *Resuspend SUD Plate* on page 106.
- Seal the plate with adhesive film and store at -20°C for up to 24 hours.

Resuspend SUD Plate

In this process, the robot adds RS1 reagent to the SUD plate to resuspend the sample.

Estimated Time

Robot:

- 1 plate: 10 minutes
- 2 plates: 20 minutes
- 3 plates: 30 minutes

Consumables

Item	Quantity	Storage	Supplied By
RS1 reagent	Bottle	Room temperature	Illumina

Preparation

- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - Robot
 - RS1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. From the robot PC, select **GTS Pre-PCR Tasks | Single Use DNA | Resuspend SUD**.
2. Select the plate type (MIDI or TCY).



CAUTION

Do not mix plate types on the robot bed.

3. In the Basic Run Parameters pane, enter the **Number of SUD Plates** (1, 2, 3) and the **Total samples in DNA** plate (16, 24, 32, 48, 96).

The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

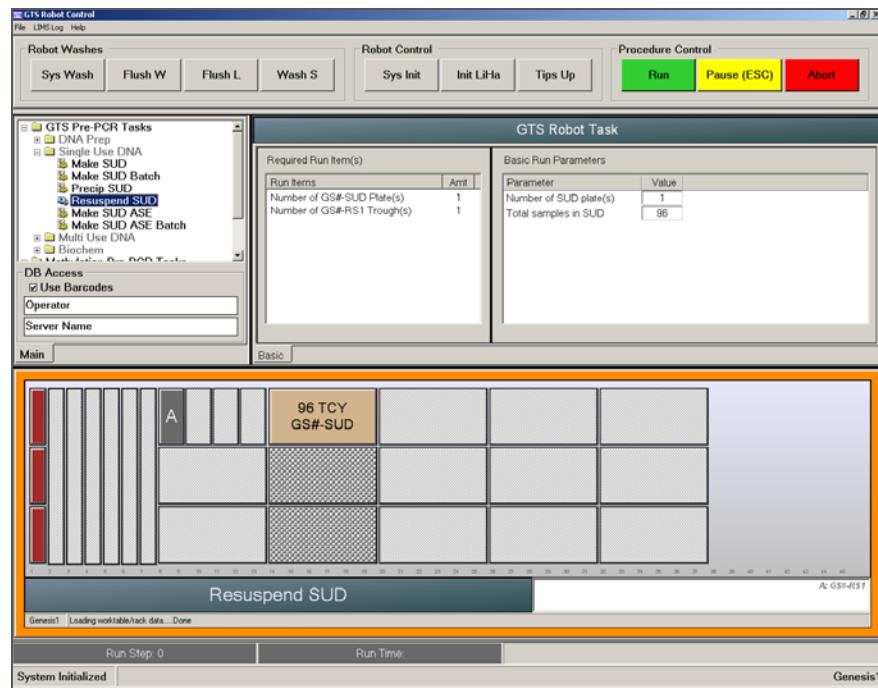


Figure 33 GTS Pre-PCR Tasks / Single Use DNA / Resuspend SUD

4. Place quarter reservoir A onto the robot bed according to the robot bed map. Dispense RS1 as follows:
 - 1 plate: 3.5 ml
 - 2 plates: 6 ml
 - 3 plates: 8.5 ml
5. Place the SUD plate on the robot bed according to the robot bed map. Remove any plate seals.

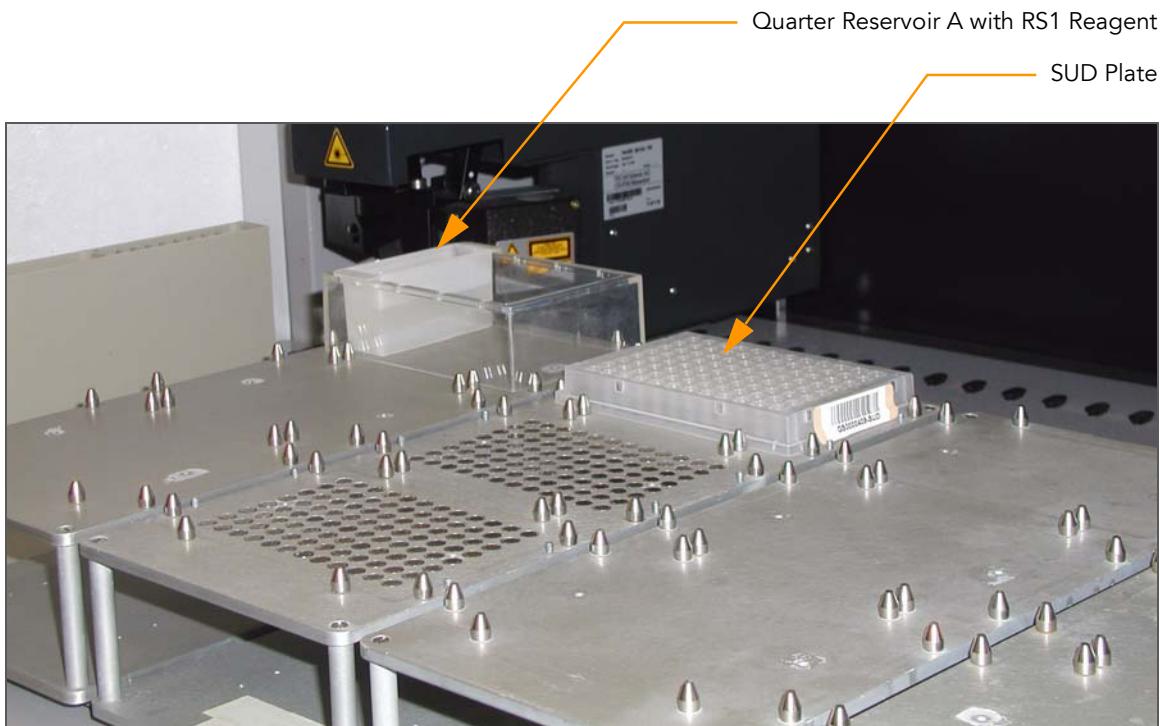


Figure 34 Robot Bed for Resuspend SUD

6. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
7. On the lab tracking form, record the position of the plate(s) on the robot bed.
8. Clear the **Use Barcodes** checkbox and click **Run**. Observe the robot start to run to ensure there are no problems.
The robot PC sounds an alert and displays a message when the process is complete.
9. When the robot finishes, seal the SUD plate with adhesive film.
10. Pulse centrifuge the plate to 280 xg for 1 minute.
11. Vortex the plate at 2300 rpm for 20 seconds, or until all wells are uniformly blue.
12. Do one of the following:
 - Proceed to *Make Allele-Specific Extension (ASE) Plate* on page 109.
 - Remove the adhesive seal from the SUD plate, and then heat-seal it with a foil heat seal. Store it at -20°C for up to 2 months.

Make Allele-Specific Extension (ASE) Plate

In this process, the robot combines the assay oligonucleotides in the OPA reagent tube and the hybridization reagents and buffers from the OB1 reagent tube in a new 96-well ASE plate. The resuspended DNA samples are transferred from the SUD plate into the ASE plate. The ASE plate is subsequently incubated to allow the activated DNA to bind with the paramagnetic particles and anneal with the oligonucleotides.

The OPA reagent contains a pool of oligonucleotide triplets designed to hybridize to the SNPs of interest: one triplet for each SNP being analyzed. The triplets consist of two allele-specific oligonucleotides (ASO1 and ASO2, one for each of two possible SNP variants) and a locus-specific oligonucleotide (LSO).

Estimated Time

Robot:

- 1 plate: 20 minutes
- 2 plates: 40 minutes
- 3 plates: 60 minutes

Incubation: 2–16 hours

Consumables

Item	Quantity	Storage	Supplied By
OPA reagent	1 tube per ASE plate	-20°C	Illumina
OB1 reagent	1 tube per ASE plate	-20°C	Illumina
96-well 0.2 ml microplate			User

Preparation

- ▶ Preheat the heat block to 70°C and allow it to equilibrate.
- ▶ Thaw the OPA and OB1 tubes to room temperature. Vortex to 280 rpm for 1 minute to mix.
- ▶ Apply an ASE barcode label to a new 96-well microplate.
- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - Robot
 - OPA reagent barcode
 - OB1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. From the robot PC, select **GTS Pre-PCR Tasks | Single Use DNA | Make SUD ASE**.
2. Select the plate type (MIDI or TCY).



Do not mix plate types on the robot bed.

3. In the Basic Run Parameters pane, enter the **Number of SUD/ASE Plates** (1, 2, 3).

The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

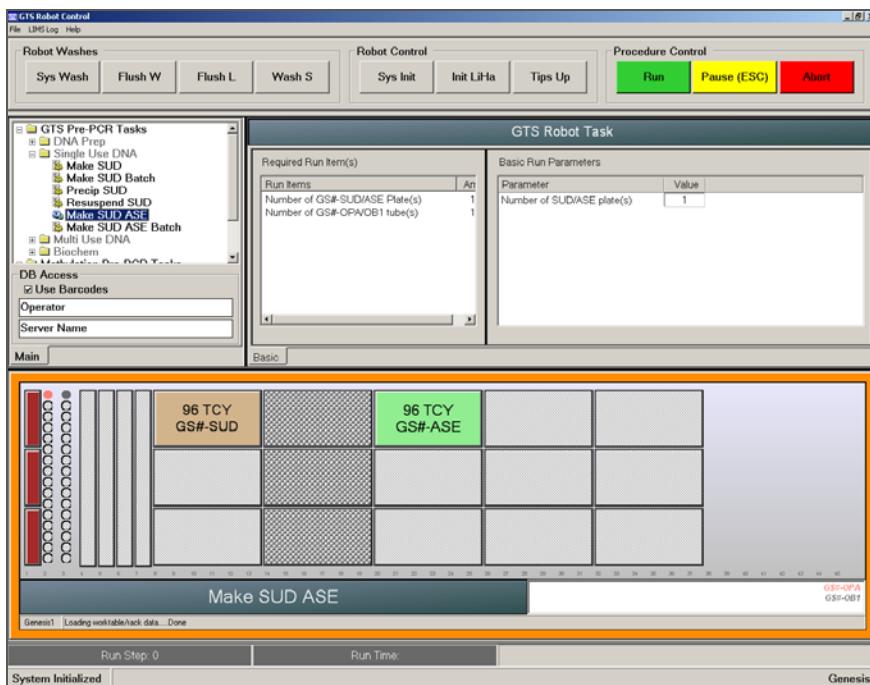


Figure 35 GTS Pre-PCR Tasks | Single Use DNA | Make SUD ASE

4. Ensure that the OPA tube lid is free of liquid. If any liquid remains, pulse centrifuge the tube to 280 xg.
5. Place the OPA and OB1 tubes in the robot tube rack according to the robot bed map. Remove the caps.
6. Pulse centrifuge the SUD plate to 280 xg for 1 minute.
7. Place the SUD and ASE plates on the robot bed according to the robot bed map. Remove any plate seals.

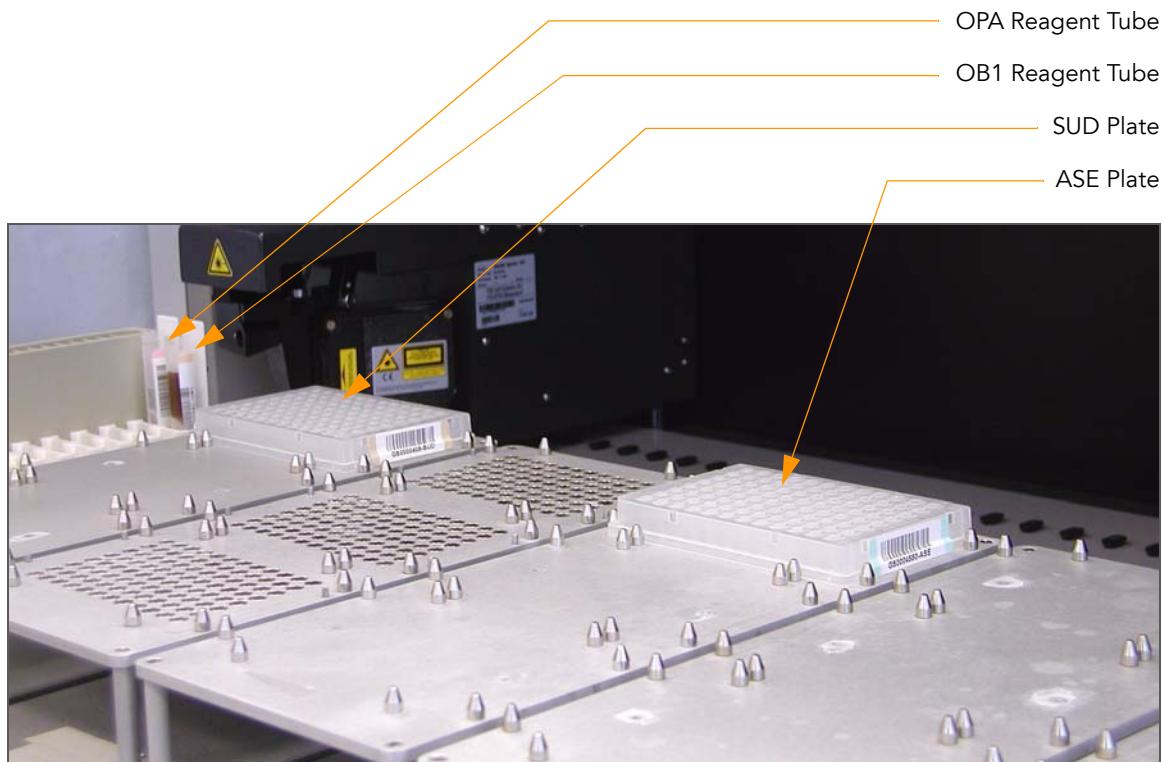


Figure 36 Robot Bed for Make ASE

8. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
9. On the lab tracking form, record the position of the plates on the robot bed.
10. Clear the **Use Barcodes** checkbox and click **Run**. Observe the robot start to run to ensure there are no problems.
The robot PC sounds an alert and displays a message when the process is complete.
11. When the robot finishes, heat-seal the ASE plate with a foil heat seal.
12. Vortex the plate at 1600 rpm for 1 minute or until all beads are completely resuspended.
13. Place the plate on the preheated 70°C heat block and close the lid.
14. Immediately reset the heat block temperature to 30°C. Incubate the ASE plate in the heat block for 2 hours while it cools. You may leave the plate in the heat block for up to 16 hours.
15. Record the start and stop times in the lab tracking form.
16. Discard unused buffers in accordance with your facility requirements.
17. Proceed to *Add Extension and Ligation Reagents (MEL)* on page 112.

Add Extension and Ligation Reagents (MEL)

In this protocol, hybridized ASO-LSO pairs on the bound gDNAs are extended and ligated. The robot first transfers AM1 and UB1 reagents to the ASE plate to wash away non-specifically hybridized and excess oligos from the DNA, and then transfers MEL (extension and ligation enzymes) reagent to each sample in the ASE plate.

Next, the ASE plate is incubated at 45°C for 15 minutes to allow (1) the 3' ends of the properly hybridized ASOs to extend downstream to the 5' ends of their paired LSO, and (2) the extended 3' ASO ends to ligate to the 5' LSO ends.

Estimated Time

Robot:

- 1 plate: 45 minutes
- 2 plates: 1 hour, 15 minutes
- 3 plates: 1 hour, 50 minutes

Incubation: 15 minutes

Consumables

Item	Quantity	Storage	Supplied By
MEL reagent	1 tube per ASE plate	-20°C	Illumina
AM1 reagent	Bottle	-20°C	Illumina
UB1 reagent	Bottle	-20°C	Illumina

Preparation

- ▶ Preheat the heat block to 45°C and allow it to equilibrate.
- ▶ Thaw all reagents to room temperature. Invert 10 times to mix.
- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - Robot
 - MEL reagent barcode
 - AM1 reagent barcode
 - UB1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

- Steps**
- From the robot PC, select **GTS Pre-PCR Tasks | Biochem | Add MEL**.
 - Select the plate type (MIDI or TCY).



Do not mix plate types on the robot bed.

- In the Basic Run Parameters pane, enter the **Number of ASE Plates** (1, 2, 3).

The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

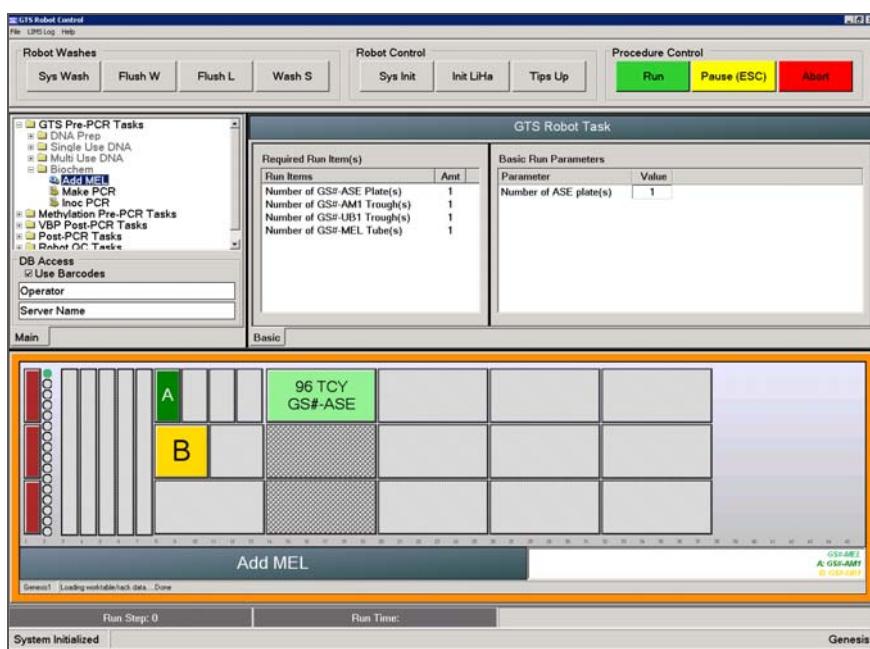


Figure 37 GTS Pre-PCR Tasks | Biochem | Add MEL

- Pulse centrifuge the MEL tube to 280 xg for 1 minute.
- Place the MEL tube in the robot tube rack according to the robot bed map. Remove the cap.
- Place quarter reservoir A onto the robot bed according to the robot bed map. Dispense AM1 reagent as follows:
 - 1 ASE plate: 15 ml
 - 2 ASE plates: 30 ml
 - 3 ASE plates: 45 ml
- Place half reservoir B onto the robot bed according to the robot bed map. Dispense UB1 reagent as follows:
 - 1 ASE plate: 15 ml
 - 2 ASE plates: 30 ml
 - 3 ASE plates: 45 ml

8. Remove the ASE plate from the heat block and visually inspect the plate wells for uniform volume.
9. Pulse centrifuge the ASE plate to 280 xg for 1 minute.
10. Place the ASE plate on the magnetic carrier on the robot bed according to the robot bed map.
11. Wait for all of the beads to be captured by the magnet, which takes about 2 minutes, and then remove the plate seal.



To avoid cross-contamination, do not remove the plate seal until the beads are fully captured.

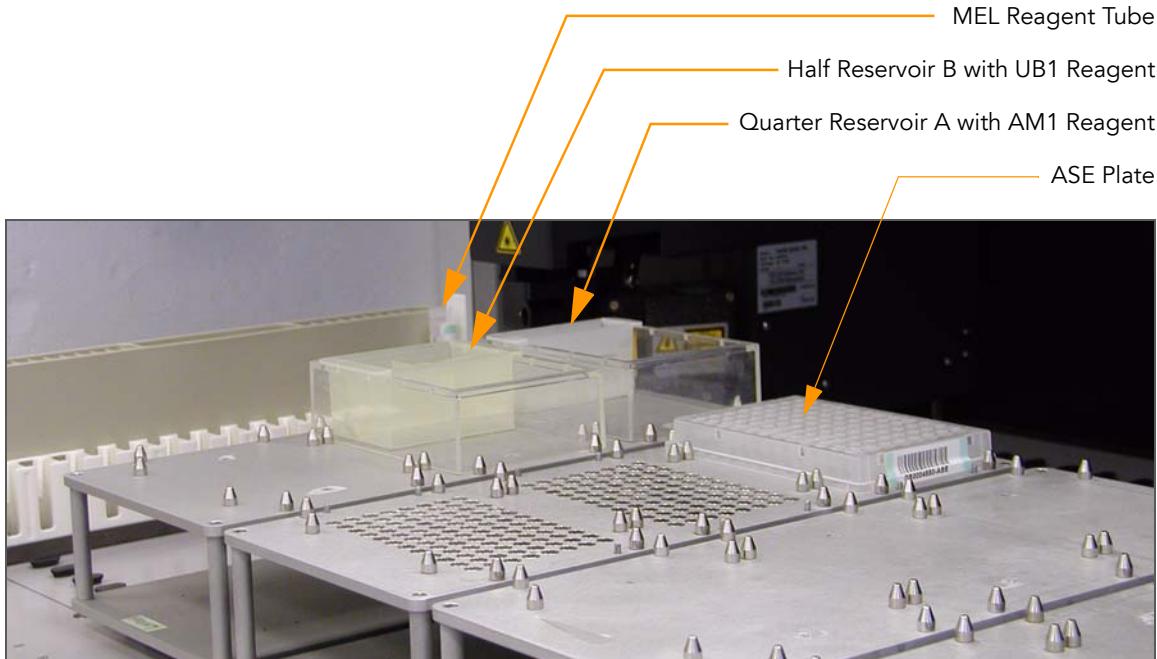


Figure 38 Robot Bed for Add MEL

12. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
13. On the lab tracking form, record the position of the plate on the robot bed.
14. Clear the **Use Barcodes** checkbox and click **Run**. Observe the robot start to run to ensure there are no problems.
15. When prompted, move the plate from the magnetic carrier to the new position shown on the bed map.



Figure 39 First Move ASE Plate Message

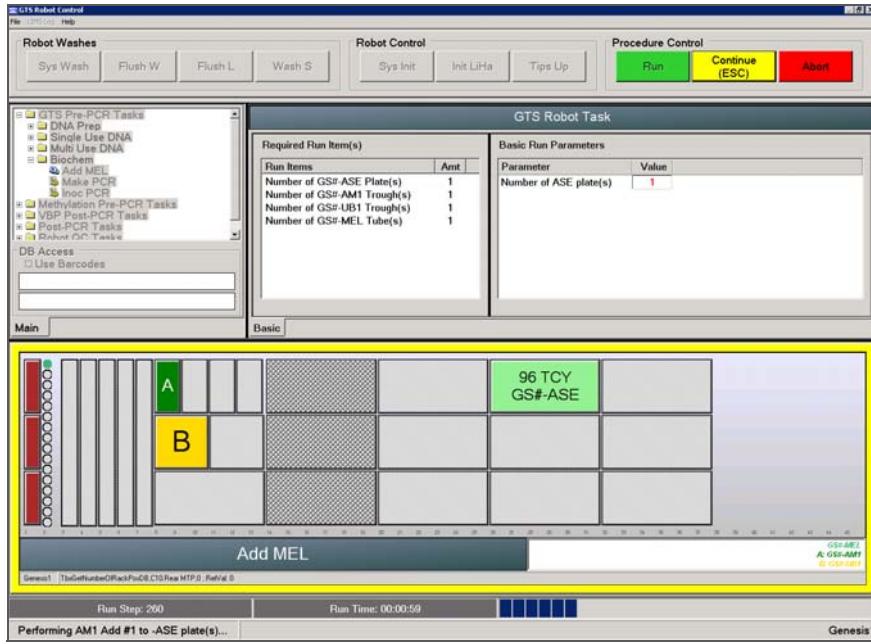


Figure 40 Bed Map After First Plate Move

- When prompted, remove the ASE plate from the robot bed.

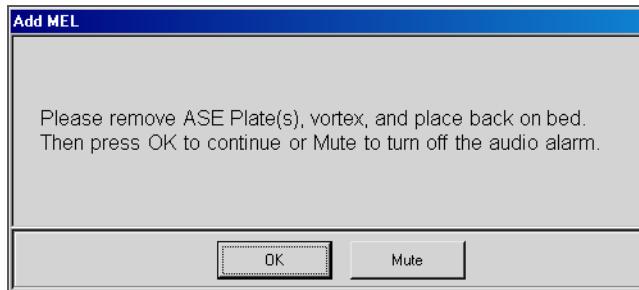


Figure 41 First Remove and Vortex ASE Plate Message

- Seal the plate with an adhesive seal.
- Secure the plate to the high-speed shaker with the Velcro straps. If you are vortexing multiple plates, position them so that the high-speed shaker is balanced.



Prevent splashing! Each vortexer in your lab should carry a unique label indicating its calibration variance for all recommended settings. Before vortexing, set the digital display value according to the appropriate values indicated on the label.

19. Vortex the sealed plate at 1600 rpm for 1 minute, or until resuspended.
20. Return the plate to the correct position on the robot bed according to the bed map. Remove the plate seal, and then click **OK**.
21. At the next prompt, move the plate to the position shown on the bed map.



Figure 42 Second Move ASE Plate Message

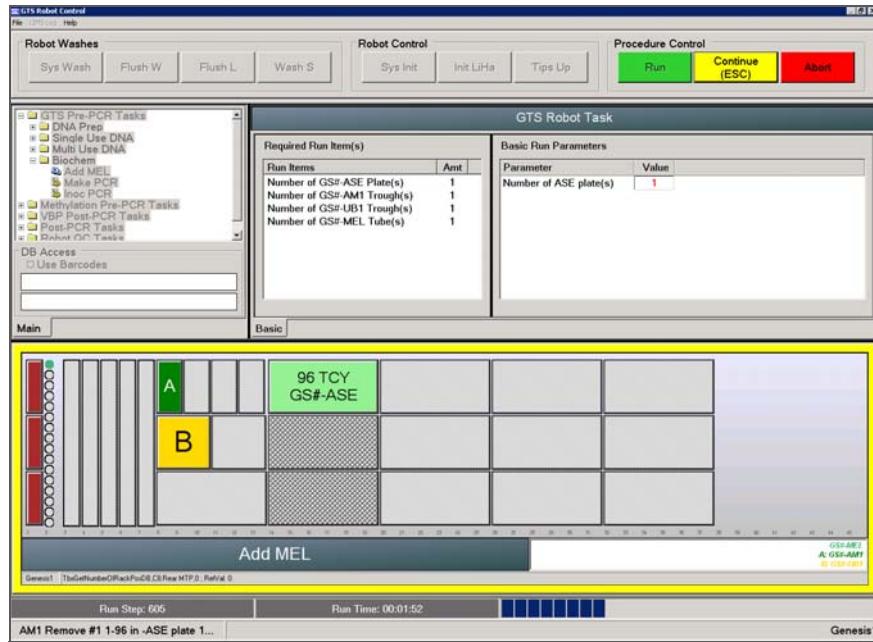


Figure 43 Bed Map After Second Plate Move

22. At the next prompt, move the plate to the position shown on the bed map (Figure 45).



Figure 44 Third Move ASE Plate Message

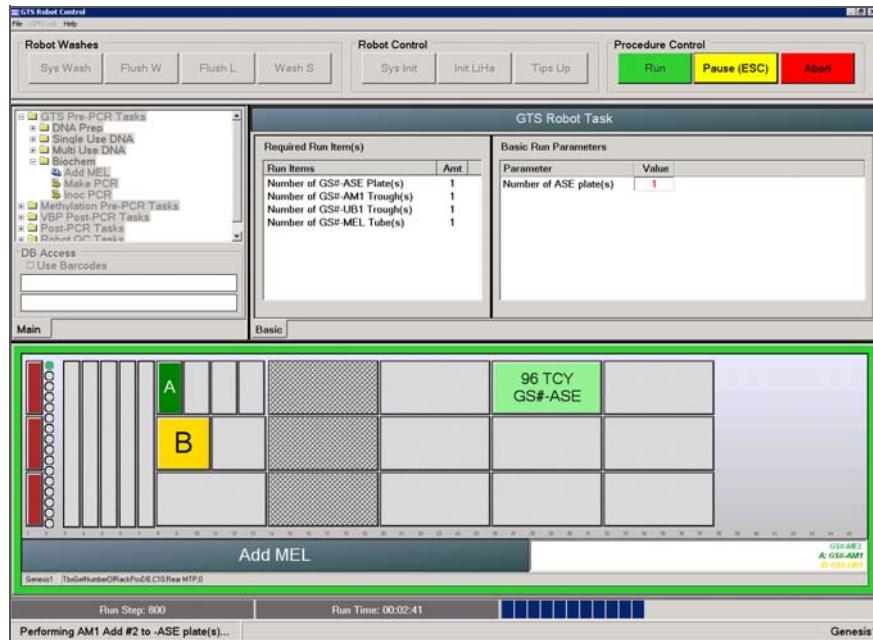


Figure 45 Bed Map After Third Plate Move

23. When prompted, remove the ASE plate from the robot bed and seal it with an adhesive seal.

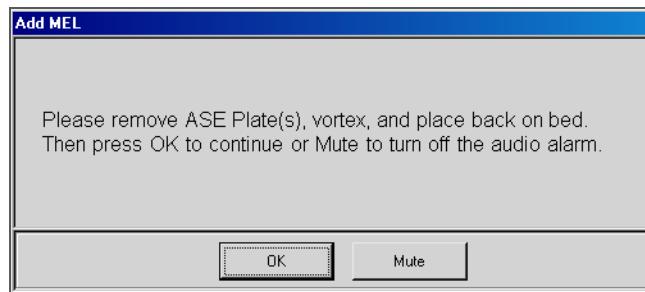


Figure 46 Second Remove and Vortex ASE Plate Message

24. Vortex the plate at 1600 rpm for 1 minute or until all beads are completely resuspended.
25. Return the plate to the correct position on the robot bed according to the bed map. Remove the plate seal, and then click **OK** in the message box.

26. When prompted, move the plate to the position shown on the bed map.



Figure 47 Fourth Move ASE Plate Message

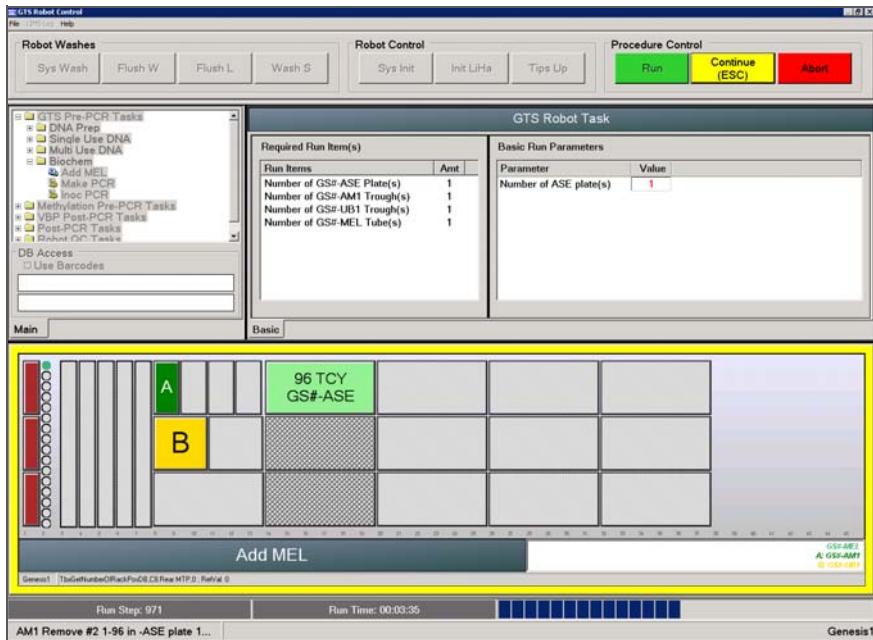


Figure 48 Bed Map After Fourth Plate Move

27. When prompted, remove the ASE plate from the robot bed.

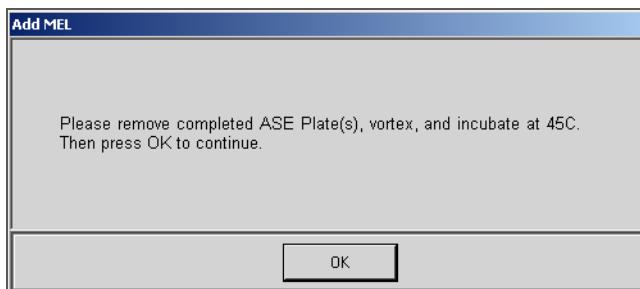


Figure 49 Final Vortex and Incubate Message

28. Seal the ASE plate with an adhesive seal.

29. Vortex the plate at 1600 rpm for 1 minute, or until the beads are resuspended.

30. Click **OK** in the message box.

31. Place the sealed plate on the preheated 45°C heat block and close the lid. Incubate the plate for exactly 15 minutes.

**CAUTION**

Do not leave the ASE plate in the heat block for more than 15 minutes.

32. While the ASE plate is incubating, perform the Make PCR Plate process. The instructions are located immediately after this set of instructions.
33. Record the start and stop times and temperatures in the lab tracking form.
34. Discard unused reagents in accordance with your facility requirements.
35. Proceed to *Inoculate PCR Plate* on page 123. Leave the ASE plate at room temperature if you proceed immediately, or store it at 4°C for up to 1 hour.

Make PCR Plate

In this process, the Illumina-recommended DNA polymerase and the optional Uracil DNA Glycosylase (UDG) are added to the master mix for PCR (the MMP reagent), creating a 96-sample plate for use in the Inoc PCR process. The PCR master mix contains buffer components, dNTPs, and PCR primers. Absent from the master mix is the polymerase enzyme, which is added to each tube before the process begins.

Estimated Time Robot: 10 minutes per plate

Consumables

Item	Quantity	Storage	Supplied By
MMP reagent	1 tube per PCR plate	-20°C	Illumina
Uracil DNA Glycosylase (UDG, Optional)	50 ml per PCR plate	-20°C	User
DNA Polymerase	64 µl per PCR plate	-20°C	Illumina
96-well 0.2 ml microplate	1 per ASE plate		User

Preparation

- ▶ Thaw the MMP reagent to room temperature. Invert 10 times to mix.
- ▶ Apply a PCR barcode label to a new 96-well microplate.
- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - Robot ID
 - MMP reagent barcode



You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. From the robot PC, select **GTS Pre-PCR Tasks | Biochem | Make PCR**.
2. Select the plate type (MIDI or TCY). All of the plates on the robot must be of the same type.
3. In the Basic Run Parameters pane, enter the **Number of PCR Plates** (1 to 9).

The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

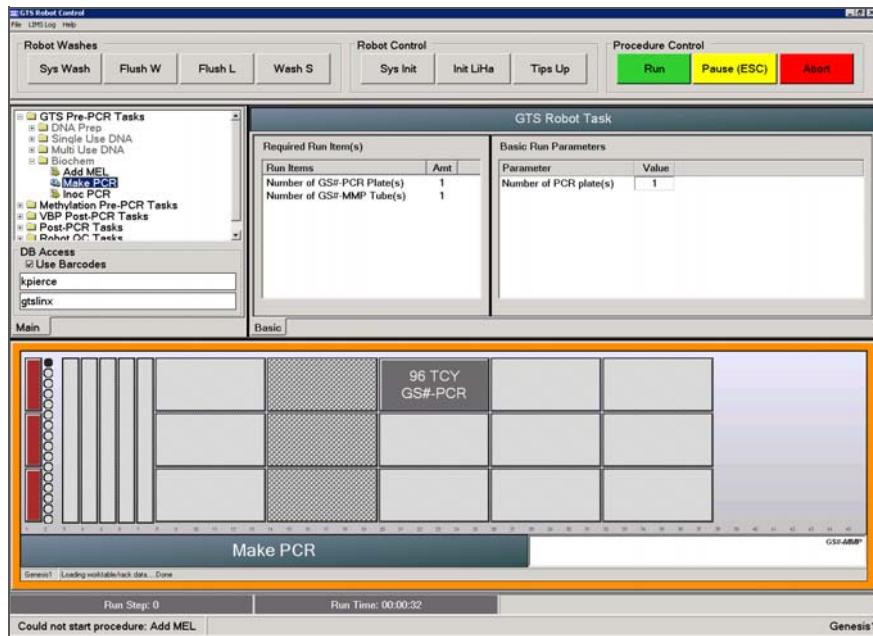


Figure 50 GTS Pre-PCR Tasks / Biochem / Make PCR

4. Add 64 µl DNA polymerase to each MMP tube.
5. [Optional] Add 50 µl Uracil DNA Glycosylase (UDG) to each MMP tube.
6. Cap the MMP tube and invert it several times to mix the contents.
7. Pulse centrifuge the MMP tube to 280 xg for 1 minute.



Do not freeze the MMP tube after adding the DNA polymerase enzyme.

8. Place the MMP tube in the robot tube rack according to the robot bed map. Remove the cap.
9. Place the PCR plate on the robot bed according to the robot bed map.

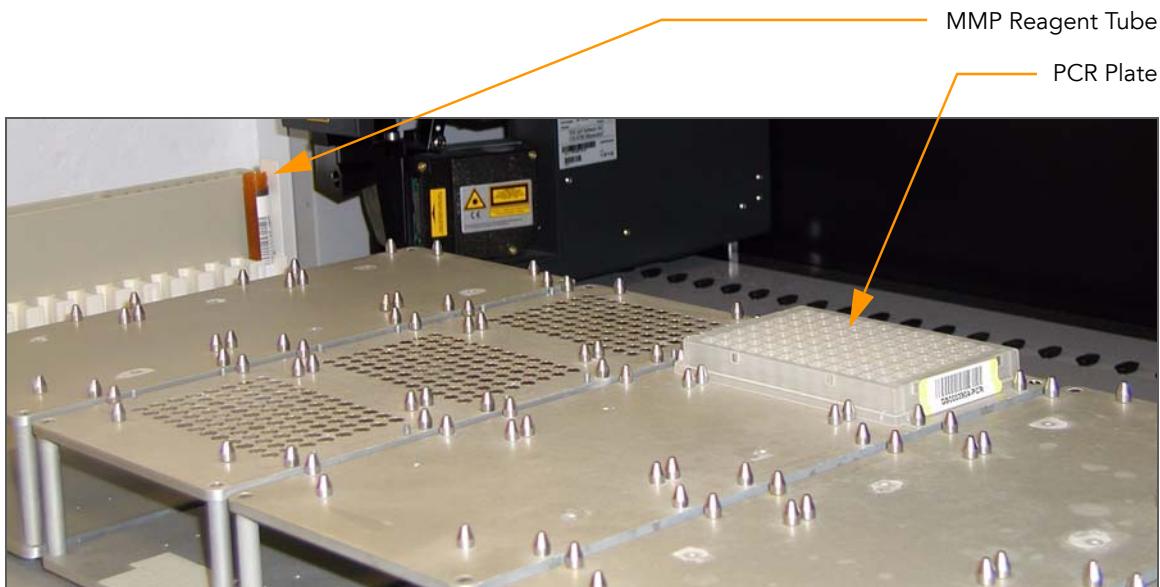


Figure 51 Robot Bed for Make PCR

10. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
11. On the lab tracking form, record the position of the plate(s) on the robot bed.
12. Clear the **Use Barcodes** checkbox and click **Run**. Observe the robot start to run to ensure there are no problems.
13. Seal the PCR plate with clear adhesive film.
14. Do one of the following:
 - Proceed to *Inoculate PCR Plate* on page 123.
 - Place the sealed PCR plate in a vacuum-sealed autobag and store it at -20°C for up to 30 days.

Inoculate PCR Plate

In this process, the PCR master mix is inoculated with the extended and ligated ASO-LSO products from the Add MEL process. First, the ASE plate is placed on the robot bed, where the ligation reaction mix is washed away and the samples are resuspended in elution buffer. Next, the plate is placed on a 95°C heat block for 1 minute to trigger heat denaturation. This releases single-stranded ASO-LSO products from the bound gDNAs back into solution. The ASE plate is returned to the robot bed, where the ASO-LSO solution is transferred to a prepared PCR plate that contains PCR master mix.

Estimated Time

Robot:

- 1 ASE plate: 40 minutes
- 2 ASE plates: 60 minutes
- 3 ASE plates: 80 minutes

Consumables

Item	Quantity	Storage	Supplied By
IP1 reagent	1 tube per PCR plate	-20°C	Illumina
UB1 reagent	Bottle	Room temperature	Illumina

Preparation

- ▶ Preheat the heat block to 95°C.
- ▶ Thaw the IP1 reagent to room temperature. Vortex to 250 rpm to mix.
- ▶ If the PCR plate is frozen, thaw for at least 20 minutes, and then pulse centrifuge to 280 xg.
- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - Robot ID
 - IP1 reagent barcode
 - UB1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. From the robot PC, select **GTS Pre-PCR Tasks | Biochem | Inoc PCR**.
2. Select the plate type (MIDI or TCY). All of the plates on the robot must be of the same type.

3. In the Basic Run Parameters pane, enter the **Number of ASE/PCR Plates** (1, 2, or 3 pairs).

The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

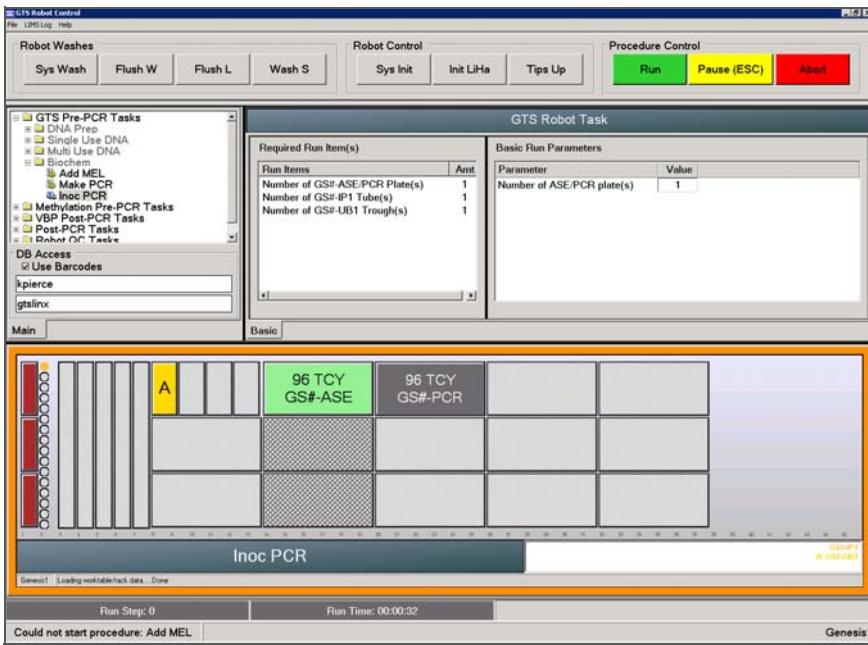


Figure 52 GTS Pre-PCR Tasks / Biochem / Inoc PCR

4. Invert the IP1 tube 10 times to mix.
5. Pulse centrifuge the IP1 tube to 280 xg for 1 minute.
6. Place the IP1 tube in the robot tube rack according to the robot bed map. Remove the cap.
7. Place quarter reservoir A onto the robot bed according to the robot bed map. Dispense UB1 as follows:
 - 1 PCR plate: 10 ml
 - 2 PCR plates: 15 ml
 - 3 PCR plates: 20 ml
8. Place the ASE plate on the magnetic carrier on the robot bed according to the robot bed map. Ensure that the plate rests securely within the pins. Remove the plate seal.
9. Place the PCR plate on the robot bed according to the robot bed map. Remove the plate seal.

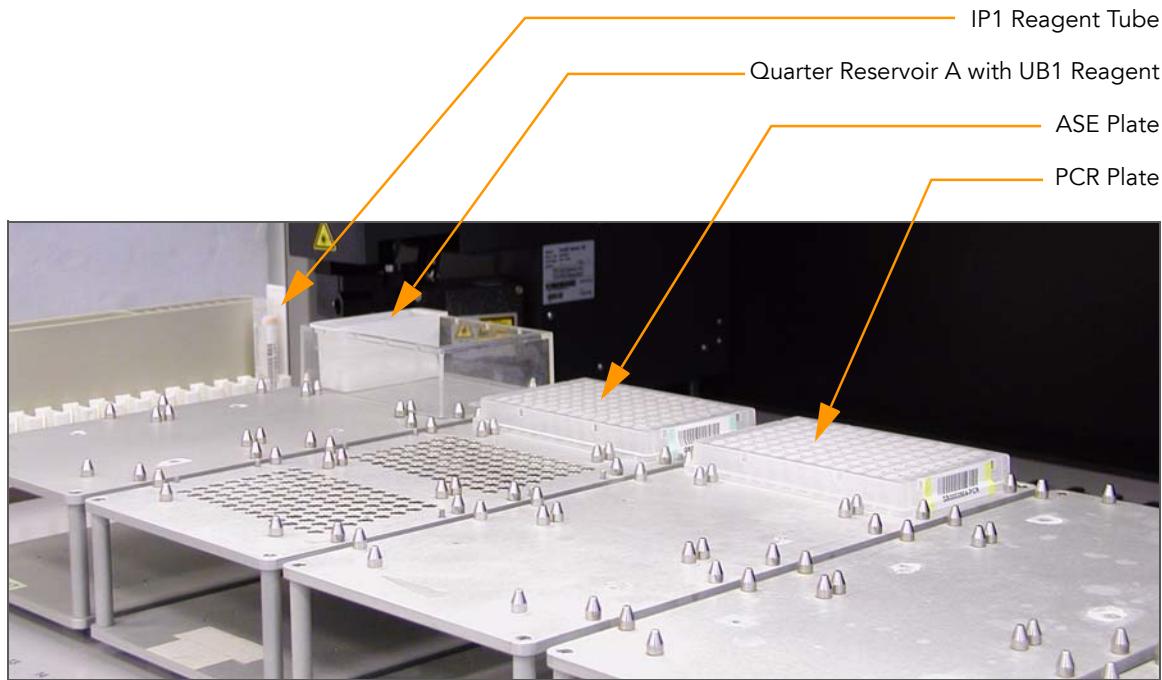


Figure 53 Robot Bed for Inoc PCR

10. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
 11. On the lab tracking form, record the position of the plates on the robot bed.
 12. Clear the **Use Barcodes** checkbox and click **Run**. The robot will pause for 1 minute to allow the paramagnetic beads to settle completely. Observe the robot start to run to ensure there are no problems.
- After the robot adds IP1 to the ASE plate, the robot PC prompts you to vortex and denature the ASE plate.

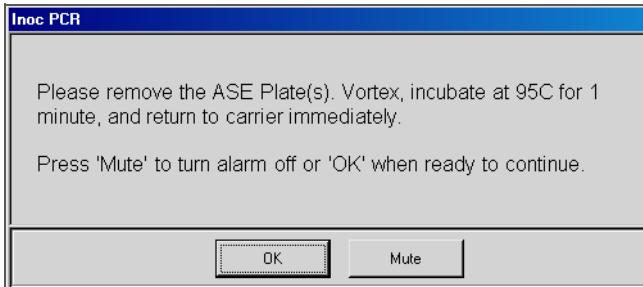


Figure 54 Inoc PCR Vortex and Incubate Message



You may click **Mute** to silence the alarm, but do not click **OK** until the ASE plate is denatured and back on the robot bed.

13. Remove the ASE plate from the robot bed and seal it with a clear adhesive seal.
14. Secure the plate to the high-speed shaker with the Velcro straps. If you are vortexing multiple plates, position them so that the high-speed shaker is balanced.

**CAUTION**

Prevent splashing! Each vortexer in your lab should carry a unique label indicating its calibration variance for all recommended settings. Before vortexing, set the digital display value according to the appropriate values indicated on the label.

15. Vortex the sealed plate at 1800 rpm for 1 minute, or until resuspended.
16. Place the sealed plate in the 95°C heat block and incubate for exactly 1 minute.
17. Return the plate to its original position on the robot bed, as shown on the bed map. Remove the plate seal, and then click **OK** in the message box.
The robot will wait for 1 minute to allow the beads to settle completely, and then inoculate the PCR plate with the denatured contents of the ASE plate.
The robot PC sounds an alert and displays a message when the process is complete.
18. Remove the ASE and PCR plates from the robot bed. Seal them with the appropriate type of PCR plate-sealing film for your thermocycler.
19. Place the plates and the lab tracking forms in a transfer box for the post-PCR area.
20. Discard unused buffers in accordance with facility requirements.
21. Immediately proceed to *Thermal Cycle PCR Plate* on page 127.

Thermal Cycle PCR Plate

This process thermal cycles the PCR plate to fluorescently label and amplify the templates generated in the pre-PCR process. Three universal primers are included in the reaction mix:

- ▶ Cy3-labelled complement to allele-specific oligo (ASO) 1
- ▶ Cy5-labelled complement to ASO 2
- ▶ Biotinylated complement to locus-specific oligo (LSO)

The fluor-labelled primers provide the signal for detection, while the biotinylated primer allows for immobilization of the PCR product. The result is highly amplified double-stranded PCR products containing a fluor-labelled strand (Cy3 or Cy5, depending on the genotype) and a biotinylated strand.

Estimated Time Thermal Cycle: ~2 hours 45 minutes

Steps 1. Place the sealed plate into the thermocycler and run the thermocycler program shown in this table.

Table 29 Thermocycler Program

	Temperature	Time
	37°C	10 minutes
	95°C	3 minutes
X 34 {	95°C	35 seconds
	56°C	35 seconds
	72°C	2 minutes
	72°C	10 minutes
	4°C	5 minutes

2. Do one of the following:

- Proceed immediately to *Bind PCR Products* on page 128.
- Store the sealed plate at room temperature in a light-protected drawer for processing later that day.
- Store the sealed plate at -20°C for up to 24 hours.

Bind PCR Products

In this process, MPB reagent and sec-butanol are added to the PCR plate. The plate is incubated at room temperature to bind the biotinylated strand to paramagnetic particles, thus immobilizing the double-stranded PCR products.

Estimated Time	Robot: 15 minutes per plate Incubation: 60 minutes
-----------------------	---

Consumables

Item	Quantity	Storage	Supplied By
MPB reagent	1 tube per PCR plate	4°C	Illumina
Sec-butanol	5 ml per PCR plate	Room temperature	User
70% EtOH	Bottle	Room temperature	User

Preparation

- ▶ Thaw the MPB reagent to room temperature.
- ▶ Remove the PCR plate from the freezer or thermocycler. Protect the plate from light until you place it on the robot bed. If the plate is frozen, thaw it at room temperature for at least 15 minutes in a light-protected location.
- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - Robot ID
 - MPB reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. From the robot PC, select **VBP Post-PCR Tasks | Bind PCR**.
2. Select the plate type (MIDI or TCY). All of the plates on the robot must be of the same type.
3. In the Basic Run Parameters pane, enter the **Number of PCR Plates** (1, 2, or 3).

The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

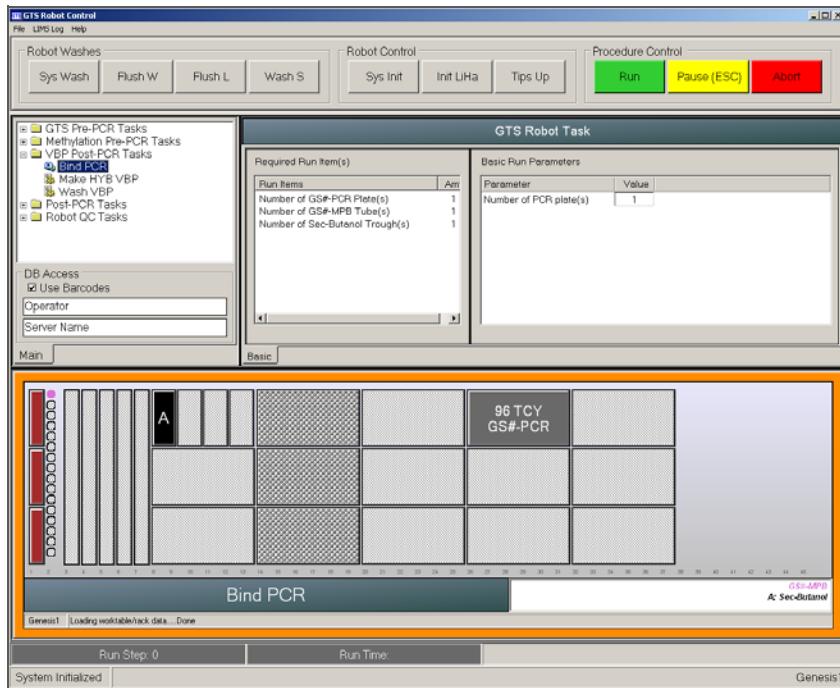


Figure 55 VBP Post-PCR Tasks / Bind PCR

4. Vortex the MPB tube at tube vortexer speed 6 for 30 seconds, until the beads are resuspended. Invert the tube to make sure that all of the beads are resuspended.
5. Place the MPB tube in the robot tube rack according to the robot bed map. Remove the cap.
6. Place quarter reservoir A onto the robot bed according to the robot bed map. Dispense sec-butanol as follows:
 - 1 PCR plate: 5 ml
 - 2 PCR plates: 10 ml
 - 3 PCR plates: 15 ml
7. Centrifuge the PCR plate to 3000 xg for 30 seconds.
8. Place the PCR plate on the robot bed according to the robot bed map. Remove any plate seals.

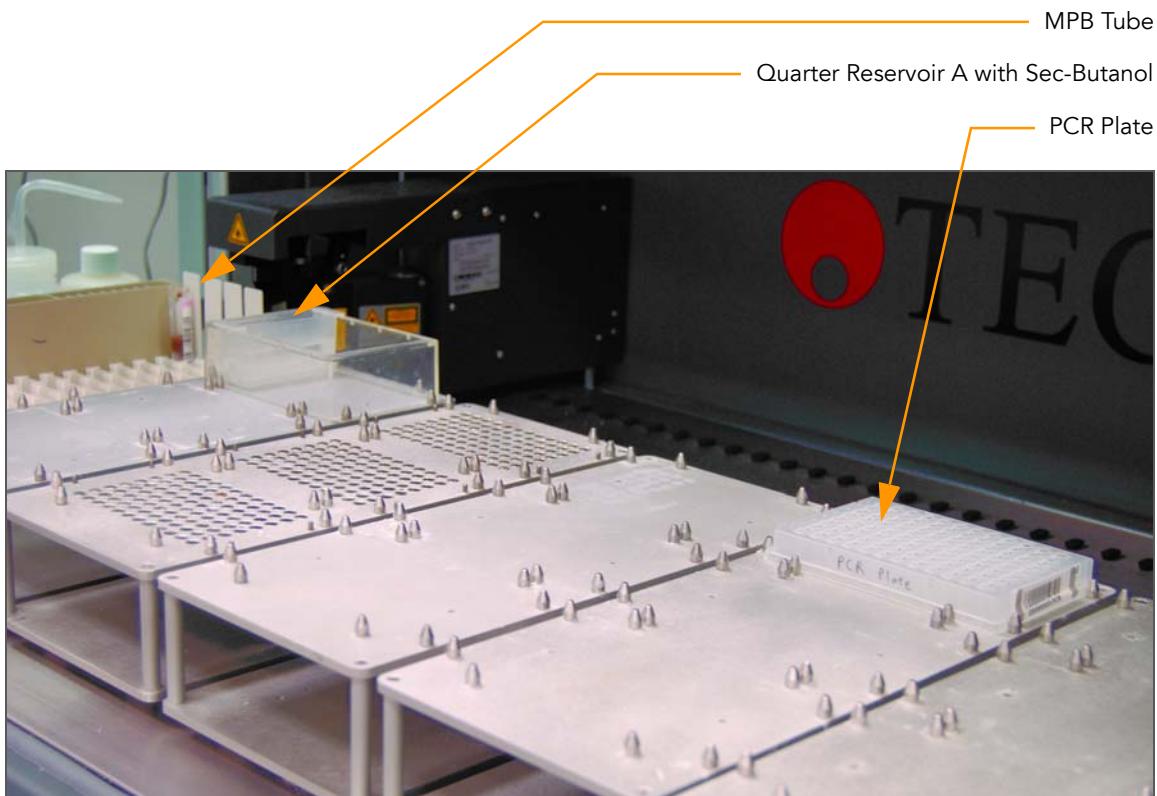


Figure 56 Robot Bed for Bind PCR

9. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
10. On the lab tracking form, record the position of the plate on the robot bed.
11. Clear the **Use Barcodes** checkbox and click **Run**. Observe the robot run to ensure there are no problems.



If you pause the robot for any reason, remove the MPB tube from the rack, re-vortex it, and return it to the rack in its original position before restarting the robot.

The robot PC sounds an alert and displays a message when the process is complete.

12. When the robot finishes, seal the PCR plate with adhesive film.
13. Incubate the PCR plate in a light-protected location for 60 minutes.
14. Do one of the following:
 - Proceed to *Make VeraCode Bead Plate* on page 131.
 - Store the sealed PCR plate at 4°C for up to 4 hours.

Make VeraCode Bead Plate

In this process, the single-stranded, fluor-labelled PCR product is washed with UB2 and NaOH, eluted into an INT plate containing MH2 reagent, and then transferred to a VBP plate.

Estimated Time Robot: 45 minutes per PCR plate

Consumables

Item	Quantity	Storage	Supplied By
MH2 reagent	2 tubes per PCR plate	Room temperature	Illumina
UB2 reagent	Bottle	Room temperature	Illumina
0.1N NaOH	Bottle	4°C	User
70% EtOH	Bottle	Room temperature	User
96-well 0.2 ml microplate	1 per PCR plate		User
VeraCode Bead Plate	1 per PCR plate	4°C	Illumina

Preparation

- ▶ Apply a INT barcode label to a new 96-well microplate.
- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - Robot ID
 - INT plate barcode
 - VBP plate barcode
 - UB2 reagent barcode
 - MH2 reagent barcode
 - NaOH lot number



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

- At the robot PC, select **VBP Post-PCR Tasks | Make HYB VBP**.
- In the Basic Run Parameters pane, enter the **Number of PCR/VBP Plates** (1, 2, or 3) and the **Total (Number of) Samples** (16 or 96) in each PCR plate.

The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

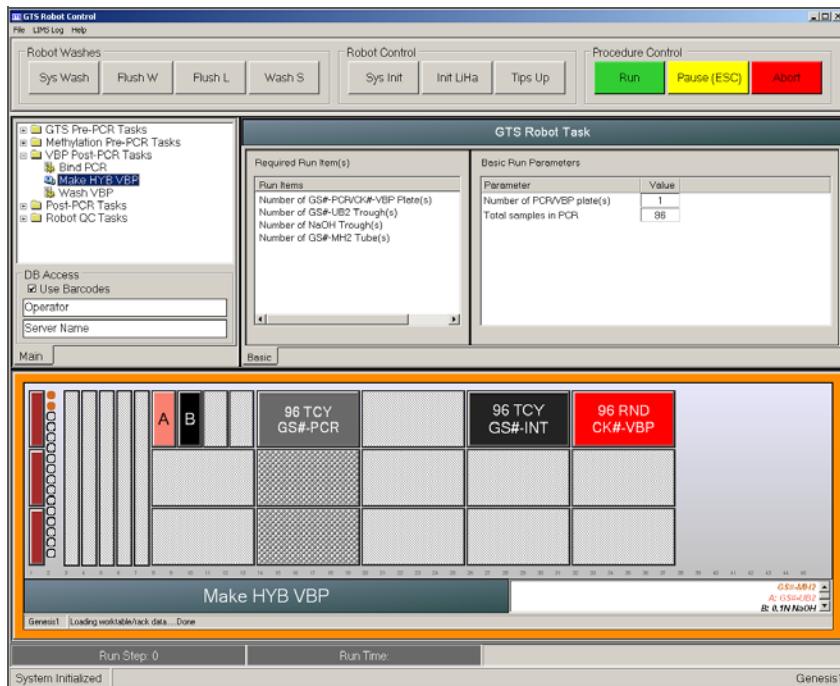


Figure 57 VBP Post-PCR Tasks | Make HYB VBP

- Pulse centrifuge each VBP plate to 250 xg, until the beads are at the bottom of the wells.
- Place each PCR, INT, and VBP plate on the robot bed according to the robot bed map. Remove any plate seals.
- Set aside the cap mat from each VBP plate for use later in the protocol.
- Place quarter reservoir A onto the robot bed according to the robot bed map. Dispense UB2 as follows:
 - 1 PCR plate: 10 ml
 - 2 PCR plates: 15 ml
 - 3 PCR plates: 22 ml
- Place quarter reservoir B onto the robot bed according to the robot bed map. Dispense 0.1N NaOH as follows:
 - 1 PCR plate: 10 ml
 - 2 PCR plates: 20 ml
 - 3 PCR plates: 30 ml
- Pulse centrifuge the MH2 tubes to 280 xg.

9. Place the MH2 tubes in the robot tube rack according to the robot bed map. Remove the caps.

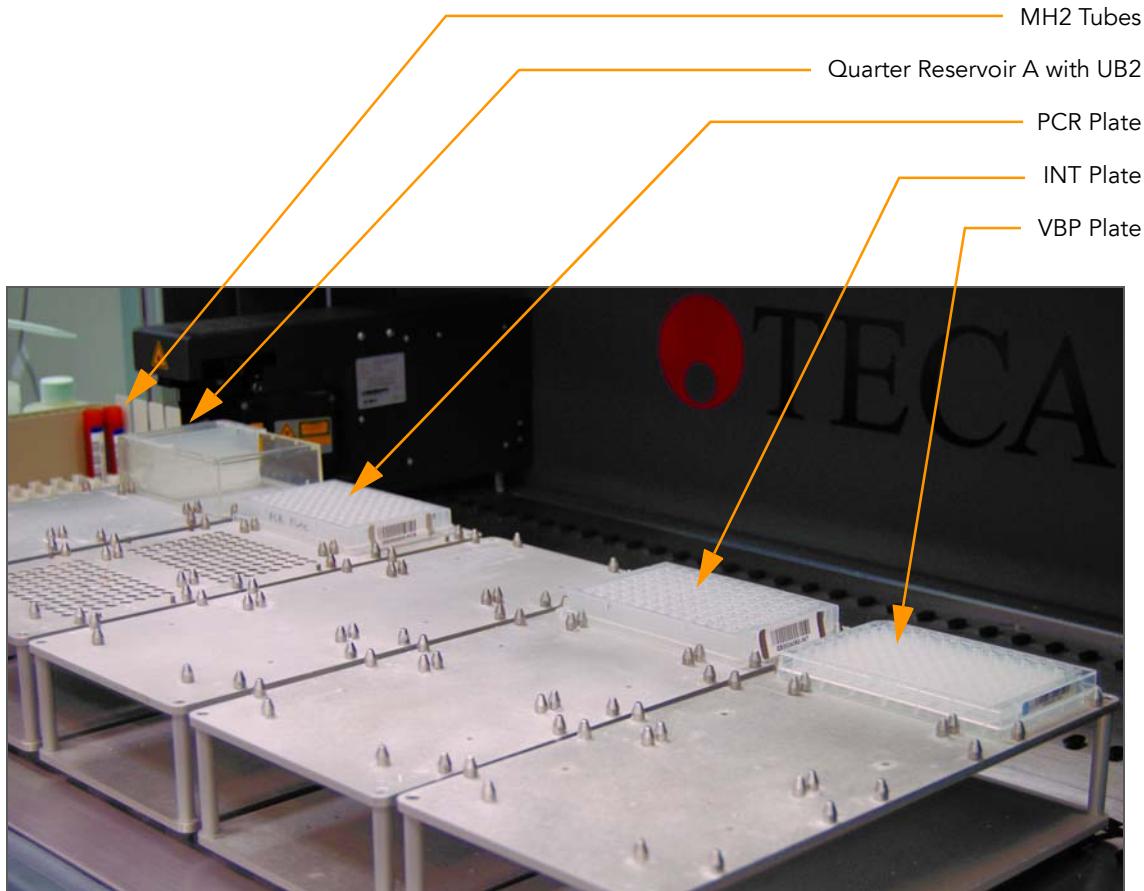


Figure 58 Robot Bed for Make VeraCode Bead Plate

10. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
11. On the lab tracking form, record the plate positions on the robot bed.
12. Clear the **Use Barcodes** checkbox and click **Run**. Observe the robot start to run to ensure that there are no problems.
13. When prompted, move the PCR plates as instructed.
14. If you plan to proceed directly to hybridization, then start preheating the Vortex Incubator to 45°C, 5 minutes before the end of the robot process. The robot PC sounds an alert and displays a message when the process is complete.
15. Carefully remove the VBP plate from the robot bed. Reseal it with the original cap mat.
16. Discard the INT and PCR plates in accordance with your facility requirements.
17. Do one of the following:

- (Strongly recommended) Proceed immediately to *Hybridize VeraCode Bead Plate* on page 135.
- Store the sealed VBP plate at 4°C for up to 4 hours, or at -20°C for up to 24 hours.

Hybridize VeraCode Bead Plate

This process uses the VeraCode Vortex Incubator, an incubating microplate shaker, to hybridize the VeraCode Bead Plate (VBP) at 45°C.

Estimated Time Incubation: 3 hours

Preparation

- ▶ If you stored the VBP plate at 4°C or -20°C after the Make VeraCode Bead Plate process, thaw it to room temperature in a light-protected location, and then pulse centrifuge to 250 xg, until the beads are at the bottoms of the wells.
- ▶ Preheat the VeraCode Vortex Incubator to 45°C and allow it to equilibrate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - VBP plate barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Place the VeraCode Bead Plate, which now contains samples, into the VeraCode Vortex Incubator. You can load up to 2 VBP plates in the vortexer.



NOTE

If you load only one plate, load an empty 96-well plate in the opposite position as a balance.

2. Close the lid and make the following settings:
 - Push the **Encoder** knob until **RPM** is highlighted. Rotate the knob to 85 (850 rpm).
 - Push the **Encoder** knob until **Time** is highlighted. Rotate the knob below 0.30 or above 99.5 until HLD appears. This sets the Vortex Incubator to run continuously.
 - Push the **Encoder** knob until **Temperature** is highlighted. Rotate the knob to 45 (45°C).
3. Press **Start/Stop** and incubate for 3 hours.
4. Proceed immediately to *Wash VeraCode Bead Plate* on page 136.

Wash VeraCode Bead Plate

In this process, the VeraCode Bead Plate is placed on a non-magnetic carrier on the robot bed. It is washed twice with the VW1 reagent to remove all unbound single-stranded hybridization products. Next, the beads are resuspended in buffer to prepare the plate for the BeadXpress Reader.

Estimated Time Robot: 20 minutes per plate

Consumables

Item	Quantity	Storage	Supplied By
VW1 reagent	Bottle	Room temperature	Illumina

Preparation

- ▶ Follow the instructions in *Prepare the Robot Before Each Use* on page 28.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - VW1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. At the robot PC, select **VBP Post-PCR Tasks | Wash VBP**.
2. In the Basic Run Parameters pane, enter the **Number of VBP Plates** (1, 2, or 3) and the **Total (Number of) Samples in VBP** (16 or 96).

The robot PC updates the Required Run Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

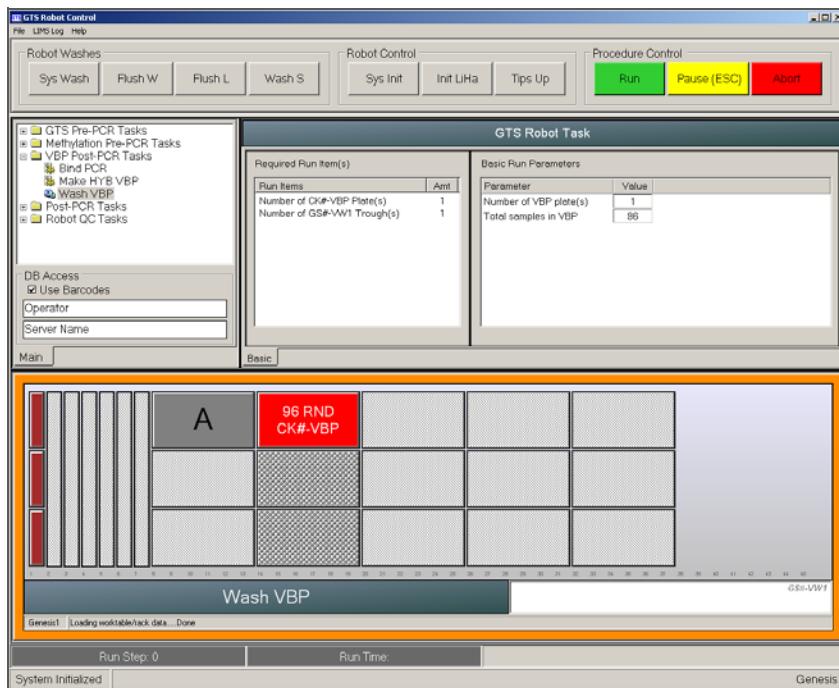


Figure 59 VBP Post-PCR Tasks / Wash VBP

3. Stop the VeraCode Vortex Incubator. When the speed indicator reaches 0, open the lid and remove the VeraCode Bead Plate.
4. Centrifuge the plate to 650 xg for 1 minute.
5. Remove the cap mat and discard.
6. Place the VBP plate on the robot bed according to the robot bed map.
7. Place full reservoir A onto the robot bed according to the robot bed map. Dispense VW1 as follows:
 - 1 plate: 50 ml
 - 2 plates: 100 ml
 - 3 plates: 150 ml

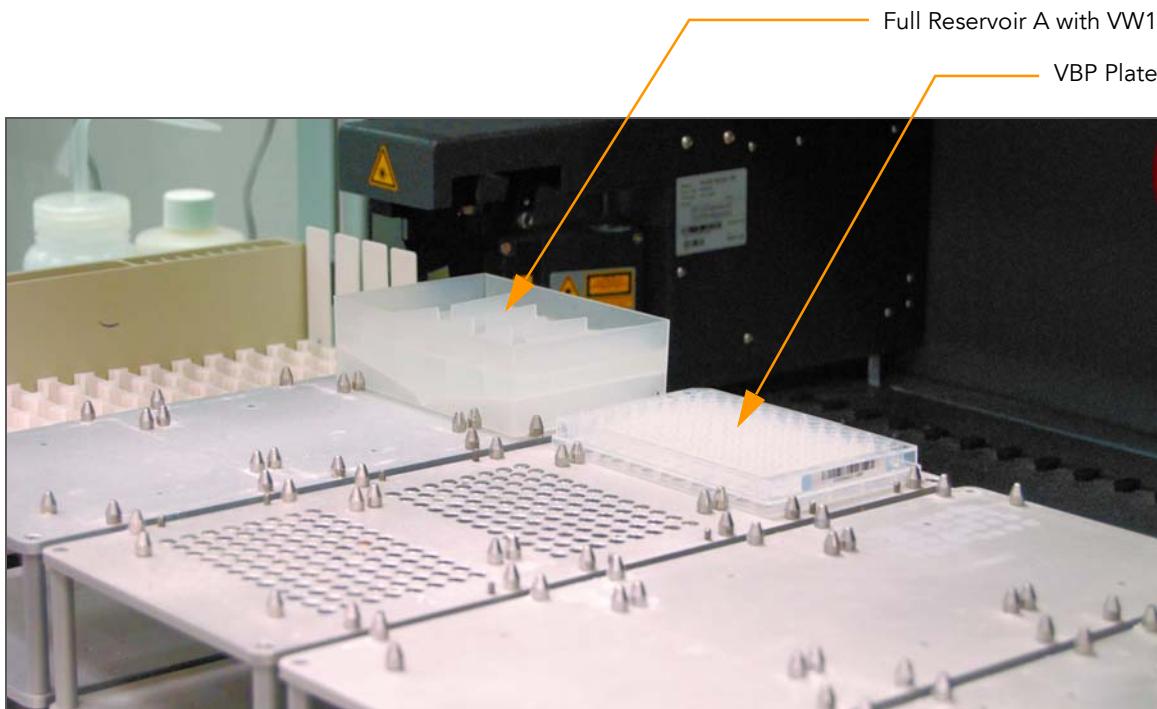


Figure 60 Robot Bed for Wash VBP

8. Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.
9. Clear the **Use Barcodes** checkbox and click **Run**. Observe the robot run to ensure there are no problems.

The robot PC sounds an alert and displays a message when the process is complete.

10. When the robot finishes, seal the VBP plate with a clear adhesive seal.
11. Discard any unused VW1 according to your facility requirements.
12. Do one of the following:
 - a. Proceed to *Scan VeraCode Bead Plate* on page 139.
 - b. Store the sealed plate in the dark at room temperature for up to 24 hours.

Scan VeraCode Bead Plate

The BeadXpress Reader uses lasers to excite the Cy3 and Cy5 fluors of the single-stranded PCR products bound to the VeraCode beads. Light emissions from these fluors are then recorded in a data file. Fluorescence data are analyzed to derive genotyping results using Illumina's GenomeStudio software package.

Estimated Time	80 samples/hour at 96-plex 27 samples/hour at 384-plex
-----------------------	---

Preparation	<ul style="list-style-type: none">▶ Prepare a scan settings file containing information about your samples, the BeadXpress Reader settings, and VeraCode beads. If you intend to analyze GoldenGate genotyping data in GenomeStudio, enter the VeraCode Bead Plate barcode (CK#-VBP) in the Plate_ID field.
--------------------	---

Steps	For instructions on scanning VeraCode Bead Plates, see the <i>BeadXpress Reader System Guide</i> .
--------------	--

Chapter 5

DASL Gene Expression Assay for VeraCode Protocols

Topics

- 142 Introduction
- 143 Workflow
- 144 Tracking Tools
- 147 Materials and Reagents for this Assay
- 149 Make RNA Quantitation Plate (Optional)
- 154 Read QRNA Plate (Optional)
- 158 Make Single-Use RNA (SUR) Plate
- 160 Make Assay Specific Extension (ASE) Plate
- 162 Add Master Mix for Extension & Ligation (MEL)
- 165 Make PCR Plate
- 167 Inoculate PCR Plate
- 169 Thermal Cycle PCR Plate
- 170 Bind PCR Products
- 172 Make Intermediate Plate for VeraCode Bead Plate
- 175 Hybridize VeraCode Bead Plate
- 177 Wash VeraCode Bead Plate
- 178 Scan VeraCode Bead Plate
- 179 Troubleshooting

Introduction

This chapter provides detailed pre- and post-PCR laboratory protocols for the DASL Gene Expression Assay for VeraCode. Perform each protocol in the order shown.

The instructions assume that you are preparing 96 RNA samples. If you are preparing fewer than 96 samples, scale down the protocols accordingly; for more information, see *Prepare Fewer than 96 Samples* on page 22.

The instructions in this chapter assume that you have already familiarized yourself with Chapter 2, *Standard Operating Procedures* and have set up the lab area appropriately.



CAUTION

It is very important to prevent PCR product contamination during this assay. To learn about safe lab practices for Illumina assays, see Chapter 2, *Standard Operating Procedures*. In addition, follow all of the safety procedures described in this chapter.

Workflow

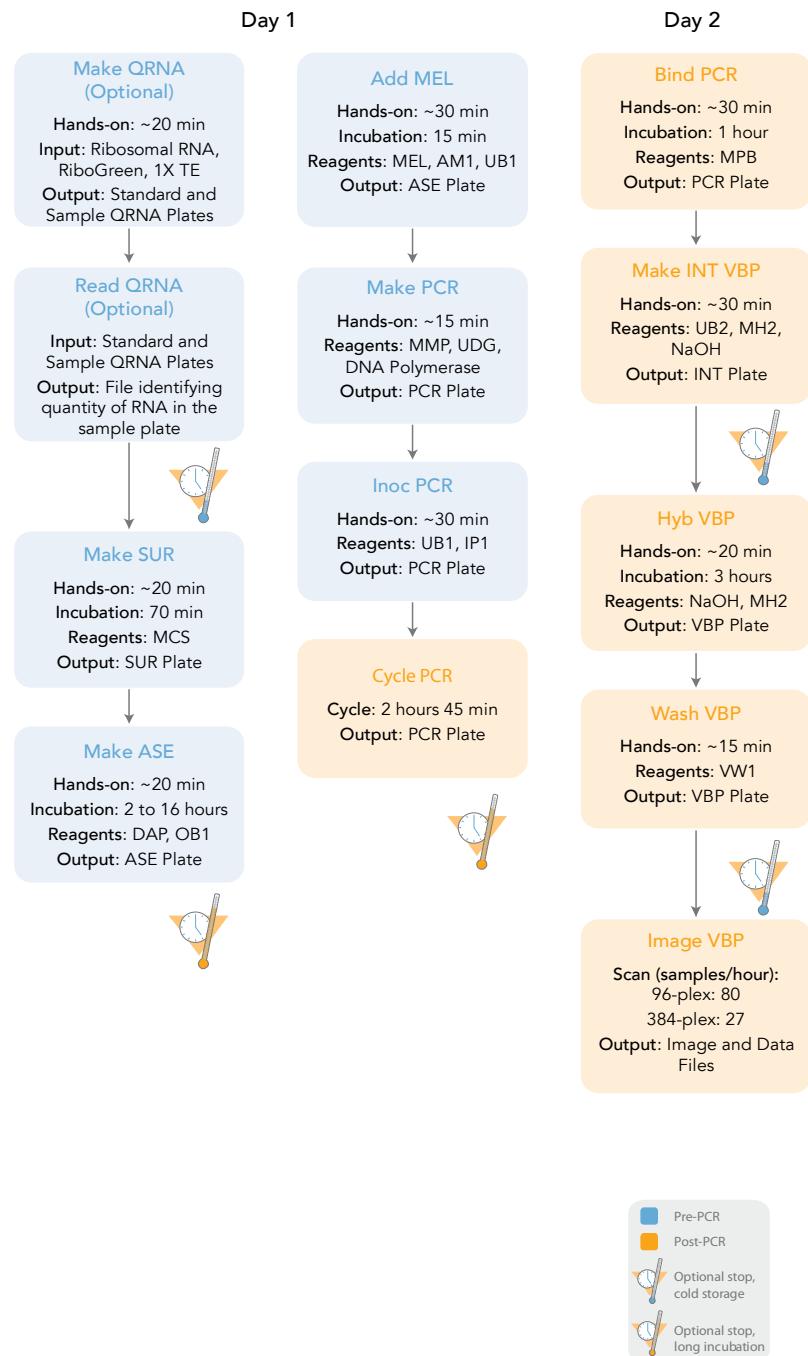


Figure 61 DASL Gene Expression Assay for VeraCode Laboratory Workflow

Tracking Tools

Lab Tracking Form

Create a copy of the two-page lab tracking form for each run (Figure 62). Use it to track information such as operator ID and reagent barcodes, and to record the sample location in each plate.



You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

DASL® Gene Expression Assay for VeraCode® Lab Tracking Form	
<p>1 Make SUR Date/Time: _____ SUR Plate: _____ Operator: _____ MCS Reagent: _____ Heat block (42°C, 60 m): Start: _____ Stop: _____</p> <p>2 Make ASE Date/Time: _____ SUR Plate: _____ Operator: _____ ASE Plate: _____ Heat block (2 h): OBT Reagent: _____ Start (0°C): Stop (30°C): DAP Reagent: _____</p> <p>3 Add MEL Date/Time: _____ ASE Plate: _____ Operator: _____ AM1 Reagent: _____ Heat block (45°C, 15 m): Start: _____ Stop: _____ UBT Reagent: _____ MEL Reagent: _____</p> <p>4 Make PCR Date/Time: _____ PCR Plate: _____ Operator: _____ MMP Reagent: _____ <input type="checkbox"/> Add Recommended DNA Polymerase <input type="checkbox"/> Add Uridine DNA Glycosylase (UDG)</p> <p>5 Inoc PCR Date/Time: _____ ASE Plate: _____ Operator: _____ PCR Plate: _____ <input type="checkbox"/> Incubate in Heat Block (95°C, 1 m) IP1 Reagent: _____ UB1 Reagent: _____</p>	
<i>Pre-PCR</i> <i>Post-PCR</i>	<i>Barcode</i> <i>Sample Plate</i>
<p>6 Cycle PCR Date/Time: _____ PCR Plate: _____ Operator: _____ Thermal Cycler: _____ Thermal Cycle Program Name: _____ Start: _____ Stop: _____</p> <p>7 Bind PCR Date/Time: _____ MPB Reagent: _____ Operator: _____ Incubate in light-protected drawer (22°C, 1 hour) Start: _____ Stop: _____</p> <p>8 Make INT Date/Time: _____ INT Plate: _____ Operator: _____ UB2 Reagent: _____ Centrifuge (1000 xg, 25°C, 5 m): MH2 Reagent: _____ <input type="checkbox"/> Start: _____ Stop: _____ NaOH Lot #: _____</p> <p>9 Hyb VBP Date/Time: _____ INT Plate: _____ Operator: _____ VBP Plate: _____ Vortex Incubator ID: _____ MH2 Reagent: _____ 850 rpm, 50°C, 3 h: Start: _____ Stop: _____ NaOH Lot #: _____</p> <p>10 Wash VBP Date/Time: _____ VBP Plate: _____ Operator: _____ VW1 Reagent: _____ <input type="checkbox"/> Vacuum-Aspirate Supernatant (50 mbar)</p> <p>11 Scan VBP Date/Time: _____ VBP Plate: _____ Operator: _____ BeadXpress® Reader ID: _____</p>	
<small>Catalog # VC-901-1001 Part # 11312747 Rev A</small>	
<small>Catalog # VC-901-1001 Part # 11312747 Rev A</small>	

Figure 62 DASL Gene Expression Assay for VeraCode Lab Tracking Form

Sample Sheet

To effectively track your samples and assay, we recommend that you create a sample sheet. The sample sheet will later be used by GenomeStudio for data analysis. See the appropriate GenomeStudio module guide for more information.

Create your sample sheet according to the guidelines provided in Table 30.

Table 30 Sample Sheet Guidelines

Section	Description	Optional (O) or Required (R)
Sample_Name	Example: S12345 If not user-specified, the GenomeStudio application will assign a default sample name, concatenating the sample plate and sample well names.	O

Table 30 Sample Sheet Guidelines (Continued)

Section	Description	Optional (O) or Required (R)
Sample_Plate	Example: GS0005623-RNA User-specified name for the plate containing RNA samples.	O
Sample_Well	Example: A01 The well containing the specific sample in the 96-well RNA plate.	O
Sample_Group	Example: Group 1 User-specified name of the sample group. If the Sample_Group is missing, GenomeStudio creates one group and assigns it a default name.	R
Pool_ID	Example: VC0007005-DAP Name of the DAP.	R
Sentrix_ID	Example: CK0004001-VBP VeraCode Bead Plate ID.	R
Sentrix_Position	Example: A01 The VeraCode Bead Plate well position to which the sample is hybridized.	R
Notes	Your sample sheet header may contain whatever information you choose. Your sample sheet may contain any number of columns you choose. Your sample sheet must be in a comma-delimited (.csv) file format. Save the sample sheet under any name you wish; for example, the user-defined experiment name.	

Figure 63 provides an example of the Sample Sheet format. The Documentation CD includes a Sample Sheet template file that you can copy and use.

The screenshot shows a Microsoft Excel window titled "Microsoft Excel - 060607_Sample_Sheet_Methyl-384_VeraCode.csv". The window displays a spreadsheet with two main sections: a header and a data table.

Header:

	A	B	C	D	E	F	G
1	[Header]						
2	Investigator Name	Steffen					
3	Project Name	VeraCode					
4	Experiment Name						
5	Date	6/6/2007					
6							

Data:

	A	B	C	D	E	F	G
8	Sample_Name	Sample_Well	Sample_Plate	Sample_Group	Pool_ID	Sentrix_ID	Sentrix_Position
9						60507	R001_C001
10						60507	R001_C002
11						60507	R001_C003
12						60507	R001_C004
13						60507	R001_C005
14						60507	R001_C006
15						60507	R001_C007
16						60507	R001_C008
17						60507	R001_C009
18						60507	R001_C010
19						60507	R001_C011
20						60507	R001_C012
21						60507	R002_C001
22						60507	R002_C002
23						60507	R002_C003
24						60507	R002_C004
25						60507	R002_C005
26						60507	R002_C006
27						60507	R002_C007
28						60507	R002_C008
29						60507	R002_C009
30						60507	R002_C010

Figure 63 VeraCode Sample Sheet

Materials and Reagents for this Assay

These materials are specifically required for the DASL Gene Expression Assay for VeraCode. For a list of equipment, materials, and reagents required for all assays in a BeadXpress Reader lab, see *Standard Equipment, Materials, and Reagents* on page 17.

User-Supplied

Table 31 User-Supplied Reagents

Item	Source
RiboGreen RNA quantification kit • RNase-free TE • Ribosomal RNA standard, 5x200 µl tubes at 100 µg/ml in TE	Molecular Probes Invitrogen, catalog # R-11490
DEPC-treated H ₂ O	
Uracil DNA Glycosylase (UDG, Optional)	

Illumina-Supplied

Table 32 Illumina-Supplied Materials and Reagents

Item	Catalog #
VeraCode 96-Plex GoldenGate Kit, 480 samples • BOX A VeraCode cDNA Activation Kit • BOX B VeraCode GoldenGate Pre-PCR #1 • BOX C VeraCode GoldenGate Pre-PCR #2 • BOX D VeraCode GoldenGate Post-PCR • BOX E 96-Plex VeraCode Bead Plates • DAP DASL Assay custom oligo pool, shipped separately <i>Description of box contents appears below.</i>	VC-201-2096
VeraCode 384-Plex GoldenGate Kit, 480 samples • BOX A VeraCode cDNA Activation Kit • BOX B VeraCode GoldenGate Pre-PCR #1 • BOX C VeraCode GoldenGate Pre-PCR #2 • BOX D VeraCode GoldenGate Post-PCR • BOX E 384-Plex VeraCode Bead Plates • DAP DASL Assay custom oligo pool, shipped separately <i>Description of box contents appears below.</i>	VC-201-2384

VeraCode GoldenGate Kit Box Contents

Table 33 Box Contents

Box	Contents
BOX A VeraCode cDNA Activation Kit	MCS —Reagent used to activate sufficient cDNA
BOX B VeraCode GoldenGate Pre-PCR #1	OB1 —Oligo hybridization and sample binding buffer MMP —Master mix for PCR reagent IP1 —Reagent used to elute extended and ligated products UB1 —Universal buffer used to wash paramagnetic beads
BOX C VeraCode GoldenGate Pre-PCR #2	MEL —Reagent used for extension and ligation AM1 —Reagent used to wash away non-specifically hybridized and excess oligos
BOX D VeraCode GoldenGate Post-PCR	MPB —Magnetic particle reagent used to bind double-stranded PCR products MH2 —Reagent used to make the VBP plate UB2 —Universal buffer used to wash magnetic particles and the SAM VW1 —Reagent used to wash the VeraCode beads
BOX E 96-Plex VeraCode Bead Plates	96-plex VeraCode Bead Plate (5)
BOX E 384-Plex VeraCode Bead Plates	384-plex VeraCode Bead Plate (5)

Other Materials

- ▶ QRNA barcode labels
- ▶ GS#-RNA barcode labels
- ▶ SUR barcode labels
- ▶ ASE barcode labels
- ▶ PCR barcode labels
- ▶ Filter plate: GS_____ -PCR labels
- ▶ INT barcode labels
- ▶ Filter plate adapter
- ▶ Vortexer calibration label

Make RNA Quantitation Plate (Optional)

This process uses the RiboGreen RNA quantitation kit to quantitate RNA samples for the DASL Gene Expression Assay for VeraCode. You can quantitate up to six plates, each containing up to 96 samples.

Illumina recommends the Molecular Probes RiboGreen assay kit to quantitate RNA samples. The RiboGreen assay can quantitate small RNA volumes, and measures RNA directly. Other techniques may pick up contamination such as small molecules and proteins. Illumina recommends using a fluorometer because fluorometry provides RNA-specific quantitation, whereas spectrophotometry might also measure DNA and yield values that are too high.



CAUTION

RiboGreen is susceptible to chemical contaminants. For more information, see the Molecular Probes website (www.probes.com).

Estimated Time Hands-on: ~30 minutes

Consumables

Item	Quantity	Storage	Supplied By
RiboGreen RNA Quantitation Kit	1 per 96 samples	4°C	User
96-well 0.65 ml microplate (MIDI)	1 per 96 samples		User
Fluotrac 200 (96-well, black, flat-bottom) plate	2 per 96 samples		User

Preparation

- ▶ Thaw all reagents to room temperature and then vortex to mix.
- ▶ Place a QRNA barcode label on each Fluotrac 200 plate. Position the labels on the skirt of the plate on the right, where the manufacturer's name appears.
- ▶ Label the microtiter plate "Standard QRNA." Label one of the Fluotrac plates "Standard QRNA."
- ▶ Label the other Fluotrac plate "Sample QRNA."

Steps **Make Standard QRNA Plate**

In this process, you create a Standard QRNA plate with serial dilutions of standard ribosomal RNA in the wells of column 1.

1. Using the 96-well microplate labelled Standard QRNA, add 10 µl 1X TE (supplied in RiboGreen kit at 20X) to the wells of rows B–H, column 1.
2. Add 20 µl ribosomal RNA to well A1.

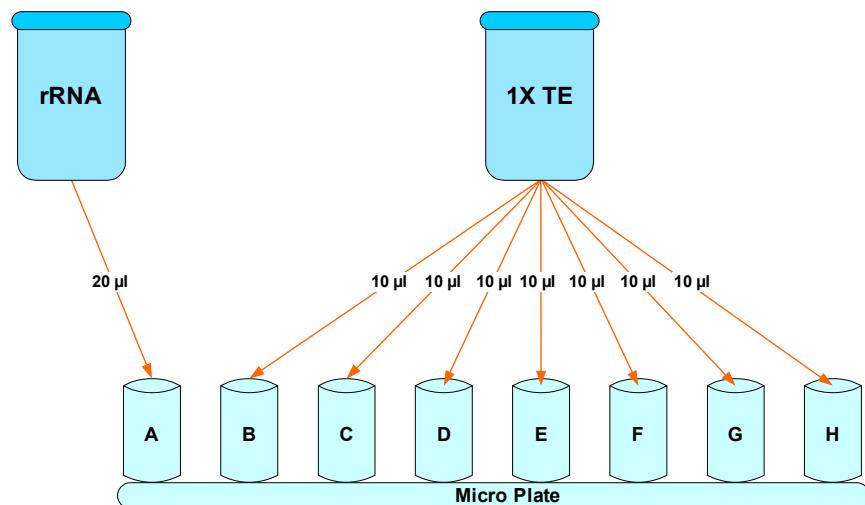


Figure 64 Dilution of Ribosomal RNA Standard

3. Transfer 10 μ l from well A1 to well B1.
4. Mix by pipetting up and down several times.
5. Change tips and transfer 10 μ l from well B1 to well C1.
6. Mix by pipetting up and down several times.
7. Repeat for wells C1, D1, E1, F1, and G1, but *do not* transfer from well G1 to H1. Change tips between dispenses.
8. Cover the plate with an adhesive seal.

Table 34 Concentration of Ribosomal RNA Standards

Row-Column	Concentration (ng/ μ l)	Final Volume in Well (μ l)
A1	100	10
B1	50	10
C1	25	10
D1	12.5	10
E1	6.25	10
F1	3.125	10
G1	1.5262	20
H1	0	10

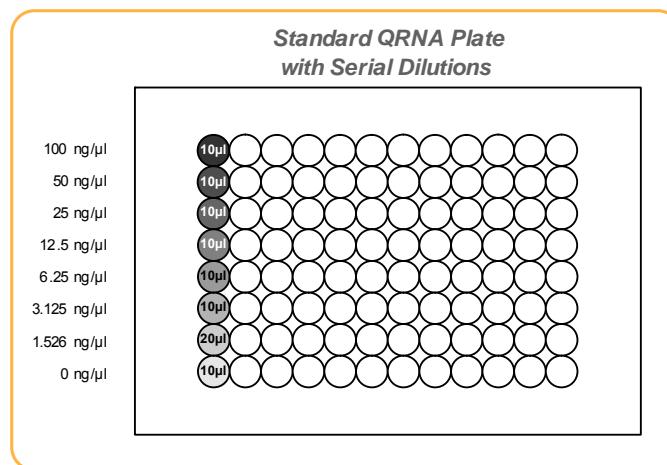


Figure 65 Ribosomal QRNA Serial Dilutions

Prepare Standard QRNA Plate with RiboGreen Dilution

In this process you create a new Standard QRNA Fluotrac plate by transferring the serial dilutions of the original Standard QRNA plate into the new plate and adding RiboGreen.

1. Prepare a 1:200 dilution of RiboGreen into 1X TE, using the kit supplies and a sealed 100 ml or 250 ml Nalgene bottle wrapped in aluminum foil. Table 35 shows the volumes needed to produce diluted reagent for multiple 96-well QRNA plates. For fewer than 96 RNA samples, scale down the volumes.

Table 35 QRNA Plate Reagent Volumes

# QRNA Plates	RiboGreen Volume (μl)	1X TE Volume (ml)
1	115	23
2	215	43
3	315	63
4	415	83
6	615	123

2. Cap the foil-wrapped bottle and vortex to mix.
3. Pour the RiboGreen/1X TE dilution into a clean reagent reservoir.
4. Using an 8-channel pipette, transfer 195 μl RiboGreen/1X TE dilution into each well of columns 1 and 2 of the Standard QRNA Fluotrac plate (Figure 66).

5. Add 2 μ l of each standard ribosomal RNA dilution from column 1 of the original Standard QRNA plate to columns 1 and 2 of the Standard QRNA Fluotrac plate.

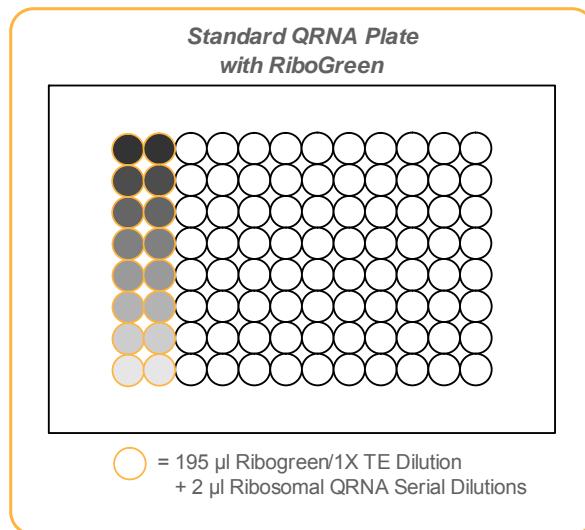


Figure 66 Standard QRNA Plate with RiboGreen

6. Immediately cover the plate with an adhesive aluminum seal.

Prepare Sample QRNA Plate with RiboGreen and RNA

In this process, you create a new Sample QRNA plate that contains RNA sample and RiboGreen.

1. Using an 8-channel pipette, transfer 195 μ l RiboGreen/1X TE dilution into each well of columns 1 and 2 of the Sample QRNA Fluotrac plate (Figure 67).
2. Add 2 μ l of RNA sample to all 96 wells of the Sample QRNA plate. Only the first two columns will also contain RiboGreen/1X TE dilution.

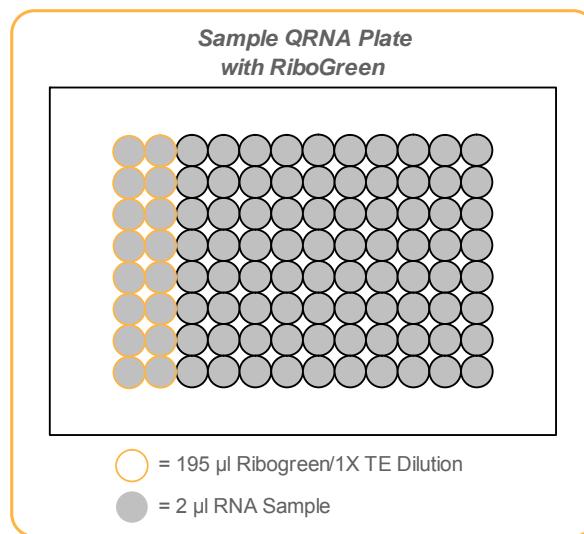


Figure 67 Sample QRNA Plate with RiboGreen



NOTE

For fewer than 96 RNA samples, add the diluted RiboGreen reagent into the number of wells needed.

3. Immediately cover the plate with an adhesive aluminum seal.
4. Proceed to *Read QRNA Plate (Optional)* on page 154.

Read QRNA Plate (Optional)

This process uses the Gemini XS or XPS Spectrofluorometer to provide specific quantitation. Illumina recommends using a fluorometer, as fluorometry provides nucleic acid-specific quantitation, whereas spectrophotometry might also measure contaminants and yield values that are too high.

Estimated Time Fluorometer: 5 minutes per plate

- Steps**
1. Turn on the fluorometer.
 2. At the PC, open the SoftMax Pro program.
 3. Load the Illumina QRNA.ppr file from the installation CD that came with your system.
 4. Select **Assays | Illumina | Illumina QRNA** (Figure 68).

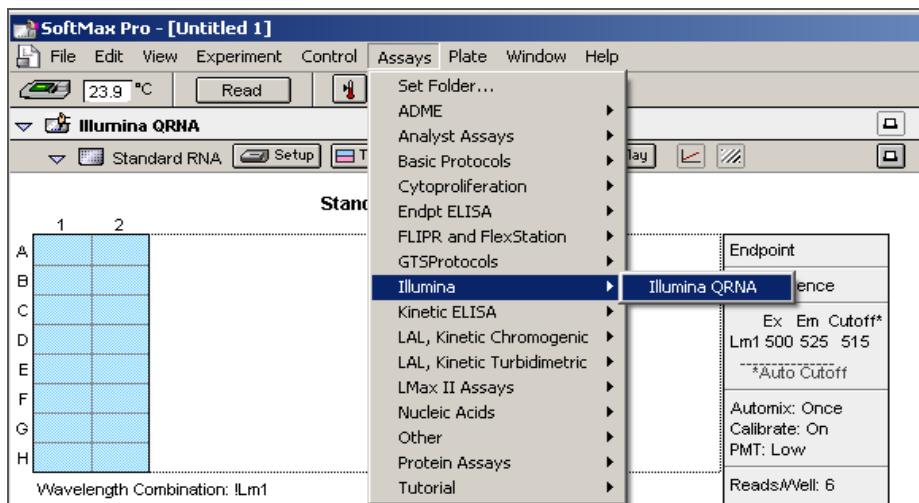


Figure 68 Load the Illumina QRNA Protocol in SoftMax Pro

5. Place the Standard QRNA Fluotrac Plate into the fluorometer loading rack with well A1 in the upper left corner.
6. Click the blue arrow next to **Standard RNA** (Figure 69).

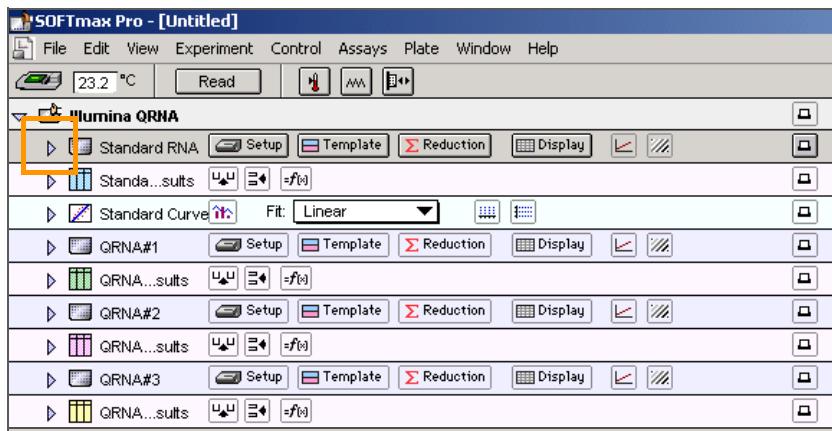


Figure 69 Select the Standard RNA Screen

- Click **Read** in the SoftMax Pro interface (Figure 70) to begin reading the Standard QRNA Plate.

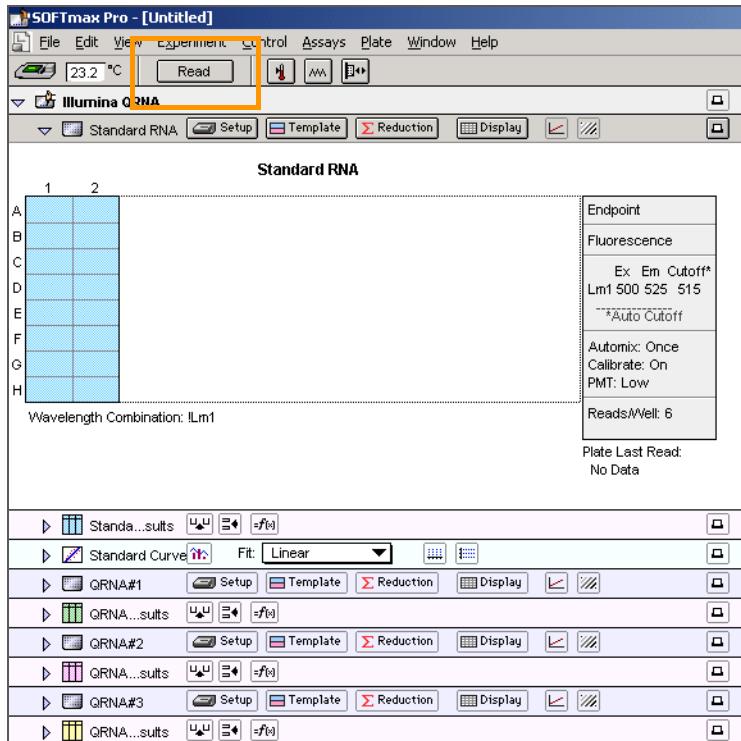


Figure 70 Read the Standard QRNA Plate

- When the software finishes reading the plate, the plate drawer opens. Remove the Standard QRNA Plate from the drawer.
- Click the blue arrow next to **Standard Curve** to view the standard curve graph (Figure 71).
- If the standard curve is acceptable, continue with the sample plate. Otherwise, click **Standard Curve** again.

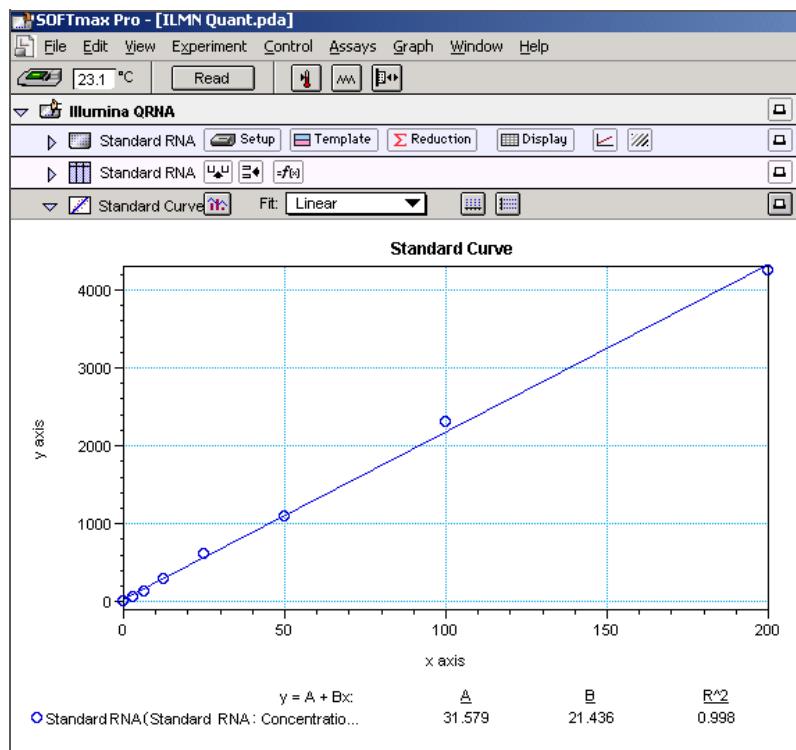


Figure 71 View Standard Curve

11. Place the first Sample QRNA plate in the reader with well A1 in the upper left corner.
12. Click the blue arrow next to **QRNA#1** and click **Read**.

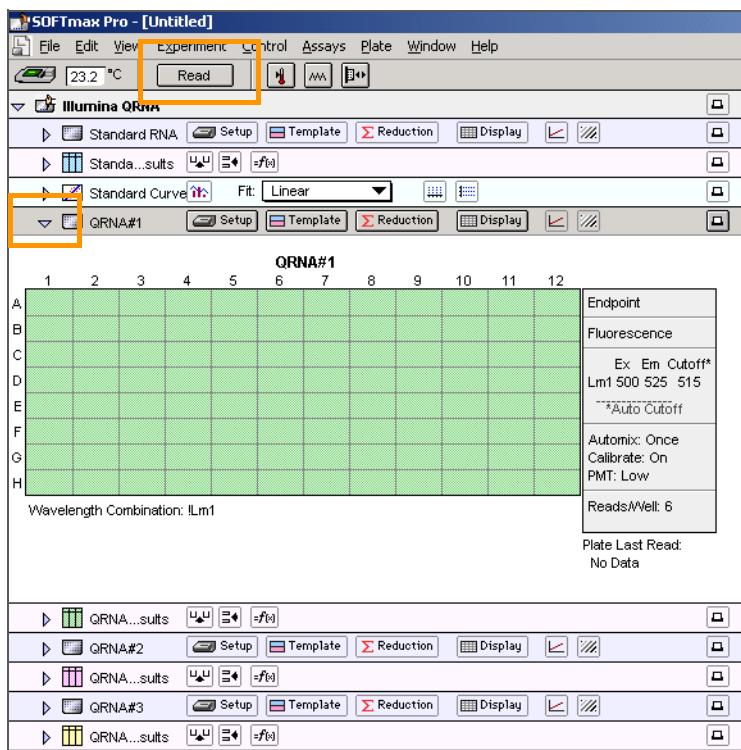


Figure 72 Read the Sample QRNA Plate

13. When the software finishes reading the plate, the plate drawer opens. Remove the plate from the drawer.
14. Repeat steps 11 through 13 for all Sample QRNA plates to be quantitated.
15. Once all plates have been read, click **File | Save** to save the output data file (*.pda).
16. When you have saved the *.pda file, click **File | Import/Export | Export** and export the file as a *.txt file. You can open the *.txt file in Microsoft Excel for data analysis.
17. Proceed to *Make Single-Use RNA (SUR) Plate* on page 158.

Make Single-Use RNA (SUR) Plate

This process reverse-transcribes sufficient RNA from each individual sample to be used once in the DASL Gene Expression Assay for VeraCode.



Be sure to use RNase-free materials and techniques throughout the Make SUR process.

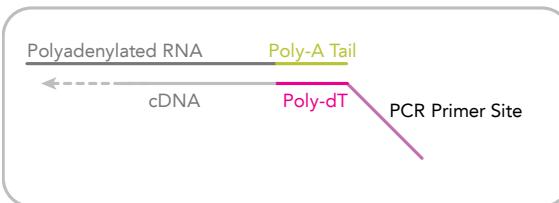


Figure 73 Make SUR



If you are performing the DASL Gene Expression Assay for VeraCode with FFPE-derived (formalin-fixed, paraffin-embedded) RNA, be aware that every previous freeze-thaw cycle that the RNA has gone through can cause a decrease of 0.01 in the correlation (R^2) between replicate samples run in parallel.

Estimated Time

Hands-on time: ~20 minutes

Incubation time: One 10 minute incubation; one 60 minute incubation

Consumables

Item	Quantity	Storage	Supplied By
MCS reagent	1 tube per plate	-20°C	Illumina
RNA samples and controls	96 or 384	-20°C	
96-well 0.2 ml skirted microplate	1 per 96 samples		User

Preparation

- ▶ In the appropriate columns of the Sample Sheet, enter the Sample_Name (optional), Sample_Plate, and Sample_Group for each Sample_Well. For more information, see *Sample Sheet* on page 144.
- ▶ Preheat a heat block to 42°C and allow the temperature to stabilize.
- ▶ Turn on the heat sealer to preheat it.
- ▶ Thaw the RNA samples and controls to room temperature and vortex to mix the contents.

- ▶ Thaw the MCS reagent tube to room temperature. Vortex briefly to mix. Pour the entire contents of the MCS tube into a new, nonsterile, disposable reservoir.
- ▶ Apply a SUR barcode label to a new 96-well microplate.

Steps

1. Add 5 µl MCS to each well of the SUR plate.
2. Normalize the RNA samples to 20–100 ng/µl with DEPC-treated H₂O.
3. Transfer 5 µl normalized RNA sample into each well of the SUR plate.
4. Seal the SUR plate with a microplate heat seal. Ensure that all wells are completely sealed.
5. Vortex the sealed plate at 2300 rpm for 20 seconds.
6. Pulse centrifuge to 250 xg.



CAUTION

It is important to centrifuge the SUR plate to 250 xg *before* the 42°C incubation to prevent the wells from drying out.

7. Incubate the SUR plate at room temperature for 10 minutes.
8. Place the SUR plate on the preheated heat block and close the lid. Incubate the plate at 42°C for 60 minutes.
9. Do one of the following:
 - Set the heat block to 70°C and proceed to *Make Assay Specific Extension (ASE) Plate* on page 160.
 - Store the SUR plate for up to 4 hours at 4°C or up to 24 hours at -20°C.

Make Assay Specific Extension (ASE) Plate

This process combines the biotinylated cDNAs from the SUR plate with DASL query oligos, hybridization reagents, and paramagnetic particles in an Assay Specific Extension (ASE) plate. The ASE plate is placed in a heat block and the query oligos for each sequence target of interest are allowed to anneal to the biotinylated cDNA samples. The cDNA is simultaneously captured by paramagnetic particles. The resulting ASE plate is ready for the extension and ligation of the hybridized oligos on the bound cDNAs.

This process is designed for one plate, using the SUR plate as input.

Estimated Time

Hands-on: ~20 minutes

Incubation: 2–16 hours

Consumables

Item	Quantity	Storage	Supplied By
OB1 reagent	1 tube per plate	-20°C	Illumina
DAP reagent	1 tube per plate	-20°C	Illumina
96-well 0.2 ml skirted microplate	1 per SUR plate		User

Preparation

- ▶ In the Pool_ID column of the Sample Sheet, enter the DAP for each Sample_Well.
- ▶ Preheat the heat block to 70°C and allow the temperature to stabilize.
- ▶ Turn on the heat sealer to preheat it. Allow 15 minutes.
- ▶ Thaw the DAP reagent tube to room temperature. Vortex the tube, and then pulse centrifuge to 250 xg. Pour the DAP reagent into a reagent reservoir.
- ▶ Thaw the OB1 reagent tube to room temperature. Vortex the tube to completely resuspend the beads. Invert tube to verify that all the paramagnetic particles are evenly suspended in solution. Pour the OB1 reagent into a second reagent reservoir.



Do not centrifuge the OB1 tube.

- ▶ Apply an ASE barcode label to a new 96-well microplate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - SUR plate barcode
 - ASE plate barcode

- OB1 reagent barcode
- DAP reagent barcode

**NOTE**

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Pulse centrifuge the SUR plate to 250 xg.
2. Add 10 µl DAP reagent to each well of the ASE plate.
3. Add 30 µl OB1 reagent to each well of the ASE plate.
4. Carefully remove the heat seal from the SUR plate.
5. Transfer 10 µl of biotinylated sample from each well of the SUR plate (approximately the entire volume) to the corresponding well of the ASE plate.
6. Using a microplate heat seal, heat-seal the ASE plate (3 seconds). Ensure that all wells are completely sealed.
7. Pulse centrifuge the ASE plate to 250 xg.
8. Vortex the ASE plate at 1600 rpm for 1 minute or until all beads are completely resuspended.
9. Place the sealed ASE plate on the 70°C heat block and close the lid.
10. Immediately reset the temperature to 30°C and allow the ASE plate to cool to 30°C. Allow about 2 hours. The ASE plate may remain in the heat block for up to 16 hours.
11. On the lab tracking form, record the start and stop times.
12. Proceed to *Add Master Mix for Extension & Ligation (MEL)* on page 162.

Add Master Mix for Extension & Ligation (MEL)

In this process, AM1 and UB1 reagents are added to the ASE plate to wash away non-specifically hybridized and excess oligos. An enzymatic extension and ligation master mix (MEL) is added to each DNA sample. The extension and ligation reaction occurs at 45°C.

Estimated Time	Hands-on: ~30 minutes
	Incubation: 15 minutes

Consumables

Item	Quantity	Storage	Supplied By
AM1 reagent	Bottle	4°C	Illumina
UB1 reagent	Bottle	4°C	Illumina
MEL reagent	1 tube per plate	-20°C	Illumina

Preparation

- ▶ Remove the ASE plate from the heat block.
- ▶ Preheat the heat block to 45°C for about one hour.
- ▶ Thaw the MEL tube to room temperature.
- ▶ Pour 11 ml AM1 into a reagent reservoir. Add 10 ml for each additional plate.
- ▶ Pour 11 ml UB1 into a second reagent reservoir. Add 10 ml for each additional plate.
- ▶ Pour the thawed MEL tube contents into a third reagent reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - ASE plate barcode
 - AM1 reagent barcode
 - UB1 reagent barcode
 - MEL reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps



CAUTION

In this procedure, you will remove all the liquid from the wells several times, leaving only the beads. Work quickly so that the beads do not dry out.

1. Centrifuge the ASE plate to 250 xg.
2. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.

If you are using the raised-bar magnetic plate from Illumina, the beads in odd-numbered columns will be pulled to the right wall of the well, and the beads in even-numbered columns will be pulled to the left wall of the well.

**NOTE**

To avoid aspirating the beads during this procedure, slant the pipette tips so that they draw liquid from the side of the well opposite the beads. Aspirate all of the odd columns first, and then rotate the plate and aspirate the even columns (or vice-versa). This enables you to keep the pipettor at the same angle throughout.

3. Carefully remove the heat seal from the ASE plate.
4. Using an 8-channel pipette with new tips, remove and discard all the liquid (50 µl) from the wells. Leave the beads in the wells.
It is not necessary to change pipette tips until you have removed the liquid from all 12 columns.
5. Visually inspect pipette tips after removing solution from each column to ensure no beads have been removed. If beads are visible in pipette tips, return the solution to the same wells, allow magnet to re-collect beads, and change the pipette tips.
6. With the ASE plate on the raised-bar magnetic plate, use an 8-channel pipette with new tips to add 50 µl AM1 to each well of the ASE plate.

**CAUTION**

To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (Figure 74, page 163). If you suspect the tips are contaminated with the contents of the well, use new tips.



Figure 74 Avoid Tip Contamination

7. Seal the ASE plate with microplate clear adhesive film.

8. Vortex the ASE plate at 1600 rpm for 20 seconds or until all beads are resuspended.
9. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
10. Remove the seal from the ASE plate, taking care to avoid splashing from the wells.
11. Using the same 8-channel pipette with the same tips, remove all AM1 reagent from each well. Leave the beads in the wells.
It is not necessary to change pipette tips until liquid has been removed from all 12 columns.
12. Repeat steps 6 through 11 once.
13. Remove the ASE plate from the raised-bar magnetic plate.
14. Using an 8-channel pipette with new tips, add 50 μ l UB1 to each well of the ASE plate.
15. Place the ASE plate onto the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
16. Using the same 8-channel pipette with the same tips, remove all UB1 reagent from each well. Leave the beads in the wells.
It is not necessary to change pipette tips until liquid has been removed from all 12 columns.
17. Repeat steps 13 through 16 once.
18. Using an 8-channel pipette with new tips, add 37 μ l MEL to each well of the ASE plate.
19. Seal the plate with microplate clear adhesive film.
20. Vortex the plate at 1600–1700 rpm for 1 minute or until the beads are resuspended.
21. Incubate the ASE plate on the preheated 45°C heat block for exactly 15 minutes.

**CAUTION**

Do not allow the ASE plate to incubate at 45°C longer than 15 minutes.

22. During the incubation, perform the *Make PCR Plate* procedure.
23. On the lab tracking form, record the start and stop times.
24. Proceed to *Inoculate PCR Plate* on page 167. Leave the ASE plate at room temperature if you proceed immediately, or store it at 4°C for up to 1 hour.

Make PCR Plate

This process adds the Illumina-recommended DNA Polymerase and optional Uracil DNA Glycosylase (UDG) to the master mix for PCR (MMP reagent) and creates a 96-well plate for the Inoc PCR process.

Estimated Time Hands-on: ~15 minutes

Consumables

Item	Quantity	Storage	Supplied By
MMP reagent	1 tube per plate	-20°C	Illumina
DNA Polymerase	64 µl	-20°C	User
Uracil DNA Glycosylase (UDG, Optional)	50 µl	-20°C	User
96-well 0.2 ml skirted microplate	1 per ASE plate		User

Preparation

- ▶ Thaw the MMP tube to room temperature.
- ▶ Apply a PCR barcode label to a new 96-well microplate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - PCR plate barcode
 - MMP reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Add 64 µl DNA Polymerase to the MMP tube. Check off this action in the lab tracking form.
2. [Optional] Add 50 µl Uracil DNA glycosylase to the MMP tube. Check off this action in the lab tracking form.
3. Invert the tube several times to mix the contents.
4. Pour the contents of the tube into a reagent reservoir.
5. Using an 8-channel pipette, add 30 µl of the mixture into each well of the PCR plate.
6. Seal the PCR plate with microplate clear adhesive film.
7. Pulse centrifuge to 250 xg.

8. Proceed to *Inoculate PCR Plate* on page 167.

Inoculate PCR Plate

This process uses the template formed in the extension and ligation process in a PCR reaction. This PCR reaction uses three universal primers (MMP reagent): two are labeled with fluorescent dyes and the third is biotinylated. The biotinylated primer allows capture of the PCR product and elution of the strand containing the fluorescent signal. The eluted samples are transferred from the ASE plate to the PCR plate.

Estimated Time	Hands-on: ~30 minutes
-----------------------	-----------------------

Consumables

Item	Quantity	Storage	Supplied By
UB1 reagent	Bottle	4°C	Illumina
IP1 reagent	1 tube per plate	-20°C	Illumina

Preparation

- ▶ Remove the ASE plate from the heat block.
- ▶ Reset the heat block to 95°C.
- ▶ Pour 6 ml UB1 into a reagent reservoir.
- ▶ Thaw the IP1 reagent to room temperature. Pour the contents of the tube into a reagent reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - ASE plate barcode
 - PCR plate barcode
 - IP1 reagent barcode
 - UB1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
2. Remove the microplate clear adhesive film from the ASE plate.
3. Using an 8-channel pipette, remove and discard the supernatant (~50 µl) from all wells of the ASE plate. Leave the beads in the wells.
It is not necessary to change pipette tips until liquid has been removed from all 12 columns.

4. Visually inspect pipette tips after removing solution from each column to ensure no beads have been removed. If beads are visible in pipette tips, return the solution to the same wells, allow magnet to re-collect beads, and change the pipette tips.
5. Leaving the plate on the magnet and using an 8-channel precision pipette with new tips, add 50 µl UB1 to each well of the ASE plate.



To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (Figure 74). If you suspect the tips are contaminated with the contents of the well, discard the tips and use new tips.

6. Leave the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
7. Remove and discard the supernatant (~50 µl) from all wells of the ASE plate. Leave the beads in the wells.
It is not necessary to change pipette tips until liquid has been removed from all 12 columns.
8. Remove the plate from the magnet.
9. Using an 8-channel pipette with new tips, add 35 µl IP1 to each column of the ASE plate.
10. Seal the plate with microplate clear adhesive film.
11. Vortex at 1800 rpm for 1 minute, or until all the beads are resuspended.
12. Place the plate on the 95°C heat block for 1 minute.
13. Place the ASE plate back onto the raised-bar magnetic plate for 2 minutes or until the beads have been completely captured.
14. Using an 8-channel pipette with new tips, transfer 30 µl supernatant from each well in the first column of the ASE plate to the first column of the PCR plate.
15. Repeat for each column of the ASE plate. Change tips between column dispenses.



Take special care not to disturb or transfer the beads when aspirating eluted product.

16. Discard the ASE plate.
17. Seal the PCR plate with the appropriate PCR plate-sealing film for your thermocycler.
18. Proceed immediately to *Thermal Cycle PCR Plate* on page 169.

Thermal Cycle PCR Plate

This process thermal cycles the PCR plate to fluorescently label and amplify the templates generated in the pre-PCR process.

Estimated Time Thermal Cycle: ~2 hours 45 minutes

Preparation

- On the lab tracking form, record:
- Date and time
 - Operator
 - PCR plate barcode
 - Thermocycler ID
 - Thermocycler program

Steps

1. Place the sealed plate into the thermocycler and run the thermocycler program shown in this table.

Table 36 Thermocycler Program

	Temperature	Time
	37°C	10 minutes
	95°C	3 minutes
X 34 {	95°C	35 seconds
	56°C	35 seconds
	72°C	2 minutes
	72°C	10 minutes
	4°C	5 minutes

2. Do one of the following:

- Proceed immediately to *Bind PCR Products* on page 170. Store the PCR plate at room temperature in a light-protected drawer.
- Seal and store the PCR plate at -20°C overnight.

Bind PCR Products

In this process, MPB reagent is added to the PCR plate and the solution is transferred to a filter plate. The filter plate is incubated at room temperature to bind the biotinylated strand to paramagnetic particles, thus immobilizing the double-stranded PCR products.

Estimated Time	Hands-on: ~30 minutes
	Incubation: 1 hour

Consumables

Item	Quantity	Storage	Supplied By
MPB reagent	1 tube per PCR plate	4°C	Illumina
0.45 µM clear Styrene filter plate with lid	1 per PCR plate		User

Preparation

- ▶ Vortex the MPB tube several times until the beads are well resuspended. Pour the contents of the tube into a non-sterile reagent reservoir.
- ▶ Write the PCR plate barcode number in the space provided on a “Filter Plate: GS _____ -PCR” label. Apply the label to the top surface of the filter plate, adjacent to column 12 (Figure 75).



This ensures that the filter plate is associated with the correct PCR plate. Do not attach the label to the lid of the filter plate, as the lid could get separated from the plate.

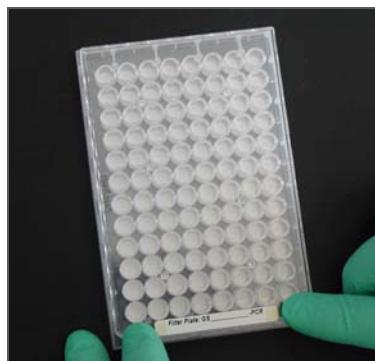


Figure 75 Apply Label to Filter Plate

- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - MPB reagent barcode
 - Filter plate barcode

**NOTE**

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Pulse centrifuge the PCR plate to 250 xg.
 2. Place new tips onto an 8-channel pipette.
 3. Add 20 µl resuspended MPB into each well of the PCR plate.
It is not necessary to change pipette tips until liquid has been transferred to all 12 columns.
-
-
- CAUTION**
- To avoid contaminating the tips, place the tips against the top edge of the wells (Figure 74, page 163). If you suspect the tips are contaminated with the contents of the well, use new tips.
-
4. Set an 8-channel pipette to 85 µl to allow space for bubbles, and attach new tips.
 5. Pipette the solution in the PCR plate up and down several times to mix the beads with the PCR product
 6. Transfer the mixed solution into the first column of the filter plate. There should be about 70 µl fluid in each well.
 7. Repeat step 6 for each column of the PCR plate. Change tips between column dispenses.
 8. Discard the empty PCR plate.
 9. Cover the filter plate with its lid and store it at room temperature, protected from light, for 60 minutes.
 10. On the lab tracking form, record the start and stop times.
 11. Proceed to *Make Intermediate Plate for VeraCode Bead Plate* on page 172.

Make Intermediate Plate for VeraCode Bead Plate

In this process, the PCR product is washed in the filter plate with UB2 and NaOH. The single-stranded, fluor-labeled material is then eluted into an INT plate containing MH2 reagent.

Estimated Time Hands-on: ~30 minutes

Consumables

Item	Quantity	Storage	Supplied By
MH2 reagent	1 tube per plate	Room temperature	Illumina
UB2 reagent	Bottle	Room temperature	Illumina
0.1N NaOH	Bottle	4°C	User
96-well V-bottom plate	2 per PCR plate		User

Preparation

- ▶ Apply a INT barcode label to a new 96-well V-bottom plate.
- ▶ Using a serological pipette, transfer 6 ml UB2 into a sterile reservoir.
- ▶ Pour 4 ml 0.1N NaOH into a second sterile reservoir.
- ▶ Pour the contents of the MH2 tube into a third sterile reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - INT plate barcode
 - UB2 reagent barcode
 - MH2 reagent barcode
 - NaOH lot number



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Place the filter plate adapter on an empty 96-well V-bottom plate (waste plate) (Figure 76).
2. Place the filter plate containing the bound PCR products onto the filter-plate adapter (Figure 76).



Figure 76 Assemble Filter Plate

3. Centrifuge to 1000 xg for 5 minutes at 25°C.
4. Remove the filter plate lid.
5. Using an 8-channel pipette with new tips, add 50 µl UB2 to each well of the filter plate.
Dispense slowly to avoid disturbing the beads.



CAUTION

To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (see Figure 74, page 163).

6. Replace the filter plate lid.
7. Centrifuge to 1000 xg for 5 minutes at 25°C.
8. Using an 8-channel pipette with new tips, add 30 µl MH2 to each well of the INT plate.
9. Replace the waste plate with the INT plate. Orient the INT plate so that well A1 of the filter plate matches well A1 of the INT plate.



WARNING

Be sure to replace the waste plate with the INT plate. Failure to replace the waste plate will result in loss of samples.

10. Discard the waste plate.
11. Using an 8-channel pipette with new tips, add 30 µl 0.1N NaOH to all wells of the filter plate.
12. Replace the filter plate lid.



NOTE

Due to the sensitivity of the dyes to 0.1N NaOH, proceed quickly. Prolonged incubation with NaOH is unnecessary; less than 5 minutes is sufficient. The DNA is denatured almost instantly.

13. Centrifuge immediately at 1000 xg for 5 minutes at 25°C. At the end, no beads should be visible in the wells of the INT plate.
14. Discard the filter plate. Save the adapter for later use in other protocols.
15. Gently mix the contents of the INT plate by moving it from side to side without splashing.
16. Cover the INT plate with clear adhesive seal.
17. Do one of the following:
 - Proceed to *Hybridize VeraCode Bead Plate* on page 175.
 - Seal the INT plate with a cap mat and store it at -20°C.

Hybridize VeraCode Bead Plate

This process uses the VeraCode Vortex Incubator, an incubating microplate shaker, to hybridize the VeraCode Bead Plate (VBP). Once the samples are transferred to the VBP, they are ready for hybridization at 50°C.

Estimated Time	Hands-on: ~20 minutes Incubation: 3 hours
-----------------------	--

Consumables

Item	Quantity	Storage	Supplied By
MH2 reagent	1 tube per plate	Room temperature	Illumina
0.1N NaOH	Bottle	4°C	User
VeraCode Bead Plate	1 per INT plate		Illumina

Preparation

- ▶ If the INT plate has been frozen, thaw to room temperature in a light-protected drawer, then pulse centrifuge to 250 xg.
- ▶ Preheat the VeraCode Vortex Incubator to 50°C and allow it to equilibrate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - INT plate barcode
 - VBP plate barcode
 - MH2 reagent barcode
 - NaOH lot number



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps Add Neutralized MH2 to INT VBP

1. Using a serological pipette, transfer 3 ml MH2 into a 15 ml conical tube.
2. Using a different serological pipette, transfer 3 ml 0.1 N NaOH to the 15 ml tube.
3. Vortex the tube until the contents are mixed.
4. Pour the mixture into a sterile reservoir.

5. Using an 8-channel pipette, add 50 µl of neutralized MH2 to each of the INT plate wells that contain sample. Be careful not to let the pipette tips touch the sample.

Hybridize VBP

1. Remove the VBP from the 4°C refrigerator and pulse centrifuge to 250 xg. If the beads are not at the bottoms of the wells, pulse centrifuge again.
2. Remove the cap mat from the VeraCode Bead Plate. Save the cap mat for subsequent use in hybridization.
3. Using an 8-channel pipette with new tips, pipette each column of sample in the INT plate up and down 4–5 times.
4. Using the same tips, transfer 100 µl of each assay product from the INT plate into the corresponding well of the VeraCode Bead Plate.
5. Place the cap mat back on the VeraCode Bead Plate.
6. Place the VeraCode Bead Plate Bead Plate, which now contains samples, into the VeraCode Vortex Incubator. You can load up to 2 VBP plates in the vortexer.



NOTE

If you load only one plate, load an empty 96-well plate in the opposite position as a balance.

7. Close the lid and make the following settings:
 - Push the **Encoder** knob until **RPM** is highlighted. Rotate the knob to 85 (850 rpm).
 - Push the **Encoder** knob until **Time** is highlighted. Rotate the knob below 0.30 or above 99.5 until HLD appears. This sets the Vortex Incubator to run continuously.
 - Push the **Encoder** knob until **Temperature** is highlighted. Rotate the knob to 50 (50°C).
8. Press **Start/Stop** and incubate for 3 hours.
9. Proceed to *Wash VeraCode Bead Plate* on page 177.

Wash VeraCode Bead Plate

In this process, the VeraCode Bead Plate is removed from the VeraCode Vortex Incubator and washed two times with the VW1 reagent.

Estimated Time Hands-on: ~15 minutes

Consumables

Item	Quantity	Storage	Supplied By
VW1 reagent	Bottle	Room temperature	Illumina

Preparation

- ▶ Pour 45 ml of VW1 into a nonsterile reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - VW1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Stop the VeraCode Vortex Incubator. When the speed indicator reaches 0, open the lid and remove the VeraCode Bead Plate.
2. Pulse centrifuge the plate to 250 xg.
3. Remove the cap mat.
4. Using an 8-channel pipette, add 200 µl VW1 buffer to each well. Make sure to agitate the bead pellet.
5. Gently swirl the plate in a circular motion on the benchtop.
6. Wait 2 minutes for the beads to collect in the bottom of the well.
7. Aspirate the supernatant with the vacuum manifold at a pressure of 50 mbar.
8. Repeat steps 4 through 7 once.
9. Do one of the following:
 - a. Proceed to *Scan VeraCode Bead Plate* on page 178.
 - b. Seal the VBP plate with an adhesive seal and store it in the dark at room temperature for up to 24 hours.

Scan VeraCode Bead Plate

The BeadXpress Reader uses lasers to excite the Cy3 and Cy5 fluors of the single-stranded PCR products bound to the VeraCode beads. Light emissions from these fluors are then recorded in a data file. Fluorescence data are analyzed to derive assay results using Illumina's GenomeStudio software package.

Estimated Time	80 samples/hour at 96-plex 27 samples/hour at 384-plex
-----------------------	---

Preparation	<ul style="list-style-type: none">▶ Prepare a scan settings file containing information about your samples, the BeadXpress Reader settings, and VeraCode beads. If you intend to analyze DASL gene expression data in GenomeStudio, you should enter the VeraCode Bead Plate serial number (CK#-VBP) in the Plate_ID field.
--------------------	---

Steps	For instructions on scanning VeraCode Bead Plate, see the <i>BeadXpress Reader System Guide</i> .
--------------	---

Troubleshooting

Use the information in this section to troubleshoot the DASL Gene Expression Assay for VeraCode.

RNA Sample Preparation

Table 37 Problems Observed During RNA Sample Preparation

Symptom	Probable Cause	Resolution	Comments
Some or all of the contents of the wells in SUR plate evaporated during the 42°C incubation.	Plate was left in heat block for longer than 60 minutes.	Repeat Make SUR.	Some condensation is normal.
	Heat block lid was not used.	Repeat Make SUR.	
	Heat seal was not completely sealed to plate, allowing evaporation.	Check heat sealer to ensure that it is functioning properly.	
	Incorrect heat seal was used to seal plate.	Use ABgene (catalog # AB-0559) foil seals for this step.	
Excessive condensation was observed on the bottom side of the heat seal after the Make ASE incubation.	Heat block lid was not used.	Centrifuge the plate to remove condensation from the seal and proceed to Add MEL.	Condensation can be minimized by using foil seals from ABgene (catalog # AB-0559).
Heat block was left at 70°C overnight for the Make ASE incubation.	Heat block temperature was not set to 30°C after loading plate in heat block.	Repeat experiment. Samples have been ruined.	Set temperature to 30°C immediately after loading the ASE plate into heat block.
Beads are difficult to resuspend during Add MEL and Inoc PCR. Note: This applies especially to gDNA samples.	High-speed shaker may be out of calibration.	Recalibrate high-speed shaker (see <i>Calibrate the Vortexer</i> on page 21).	Particularly difficult samples can be resuspended manually using a pipettor.

Hybridize VeraCode Bead Plate

Table 38 Problems Observed During Hyb VBP

Symptom	Probable Cause	Resolution	Comments
The cap mat came up in parts of the VBP plate during the 50°C incubation.	The cap mat was not completely sealed and the sample solution dried out.	Repeat experiment on the sample that was dried out.	Use cap mat applicator to ensure the cap mat is sealed completely to the plate.

Signal Intensity

Table 39 Problems with Signal Intensity

Symptom	Probable Cause	Resolution	Comments
Low intensity was observed in all the bead types of the VBP while scanning.	Wrong wash buffer was used.	Repeat experiment.	Use VW1 to wash the sample plate.
	Wrong pMT setting was used.	Stop the current scan and rescan the VBP using a higher pMT setting.	
Low intensity was observed. However, second hyb controls were fine.	Failures occurred in one of the experimental steps upstream of second hyb.	Check the control panel to assess data quality.	As a process control, including activated gDNA samples in the assay can help determine whether the problem comes from the VeraCode GoldenGate steps as opposed to the VeraCode DASL steps.
Low signal was observed for RNA samples only.	RNA concentration was too low.	Re-check RNA concentration with RiboGreen.	
	Failure occurred during Make SUR preparation or in the heat block temperature settings.	Calibrate heat block.	
	Possible RNase contamination of sample.	Maintain RNase-free best practices. Check working equipment for RNase contamination.	Illumina recommends the Ambion RNaseAlert Lab Test Kit (catalog # 1964).

Analysis

Table 40 Problems Observed During Analysis

Symptom	Probable cause	Resolution	Comments
Low correlation between RNA sample replicates.	Incorrect cycler program or temperature control problems on the cycler.	Measure the cycle time for PCR plates. Expected times range from 2 hours and 45 minutes to 3 hours and 5 minutes depending on the cycler.	Always record the times and compare them to historical norms.
	VBP plate was not incubated at correct temperature (50°C).	Repeat the experiment.	
	Post-hyb wash was not done after the 50°C 3-hour incubation.	Repeat the experiment.	
	Possible RNase contamination of sample.	Maintain RNase-free best practices.	Illumina recommends Ambion RNaseAlert Lab Test Kit (catalog # 1964).
Strong signal from multiple contamination controls observed in control panel.	DAP tubes were pooled during the experiment.	For the contamination controls to be informative, do not pool contents of multiple DAP tubes.	DAP tubes with the same barcode may have different contamination controls.
	Cross-contamination may have occurred.	Use the VeraCode DASL kit with UDG to control amplicon contamination. In addition, treat lab work surfaces with 10% bleach and allow them to air-dry.	
Cy3/Cy5 ratio was higher than usual in the second hybridization controls.	PCR plate with MPB beads in the Bind PCR process were subjected to excessive light.	Protect PCR plate from light. Fluorescent lighting is permissible, but keep plates in the dark when not in use.	
	Bleach or bleach fumes might have been present.	Remove bleach container during procedure; allow bleach fumes to dissipate after cleaning lab surfaces.	In sufficient concentrations, bleach will also affect Cy3.
	The washed VBP plate was subjected to excessive light before scanning.	Protect the washed VBP plate from light while waiting for the scanner.	It is normal to see a slightly increased Cy3/Cy5 ratio with a washed VBP plate that has been staged for longer than 1 day.

Table 40 Problems Observed During Analysis (Continued)

Symptom	Probable cause	Resolution	Comments
Gene expression results did not correlate with samples.	Plate orientations were reversed.	Re-sort data in inverse order (H12 to A1) and re-analyze.	Add a positive known control sample in a standardized, non-symmetric well position.
	Wrong scan settings file was loaded into BeadXpress Reader.	Load the correct scan settings file.	
	Wrong DAP manifest was loaded into GenomeStudio or associated with the Sample Sheet.	Load the correct DAP manifest.	
	The samples were copied directly from the scan settings file to the Sample Sheet or vice versa.	Rearrange the sample names according to the templates for row-major or column-major file.	
	Wrong RNA was loaded in well.	There is no solution for this problem. Monitor RNA input carefully.	
r^2 correlation between intact control RNA and formalin-fixed, paraffin-embedded (FFPE) samples was low.	The degradation of FFPE samples varied compared to the control RNA.	Do not use the VeraCode DASL Assay to compare intact RNA with degraded RNA.	

Chapter 6

GoldenGate Methylation Assay for VeraCode Protocols

Topics

- 184 Introduction
- 185 Workflow
- 186 Tracking Tools
- 189 Materials and Reagents for this Assay
- 191 Make DNA Quantitation Plate (Optional)
- 196 Read QDNA Plate (Optional)
- 200 Make Bisulfite-Converted DNA (BCD) Plate
- 204 Make Bisulfite-Converted Single-Use DNA (BCS) Plate
- 206 Precipitate BCS Plate
- 208 Resuspend BCS Plate
- 209 Make Allele-Specific Extension (ASE) Plate
- 211 Add Master Mix for Extension & Ligation (MEL)
- 214 Make PCR Plate
- 216 Inoculate PCR Plate
- 218 Thermal Cycle PCR Plate
- 219 Bind PCR Products
- 221 Make Intermediate Plate for VeraCode Bead Plate
- 224 Hybridize VeraCode Bead Plate
- 226 Wash VeraCode Bead Plate
- 227 Scan VeraCode Bead Plate

Introduction

This chapter provides detailed pre- and post-PCR laboratory protocols for the GoldenGate Methylation Assay for VeraCode. Perform each protocol in the order shown.

The instructions assume that you are preparing 96 samples. If you are preparing fewer than 96 samples, scale down the protocols accordingly. For more information, see *Prepare Fewer than 96 Samples* on page 22.

The instructions in this chapter assume that you have already familiarized yourself with Chapter 2, *Standard Operating Procedures* and have set up the lab area appropriately.



CAUTION

It is very important to prevent PCR product contamination during this assay. To learn about safe lab practices for Illumina assays, see Chapter 2, *Standard Operating Procedures*. In addition, follow all of the safety procedures described in this chapter.

Workflow

The following diagram illustrates the workflow for the GoldenGate Methylation Assay for VeraCode (Figure 77).

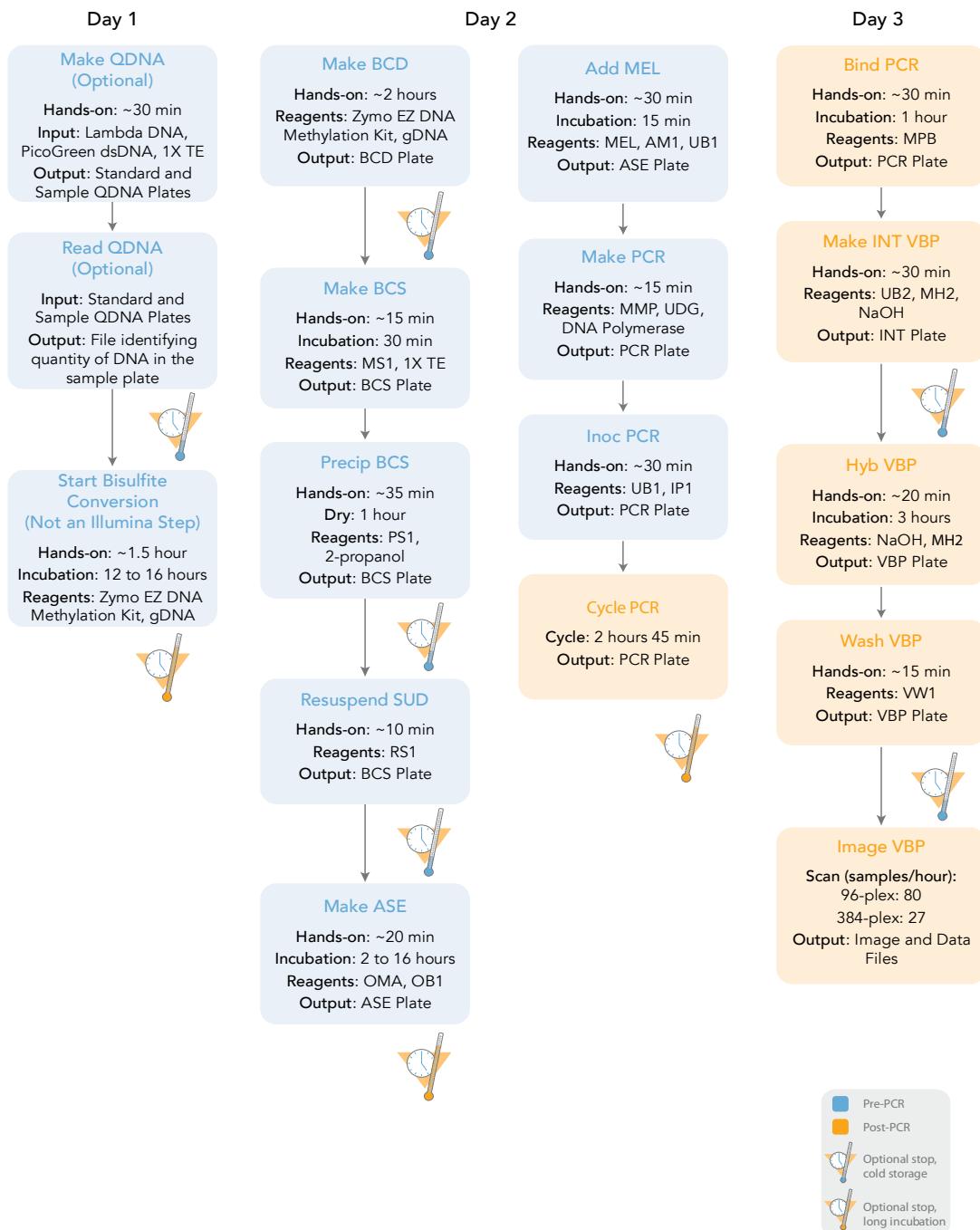


Figure 77 GoldenGate Methylation Assay for VeraCode Workflow

Tracking Tools

Lab Tracking Form

Create a copy of the two-page lab tracking form for each run (Figure 78). Use it to track information such as operator ID and reagent barcodes, and to record the sample location in each plate.



You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

<div style="border: 1px solid black; padding: 10px;"> <p>illumina®</p> <p style="text-align: center;">GoldenGate® Methylation Assay for VeraCode® Lab Tracking Form</p> <p>1 Bisulfite-Convert gDNA Date/Time: _____ GS4 DNA Plate: _____ Operator: _____ BCD Plate: _____ <input type="checkbox"/> Bisulfite-convert with Zymo EZ DNA Methylation Kit</p> <p>2 Activate DNA (Make, Precipitate, Resuspend) Date/Time: _____ BCS Plate: _____ Operator: _____ Robot: _____ MS1 Reagent: _____ Plate Positions on Robot: _____ PS1 Reagent: _____ (Make) _____ (Precip) _____ (Resud) _____ RS1 Reagent: _____ Heat block (95°C, 30 m): Start: _____ Stop: _____ Centrifuge (3000 xg, 20 m): Start: _____ Stop: _____ Air dry (22°C, 15 m): Start: _____ Stop: _____</p> <p>3 Make ASE Date/Time: _____ BCS Plate: _____ Operator: _____ Robot: _____ ASE Plate: _____ Plate Positions on Robot: _____ OB1 Reagent: _____ Heat block (2 h): _____ OMA Reagent: _____ Start (70°C): _____ Stop (30°C): _____</p> <p>4 Add MEL Date/Time: _____ ASE Plate: _____ Operator: _____ Robot: _____ AM1 Reagent: _____ Plate Positions on Robot: _____ UB1 Reagent: _____ Heat block (45°C, 15 m): Start: _____ Stop: _____ MEL Reagent: _____</p> <p>5 Make PCR Date/Time: _____ PCR Plate: _____ Operator: _____ Robot: _____ MMP Reagent: _____ Plate Position on Robot: _____ <input type="checkbox"/> Add Recommended DNA Polymerase <input type="checkbox"/> Add Uracil DNA Glycosylase (UDG)</p> <p>6 Inoc PCR Date/Time: _____ ASE Plate: _____ Operator: _____ Robot: _____ PCR Plate: _____ Plate Positions on Robot: _____ IP1 Reagent: _____ <input type="checkbox"/> Incubate in Heat Block (95°C, 1 m) UB1 Reagent: _____</p> <p style="text-align: right;">Pre-PCR Post-PCR</p> <p style="text-align: center;">Page 1 of 2 Catalog # VC-901-1001 Part # 11312739 Rev A</p> </div>	<div style="border: 1px solid black; padding: 10px;"> <p>illumina®</p> <p style="text-align: center;">GoldenGate® Methylation Assay for VeraCode® Lab Tracking Form</p> <p>7 Cycle PCR Date/Time: _____ PCR Plate: _____ Operator: _____ Thermal Cycler: _____ Thermal Cycle Program Name: _____ Start: _____ Stop: _____</p> <p>8 Bind PCR Date/Time: _____ MPB Reagent: _____ Operator: _____ Robot: _____ Plate Position on Robot: _____ Incubate in light-protected drawer (22°C, 1 hour): Start: _____ Stop: _____</p> <p>9 Make INT Date/Time: _____ INT Plate: _____ Operator: _____ Robot: _____ UB2 Reagent: _____ Plate Positions on Robot: _____ MH2 Reagent: _____ Centrifuge (1000 xg, 25°C, 5 m): NaOH Lot #: _____ <input type="checkbox"/> Start: _____ Stop: _____</p> <p>10 Hyb VBP Date/Time: _____ INT Plate: _____ VBP Plate: _____ Operator: _____ VBP Plate: _____ Vortex Incubator ID: _____ MH2 Reagent: _____ (850 rpm, 45°C, 3 h): Start: _____ Stop: _____ NaOH Lot #: _____</p> <p>11 Wash VBP Date/Time: _____ VBP Plate: _____ WV1 Reagent: _____ Operator: _____ <input type="checkbox"/> Vacuum-Aspirate Supernatant (50 mb)</p> <p>12 Scan VBP Date/Time: _____ VBP Plate: _____ BeadXpress® Reader ID: _____ Operator: _____</p> <p style="text-align: right;">Pre-PCR Post-PCR</p> <p style="text-align: center;">Page 2 of 2 Catalog # VC-901-1001 Part # 11312739 Rev A</p> </div>
---	--

Figure 78 GoldenGate Methylation Assay for VeraCode Lab Tracking Form

Sample Sheet

To effectively track your samples and assay, we recommend that you create a Sample Sheet. The Sample Sheet will later be used by the GenomeStudio Methylation Module for data analysis. See the *GenomeStudio Methylation Module User Guide* for more information.

Create your Sample Sheet according to the guidelines provided in Table 41.

Table 41 Sample Sheet Guidelines

Section	Description	Optional (O) or Required (R)
Sample_Name	Example: S12345 If not user-specified, the GenomeStudio application will assign a default sample name, concatenating the sample plate and sample well names.	O
Sample_Well	Example: A01 The well containing the specific sample in the 96-well bisulfite-converted DNA plate.	O
Sample_Plate	Example: GS0005623-BCD User-specified name for the plate containing bisulfite-converted DNA samples.	O
Sample_Group	Example: Group 1 User-specified name of the sample group. If the Sample_Group is missing, GenomeStudio creates one group and assigns it a default name.	R
Pool_ID	Example: VC0007005-OMA Name of the methylation oligo pool.	R
Sentrix_ID	Example: CK0004001-VBP VeraCode Bead Plate ID.	R
Sentrix_Position	Example: A01 The VeraCode Bead Plate well position to which the sample is hybridized.	R
Notes	Your sample sheet header may contain whatever information you choose. Your sample sheet may contain any number of columns you choose. Your sample sheet must be in a comma-delimited (.csv) file format. Save the sample sheet under any name you wish; for example, the user-defined experiment name.	

Figure 79 provides an example of the Sample Sheet format. The VeraCode Assay Documentation CD includes a Sample Sheet template file that you can copy and use.

The screenshot shows a Microsoft Excel window titled "Microsoft Excel - 060607_Sample_Sheet_Methyl-384_VeraCode.csv". The window displays a table with two main sections: a header section and a data section.

Header Section:

	A	B	C	D	E	F	G
1	[Header]						
2	Investigator Name	Steffen					
3	Project Name	VeraCode					
4	Experiment Name						
5	Date	6/6/2007					
6							

Data Section:

	A	B	C	D	E	F	G
8	Sample_Name	Sample_Well	Sample_Plate	Sample_Group	Pool_ID	Sentrix_ID	Sentrix_Position
9						60507	R001_C001
10						60507	R001_C002
11						60507	R001_C003
12						60507	R001_C004
13						60507	R001_C005
14						60507	R001_C006
15						60507	R001_C007
16						60507	R001_C008
17						60507	R001_C009
18						60507	R001_C010
19						60507	R001_C011
20						60507	R001_C012
21						60507	R002_C001
22						60507	R002_C002
23						60507	R002_C003
24						60507	R002_C004
25						60507	R002_C005
26						60507	R002_C006
27						60507	R002_C007
28						60507	R002_C008
29						60507	R002_C009
30						60507	R002_C010

Figure 79 VeraCode Sample Sheet

Materials and Reagents for this Assay

These items are specifically required for the GoldenGate Methylation Assay for VeraCode. For a list of equipment, materials, and reagents required for all assays in a BeadXpress Reader lab, see *Standard Equipment, Materials, and Reagents* on page 17.

User-Supplied

Table 42 User-Supplied Materials and Reagents

Item	Source
Quant-iT PicoGreen DNA quantification reagent	Molecular Probes Invitrogen, catalog # P7581
Lambda DNA	Invitrogen, catalog # 25250-028
Zymo Research EZ DNA Methylation kit • Bisulfite-conversion reagent • Dilution buffer • Desulphonation buffer • Elution buffer	Zymo Research, catalog # D5001 (capped columns, 50 DNA reactions) Zymo Research, catalog # D5002, 200 DNA reactions Zymo Research, catalog # D5003 (96-well format)
Uracil DNA Glycosylase (UDG, Optional)	

Illumina-Supplied

Table 43 Illumina-Supplied Materials and Reagents

Item	Catalog #
VeraCode 96-Plex GoldenGate Kit, 480 samples • BOX A VeraCode DNA Activation Kit • BOX B VeraCode GoldenGate Pre-PCR #1 • BOX C VeraCode GoldenGate Pre-PCR #2 • BOX D VeraCode GoldenGate Post-PCR • BOX E 96-Plex VeraCode Bead Plates • OMA GoldenGate Methylation Assay custom oligo pool, shipped separately <i>Description of box contents appears below.</i>	VC-201-0096
VeraCode 384-Plex GoldenGate Kit, 480 samples • BOX A VeraCode DNA Activation Kit • BOX B VeraCode GoldenGate Pre-PCR #1 • BOX C VeraCode GoldenGate Pre-PCR #2 • BOX D VeraCode GoldenGate Post-PCR • BOX E 384-Plex VeraCode Bead Plates • OMA GoldenGate Methylation Assay custom oligo pool, shipped separately <i>Description of box contents appears below.</i>	VC-201-0384

VeraCode GoldenGate Kit Box Contents**Table 44** Box Contents

Box	Contents
BOX A VeraCode DNA Activation Kit	MS1 —Reagent used to activate sufficient DNA PS1 —Precipitation solution for DNA activation RS1 —Resuspension solution for DNA activation
BOX B VeraCode GoldenGate Pre-PCR #1	OB1 —Oligo hybridization and cDNA and gDNA binding buffer MMP —Master mix for PCR reagent IP1 —Reagent used to elute extended and ligated products UB1 —Universal buffer used to wash paramagnetic beads
BOX C VeraCode GoldenGate Pre-PCR #2	MEL —Reagent used for extension and ligation AM1 —Reagent used to wash away non-specifically hybridized and excess oligos from the gDNA
BOX D VeraCode GoldenGate Post-PCR	MPB —Magnetic particle reagent used to bind double-stranded PCR products MH2 —Reagent used to make the VBP plate UB2 —Universal buffer used to wash magnetic particles and the SAM VW1 —Reagent used to wash the VeraCode beads
BOX E 96-Plex VeraCode Bead Plates	96-plex VeraCode Bead Plate (5)
BOX E 384-Plex VeraCode Bead Plates	384-plex VeraCode Bead Plate (5)

Other Materials

- ▶ QDNA barcode labels
- ▶ GS#-DNA barcode labels
- ▶ BCD barcode labels
- ▶ BCS barcode labels
- ▶ ASE barcode labels
- ▶ PCR barcode labels
- ▶ Filter plate: GS_____ -PCR labels
- ▶ INT barcode labels
- ▶ Filter plate adapter
- ▶ Vortexer calibration label

Make DNA Quantitation Plate (Optional)

This process uses the PicoGreen dsDNA quantitation reagent to quantitate double-stranded DNA samples before bisulfite conversion.

Illumina recommends using the Invitrogen Molecular Probes PicoGreen assay to quantitate dsDNA samples. The PicoGreen assay can quantitate small DNA volumes, and measures DNA directly. Other techniques may pick up contaminants such as RNA and protein.

Estimated Time

Hands-on: 20 minutes per plate, plus 10 minutes to prepare the PicoGreen

Consumables

Item	Quantity	Storage	Supplied By
PicoGreen dsDNA quantitation reagent	See instructions	-20°C	User
1X TE	See instructions	Room temperature	User
Lambda DNA	See instructions	-20°C	User
96-well 0.65 ml microplate (MIDI)	1 per 96 samples		User
Fluotrac 200 (96-well black flat-bottom) plate	2 per 96 samples		User

Preparation

- ▶ Remove PicoGreen reagent from freezer and thaw at room temperature for 60 minutes in a light-impermeable container.
- ▶ Label a 96-well MIDI plate "Standard QDNA."
- ▶ Label a 96-well black flat-bottom plate "Standard QDNA."
- ▶ Label a 96-well black flat-bottom plate "Sample QDNA."

Steps

Make Standard QDNA MIDI Plate

In this process, you create a Standard QDNA plate with serial dilutions of stock Lambda DNA in the wells of column 1.

1. Place stock Lambda DNA in well A1 of the Standard QDNA MIDI plate and dilute it to 75 ng/µl in a final volume of 233.3 µl.
 - a. Use the following formula to calculate the amount of stock Lambda DNA to add to A1:

$$\frac{(233.3 \mu\text{l}) \times (75 \text{ ng}/\mu\text{l})}{(\text{stock Lambda DNA concentration})} = \mu\text{l of stock Lambda DNA to add to A1}$$

- b. Dilute the stock DNA in well A1 using the following formula:

$$\mu\text{l of 1X TE to add to A1} = 233.3 \mu\text{l} - \mu\text{l of stock Lambda DNA in well A1}$$

2. Add 66.7 μl 1X TE to well B1 of the same plate.
3. Add 100 μl 1X TE to wells C, D, E, F, G, and H of column 1 of the same plate.

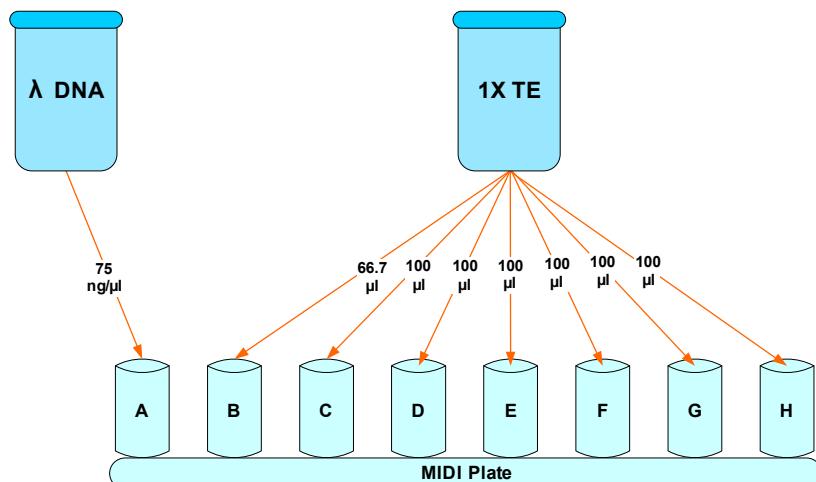


Figure 80 MIDI Plate Wells

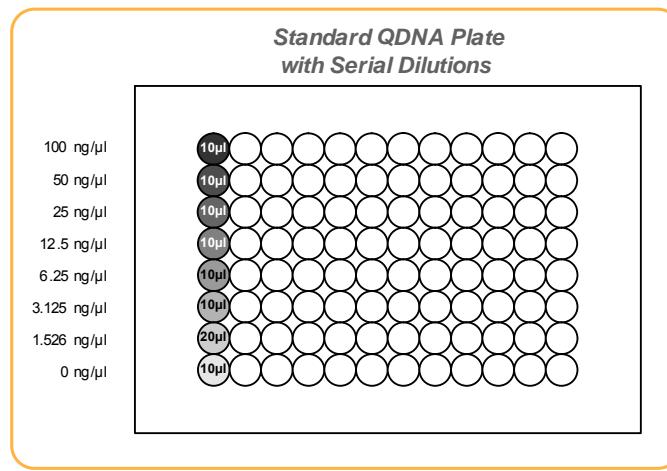
4. Pipette the contents of A1 up and down 10 times to mix.
5. Transfer 133.3 μl of Lambda DNA from well A1 into well B1, and then pipette the contents of well B1 up and down 10 times.
6. Change pipette tips. Transfer 100 μl from well B1 into well C1, and then pipette the contents of well C1 up and down 10 times.
7. Change pipette tips. Transfer 100 μl from well C1 into well D1, and then pipette the contents of well D1 up and down 10 times.
8. Change pipette tips. Transfer 100 μl from well D1 into well E1, and then pipette the contents of well E1 up and down 10 times.
9. Change pipette tips. Transfer 100 μl from well E1 into well F1, and then pipette mix the contents of well F1 up and down 10 times.
10. Change pipette tips. Transfer 100 μl from well F1 into well G1, and then pipette the contents of well G1 up and down 10 times.
11. **Do not transfer solution from well G1 to well H1.** Well H1 serves as the blank 0 $\mu\text{g}/\mu\text{l}$ Lambda DNA.

Table 45 Concentration of Lambda DNA Standards

Row-Column	Conc. ($\mu\text{g}/\mu\text{l}$)	Final Volume in Well (μl)
A1	75	100
B1	50	100

Table 45 Concentration of Lambda DNA Standards

Row-Column	Conc. (ng/ μ l)	Final Volume in Well (μ l)
C1	25	100
D1	12.5	100
E1	6.25	100
F1	3.125	100
G1	1.5625	200
H1	0	100

**Figure 81** QDNA Plate with Serial Dilutions of Lambda DNA

12. Cover the plate with a cap mat.
13. Do one of the following:
 - Proceed to *Prepare Standard QDNA Fluotrac Plate with PicoGreen Dilution*.
 - Store the plate at 4°C for future use.

Prepare Standard QDNA Fluotrac Plate with PicoGreen Dilution

In this process you create a new Standard QDNA Fluotrac plate by transferring the serial dilutions of the Standard QDNA MIDI plate into the new plate and adding PicoGreen.



PicoGreen reagent degrades quickly in the presence of light. Do not use glass containers for PicoGreen reagent.

1. Prepare a 1:200 dilution of PicoGreen to 1X TE, using the kit supplies and a sterile 100 ml plastic container wrapped in aluminum foil. Table 46 shows the volumes needed to produce diluted reagent for multiple 96-well QDNA plates. For fewer than 96 samples, scale down the volumes.

Table 46 QDNA Plate Reagent Volumes

# QDNA Plates	PicoGreen Volume (μ l)	1X TE Volume (ml)
1	115	23
2	215	43
3	315	63

2. Cap the sterile plastic container and vortex to mix.
3. Pour the PicoGreen/1X TE dilution into a sterile reservoir.
4. Using an 8-channel pipette, transfer 195 μ l PicoGreen/1X TE dilution into each well of columns 1 and 2 of the Standard QDNA Fluotrac plate (Figure 82).
5. Add 2 μ l of each stock Lambda DNA dilution from column 1 of the original Standard QDNA MIDI plate into the corresponding wells of columns 1 and 2 in the Standard QDNA Fluotrac plate.

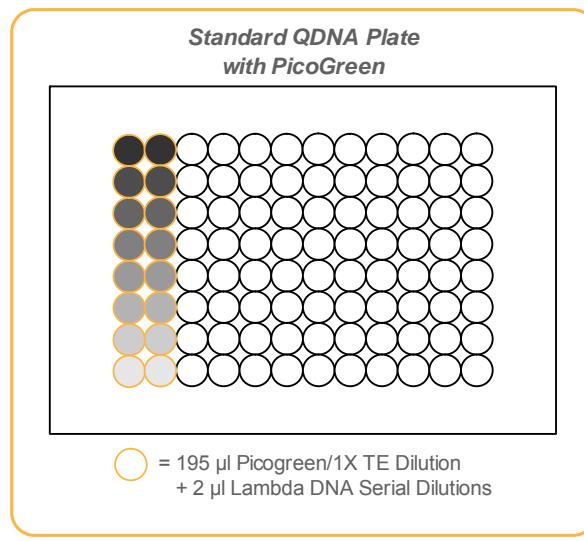


Figure 82 Standard QDNA Plate with PicoGreen

6. Pipette mix the contents of the new Standard QDNA plate.
7. Immediately cover the plate with an aluminum adhesive seal.

Prepare Sample QDNA Fluotrac plate with PicoGreen and DNA

In this process, you create a new Sample QDNA Fluotrac plate that contains DNA sample and PicoGreen.

1. Transfer 195 µl of the PicoGreen/1X TE dilution that you made earlier into each well of the new black flat-bottom plate labelled "Sample QDNA" (Figure 83).
2. Add 2 µl sample DNA to each well of the Sample QDNA plate.

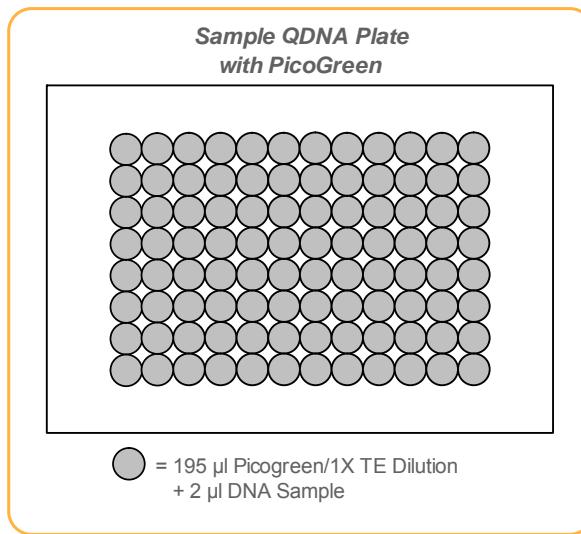


Figure 83 Sample QDNA Plate with PicoGreen

3. Pipette mix the contents of the Sample QDNA plate.
4. Immediately cover the plate with an aluminum adhesive seal.
5. Proceed to *Read QDNA Plate (Optional)* on page 196.

Read QDNA Plate (Optional)

This process uses the Gemini XS or XPS Fluorometer to provide DNA-specific quantitation. Illumina recommends using a fluorometer, because fluorometry provides DNA-specific quantitation, whereas spectrophotometry may also measure RNA and yield values that are too high.

Estimated Time Fluorometer: 5 minutes per plate

- Steps**
1. Turn on the fluorometer.
 2. At the PC, open the SoftMax Pro program.
 3. Load the Illumina QDNA.ppr file (available on the installation CD that came with your system).
 4. Select **Assays | Nucleic Acids | Illumina QDNA** (Figure 84).

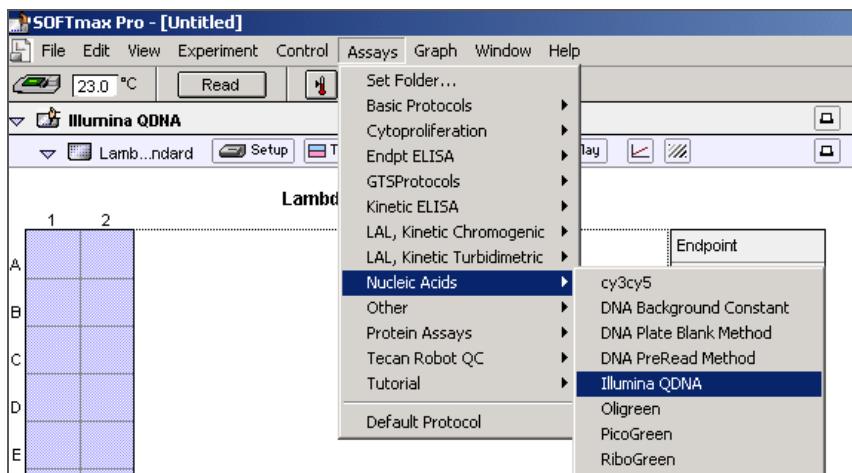


Figure 84 Load the PicoGreen Protocol in SoftMax Pro

5. Place the Standard QDNA Plate into the fluorometer loading rack with well A1 in the upper-left corner.
6. Click the blue arrow next to **Lambda Standard** (Figure 85).

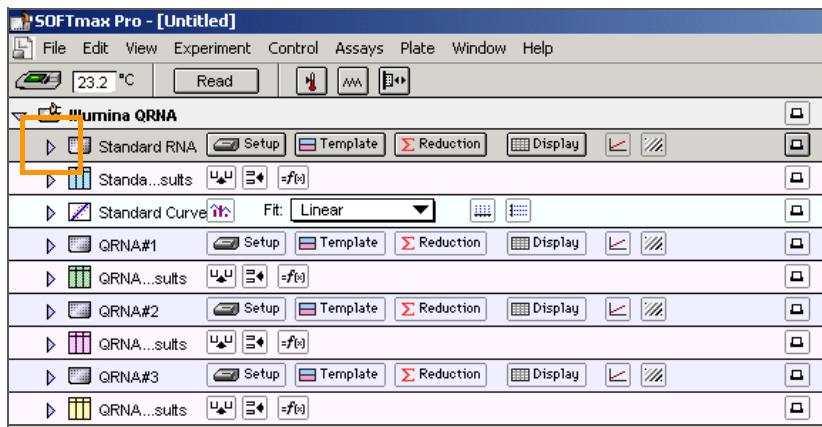


Figure 85 Select Lambda Standards Screen

- Click **Read** in the SoftMax Pro interface (Figure 86) to begin reading the Standard QDNA Plate.

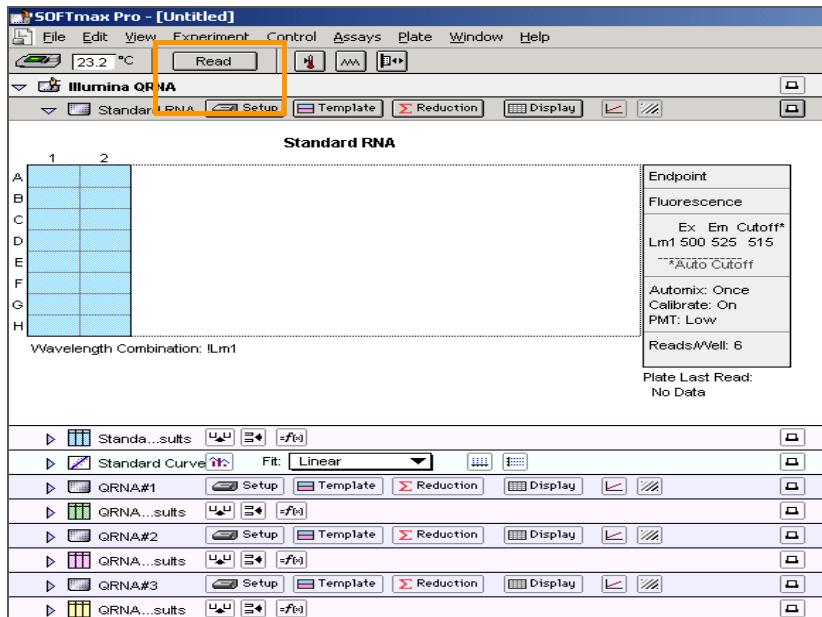


Figure 86 Read Standard QDNA Plate

- When the software finishes reading the plate, the plate drawer opens. Remove the Standard QDNA Plate from the drawer.
- Click the blue arrow next to **Standard Curve** to view the standard curve graph (Figure 87).
- If the standard curve is acceptable, continue with the sample plate. Otherwise, click **Standard Curve** again.

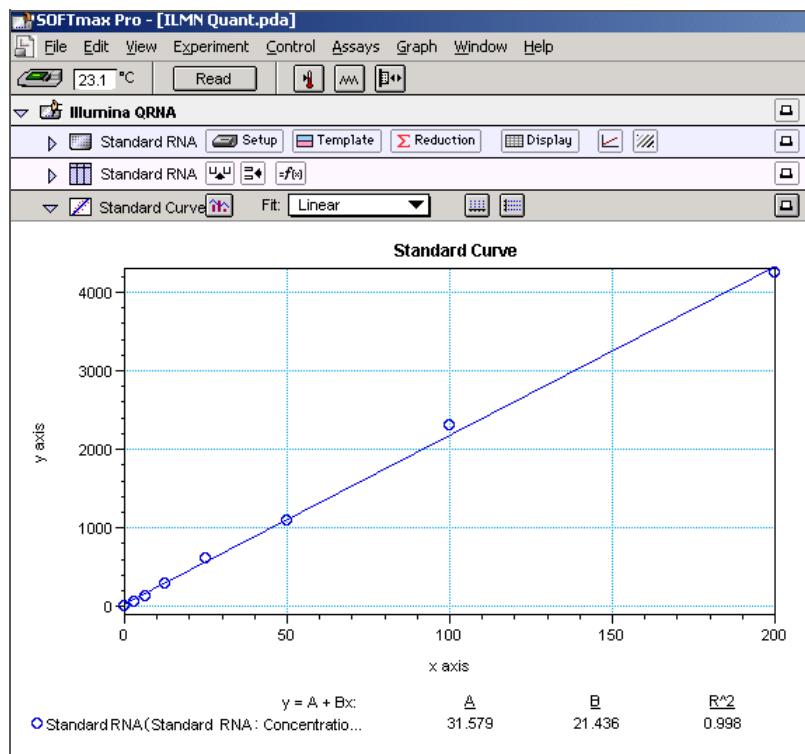


Figure 87 View Standard Curve

11. Place the first Sample QDNA plate in the reader with well A1 in the upper left corner.
12. Click the blue arrow next to **QDNA#1** and click **Read**.

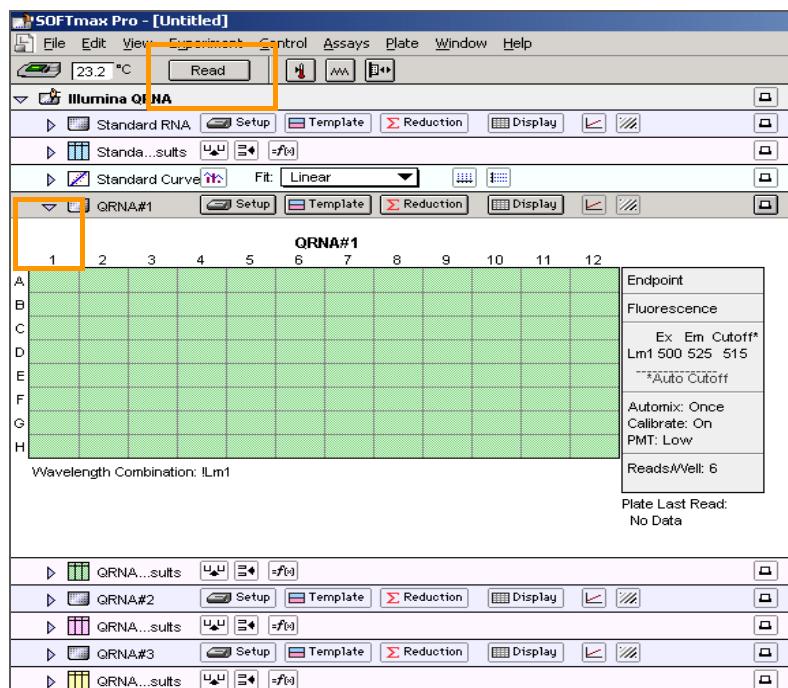


Figure 88 Read Sample QDNA Plate

13. When the software finishes reading the plate, the plate drawer opens.
Remove the plate from the drawer.
14. Repeat steps 11 through 13 for all sample plates that you want to quantitate.
15. Once all plates have been read, click **File | Save** to save the output data file (*.pda).
16. Once you save the *.pda file, click **File | Import/Export | Export** and export the file as a *.txt file. You can open the *.txt file in Microsoft Excel for data analysis.
17. Proceed to *Make Bisulfite-Converted DNA (BCD) Plate* on page 200.

Make Bisulfite-Converted DNA (BCD) Plate

This process uses the EZ DNA Methylation Kit to convert unmethylated cytosines (C) in genomic DNA to uracil (U), while leaving methylated cytosines (C) unchanged for methylation analysis.

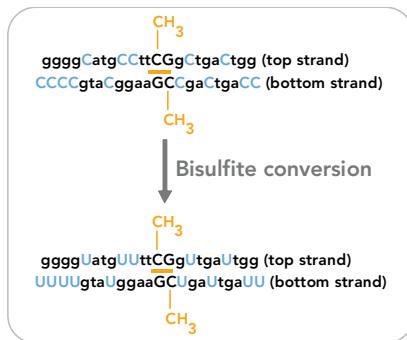


Figure 89 Bisulfite Conversion

Methylation detection in bisulfite-converted DNA is based on the different sensitivity of cytosine and 5-methylcytosine to deamination by bisulfite. Under acidic conditions, cytosine undergoes conversion to uracil, while methylated cytosine remains unreactive. An effective bisulfite-conversion protocol is a necessary prerequisite for a successful assay for methylation. Incomplete conversion of cytosine to uracil can result in false-positive methylation signals, and can reduce the overall quality of the assay data.



Always perform bisulfite conversion of DNA in the pre-PCR area.

Bisulfite-Conversion Workflow

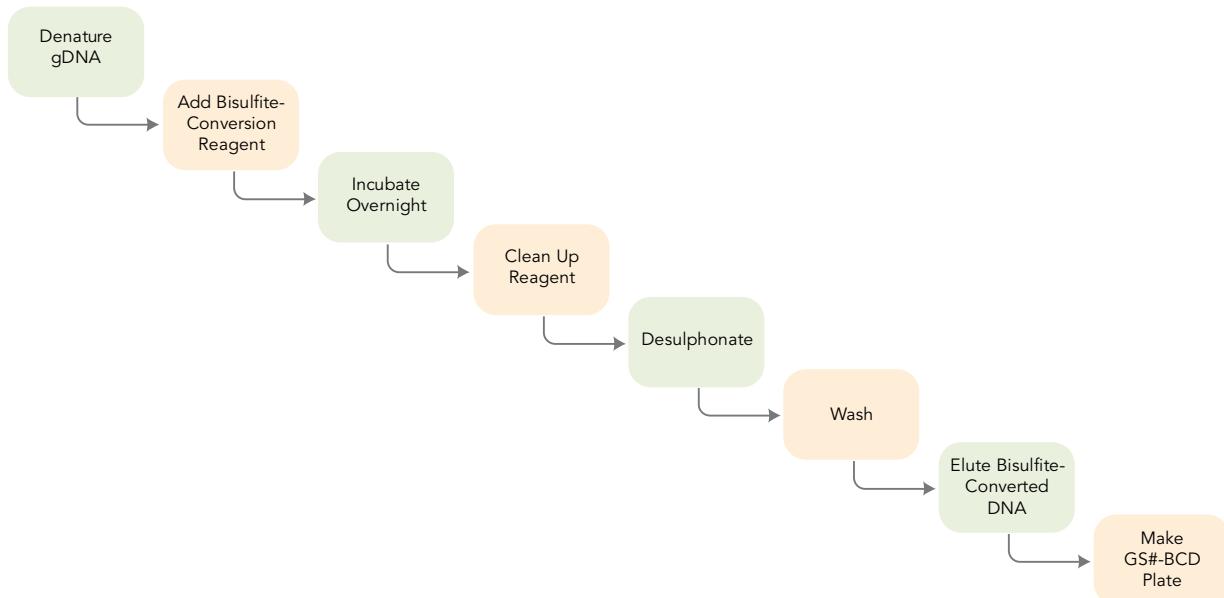


Figure 90 Bisulfite Conversion Workflow

Estimated Time

Hands-on: ~1.5 hours on Day 1, ~2 hours on Day 2

Incubation: 12–16 hours

Consumables

Item	Quantity	Storage	Supplied By
Zymo Research EZ DNA Methylation kit (includes bisulfite-conversion reagent, dilution buffer, desulphonation buffer, elution buffer)	1 per 2 plates	-20°C	User
96-well 0.2 ml skirted microplate	1 to 3 plates		User
Genomic DNA (gDNA)	> 500 ng in 12 µl elution buffer for two single-use activation reactions		User

Preparation

- ▶ Apply a BCD barcode label to each new 96-well microplate.
- ▶ Prepare the conversion reagent according to the manufacturer's instructions. For best results, use it immediately.

**CAUTION**

The conversion reagent is photosensitive, so minimize its exposure to light.

- ▶ Prepare the wash buffer according to the manufacturer's instructions.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator

**NOTE**

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps Day 1, Bisulfite Conversion

The following steps are intended only to provide an overview of the process. Follow the manufacturer's instructions, because the protocols vary significantly for different kits.

**CAUTION**

Bisulfite-conversion kits that are not specified in this guide are not recommended for use with the GoldenGate Methylation Assay for VeraCode.

1. Denature the genomic DNA and add conversion reagent.
Denaturation is necessary for bisulfite conversion, since the conversion reagent only works on single-stranded DNA.
2. Incubate in a light-protected area for 12 to 16 hours at 50°C.

Day 2, Bisulfite Conversion

1. Follow the instructions in the EZ DNA Methylation Kit to do the following:
 - a. Clean the samples using the provided spin columns or filter plate. Wash off the remaining conversion reagent.
 - b. Desulphonate the column or plate with desulphonation buffer. Incubate at room temperature (22°C) for 15 minutes.
 - c. Clean the samples and wash twice to remove the desulphonation buffer.
 - d. Add 12 µl elution buffer.
 - e. Centrifuge to elute.
2. Transfer the bisulfite-converted DNA samples to the BCD plate.
3. On the lab tracking form, record the GS#-DNA and BCD plate barcodes.
4. Heat-seal the plate and store it at -20°C for up to one month. Thaw the plate completely and vortex to mix contents before using it in an assay.

5. Proceed to *Make Bisulfite-Converted Single-Use DNA (BCS) Plate* on page 204.

Make Bisulfite-Converted Single-Use DNA (BCS) Plate

This process activates enough bisulfite-converted DNA of each individual sample to be used once in the GoldenGate Methylation Assay for VeraCode.

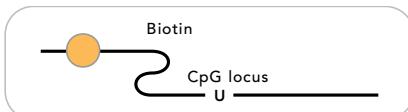


Figure 91 Activate Bisulfite-Converted Single-Use DNA

Estimated Time	Hands-on: ~15 minutes
	Incubation: 30 minutes

Consumables

Item	Quantity	Storage	Supplied By
MS1 reagent	1 tube per BCS plate	-20°C	Illumina
96-well 0.2 ml skirted microplate	1 per BCD plate		User

Preparation

- ▶ In the Sample Sheet, enter the Sample_Name (optional) and Sample_Plate for each Sample_Well.
- ▶ Preheat the heat block to 95°C and allow the temperature to stabilize. Allow 45 minutes.
- ▶ Turn on the heat sealer to preheat it. Allow 15 minutes.
- ▶ Thaw the MS1 reagent tube to room temperature. Vortex to fully mix contents, and pour the entire tube into a reagent reservoir.
- ▶ Apply a BCS (Bisulfite-Converted Single-Use DNA) barcode label to a new 96-well microplate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - BCS plate barcode
 - MS1 reagent barcode



You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Adjust the volume of bisulfite-converted DNA samples in the BCD plate to 5 µl for each 250 ng DNA in the conversion reaction.

Example: If you started out with 500 ng gDNA, adjust the volume to 10 µl.

2. Pulse centrifuge the BCD plate to 250 xg.
3. Add 5 µl MS1 reagent to each well of the BCS plate.
4. Add 5 µl bisulfite-converted DNA sample to each well of the BCS plate.
5. Heat-seal the BCS plate with a microplate foil heat seal. Ensure that all wells are completely sealed.
6. Pulse centrifuge to 250 xg.
7. Vortex at 2300 rpm for 20 seconds, making sure that plate is firmly strapped to the vortexer platform.
8. Pulse centrifuge to 250 xg.

**CAUTION**

Be sure to centrifuge the BCS plate to 250 xg *before* the 95°C incubation to prevent wells from drying out during the incubation.

9. Place the BCS plate in the preheated heat block and close the lid.
10. Incubate the BCS plate at 95°C for exactly 30 minutes.

**CAUTION**

Do not allow the 95°C incubation period to exceed 30 minutes.

11. On the lab tracking form, record the start and stop times.
12. Pulse centrifuge the plate to 250 xg.
13. If you plan to perform the Make ASE protocol today, then immediately set the heat block to 70°C.
14. Proceed to *Precipitate BCS Plate* on page 206.

Precipitate BCS Plate

This process precipitates the DNA in the BCS plate and removes excess DNA activation reagent (MS1).

Estimated Time	Hands-on: ~30 minutes
	Dry: 15 minutes

Consumables

Item	Quantity	Storage	Supplied By
PS1 reagent	Bottle	4°C	Illumina
2-propanol	Bottle	Room temperature	User

Preparation

- ▶ Pour 1 ml PS1 into a reagent reservoir.
- ▶ Pour 2 ml 2-propanol into a second reagent reservoir.
- ▶ On the lab tracking form, record the PS1 reagent barcode.



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Remove the heat seal from the heated BCS plate, taking care to avoid splashing from the wells.
2. Add 5 µl PS1 reagent to each well of the BCS plate.



CAUTION

To avoid contaminating the pipette tips, place the tips against the top edge of the well. If you suspect the tips are contaminated with the contents of the well, discard them and use new tips.

3. Seal the BCS plate with clear adhesive film.
4. Pulse centrifuge the plate to 250 xg.
5. Vortex at 2300 rpm for 20 seconds or until the solution is uniformly blue.
6. Remove the clear adhesive film and add 15 µl 2-propanol to each well of the BCS plate.
7. Seal the BCS plate with microplate clear adhesive film.
8. Vortex at 1600 rpm for 20 seconds or until the solution is uniformly blue.
9. Centrifuge the sealed BCS plate to 3000 xg for 20 minutes. A faint blue pellet should be at the bottom of each well.

**NOTE**

If you do not see a faint blue pellet at the bottom of each well, the DNA has not precipitated. In some cases, depending on DNA quality, the blue pellet may appear diffuse.

10. On the lab tracking form, record the start and stop times.

Perform the next step immediately to avoid dislodging the activated DNA pellets. If any delay occurs, re-centrifuge to 3000 xg for 10 minutes before proceeding.

11. Remove the BCS plate seal and decant the supernatant by inverting the BCS plate and smacking it down onto an absorbent pad.**CAUTION**

Do not tilt the plate, as this can cause cross-contamination between wells. Be sure to tap the plate firmly enough to decant all the supernatant; tapping lightly will not work as well.

12. Tap the inverted plate onto the pad to blot excess supernatant.**13.** Place the inverted BCS plate on an absorbent pad and centrifuge to 8 xg for 1 minute.**WARNING**

Do not spin the inverted plate to more than 8 xg, or the sample will be lost!

14. Remove the BCS plate from the centrifuge.**15.** Set the plate upright and allow it to dry at room temperature for 15 minutes.**16.** On the lab tracking form, record the start and stop times.**17.** Do one of the following:

- Proceed to Resuspend BCS Plate on page 208.
- Seal the plate with adhesive film and store at -20°C for up to 24 hours.

Resuspend BCS Plate

In this process, the DNA in the BCS plate is resuspended.

Estimated Time Hands-on: ~15 minutes

Consumables

Item	Quantity	Storage	Supplied By
RS1 reagent	Bottle	Room temperature	Illumina

Preparation

- ▶ Pour 1.2 ml RS1 into a reagent reservoir.
- ▶ On the lab tracking form, record the RS1 reagent barcode.



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Add 10 μ l RS1 reagent to each well of the BCS plate.
2. Seal the BCS plate with microplate clear adhesive film.
3. Pulse centrifuge to 250 xg.
4. Vortex at 2300 rpm for 1 minute or until the blue pellet is completely dissolved. Ensure that the plate is firmly strapped to the vortexer platform to prevent plate movement.
BCS sample plate activation is complete.
5. Do one of the following:
 - Proceed to *Make Allele-Specific Extension (ASE) Plate* on page 209.
 - Store the BCS plate at 4°C overnight or at -20°C for one month. Thaw the DNA completely and vortex to mix contents before using it in an assay.

Make Allele-Specific Extension (ASE) Plate

This process combines the biotinylated gDNAs from the BCS plate with query oligos, hybridization reagents, and paramagnetic particles in an Allele Specific Extension (ASE) plate. The ASE plate is placed in a heat block and the query oligos for each sequence target of interest are allowed to anneal to the biotinylated gDNA samples. The gDNA is simultaneously captured by paramagnetic particles. The resulting ASE plate is ready for the extension and ligation of the hybridized oligos on the bound gDNAs.

This process is designed for one plate, using the BCS plate as input.

Estimated Time

Hands-on: ~20 minutes

Incubation: 2–16 hours

Consumables

Item	Quantity	Storage	Supplied By
OB1 reagent	1 tube per plate	-20°C	Illumina
OMA reagent	1 tube per plate	-20°C	Illumina
96-well 0.2 ml skirted microplate	1 per BCS plate		User

Preparation

- ▶ In the Pool_ID column of the Sample Sheet, enter the OMA for each Sample_Well.
- ▶ Preheat the heat block to 70°C and allow the temperature to stabilize.
- ▶ Turn on the heat sealer to preheat it. Allow 15 minutes.
- ▶ Thaw the OMA reagent tube to room temperature. Vortex the tube, and then pulse centrifuge to 250 xg. Pour the OMA reagent into a reagent reservoir.
- ▶ Thaw the OB1 reagent tube to room temperature. Vortex the tube to completely resuspend the beads. Invert tube to verify that all the paramagnetic particles are evenly suspended in solution. Pour the OB1 reagent into a second reagent reservoir.



Do not centrifuge the OB1 tube.

- ▶ Apply an ASE barcode label to a new 96-well microplate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - BCS plate barcode
 - ASE plate barcode

- OB1 reagent barcode
- OMA reagent barcode

**NOTE**

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Pulse centrifuge the BCS plate to 250 xg.
2. Add 10 µl OMA reagent to each well of the ASE plate.
3. Add 30 µl OB1 reagent to each well of the ASE plate.
4. Carefully remove the heat seal from the BCS plate.
5. Transfer 10 µl of biotinylated sample from each well of the BCS plate (approximately the entire volume) to the corresponding well of the ASE plate.
6. Using a microplate heat seal, heat-seal the ASE plate (3 seconds). Ensure that all wells are completely sealed.
7. Pulse centrifuge the ASE plate to 250 xg.
8. Vortex the ASE plate at 1600 rpm for 1 minute or until all beads are completely resuspended.
9. Place the sealed ASE plate on the 70°C heat block and close the lid.
10. Immediately reset the temperature to 30°C.
11. Allow the ASE plate to cool to 30°C, which takes about 2 hours. The ASE plate may remain in the heat block for up to 16 hours.
12. On the lab tracking form, record the start and stop times.
13. Proceed to *Add Master Mix for Extension & Ligation (MEL)* on page 211.

Add Master Mix for Extension & Ligation (MEL)

In this process, AM1 and UB1 reagents are added to the ASE plate to wash away non-specifically hybridized and excess oligos. An enzymatic extension and ligation master mix (MEL) is added to each DNA sample. The extension and ligation reaction occurs at 45°C.

Estimated Time	Hands-on: ~30 minutes Incubation: 15 minutes
-----------------------	---

Consumables

Item	Quantity	Storage	Supplied By
AM1 reagent	Bottle	4°C	Illumina
UB1 reagent	Bottle	4°C	Illumina
MEL reagent	1 tube per plate	-20°C	Illumina

Preparation

- ▶ Remove the ASE plate from the heat block.
- ▶ Preheat the heat block to 45°C for about one hour.
- ▶ Thaw the MEL tube to room temperature.
- ▶ Pour 11 ml AM1 into a reagent reservoir. Add 10 ml for each additional plate.
- ▶ Pour 11 ml UB1 into a second reagent reservoir. Add 10 ml for each additional plate.
- ▶ Pour the thawed MEL tube contents into a third reagent reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - ASE plate barcode
 - AM1 reagent barcode
 - UB1 reagent barcode
 - MEL reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps



CAUTION

In this procedure, you will remove all the liquid from the wells several times, leaving only the beads. Work quickly so that the beads do not dry out.

1. Centrifuge the ASE plate to 250 xg.
2. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.

If you are using the raised-bar magnetic plate from Illumina, the beads in odd-numbered columns will be pulled to the right wall of the well, and the beads in even-numbered columns will be pulled to the left wall of the well.

**NOTE**

To avoid aspirating the beads during this procedure, slant the pipette tips so that they draw liquid from the side of the well opposite the beads. Aspirate all of the odd columns first, and then rotate the plate and aspirate the even columns (or vice-versa). This enables you to keep the pipettor at the same angle throughout.

3. Carefully remove the heat seal from the ASE plate.
4. Using an 8-channel pipette with new tips, remove and discard all the liquid (50 µl) from the wells. Leave the beads in the wells.
It is not necessary to change pipette tips until you have removed the liquid from all 12 columns.
5. Visually inspect pipette tips after removing solution from each column to ensure no beads have been removed. If beads are visible in pipette tips, return the solution to the same wells, allow magnet to re-collect beads, and change the pipette tips.
6. With the ASE plate on the raised-bar magnetic plate, use an 8-channel pipette with new tips to add 50 µl AM1 to each well of the ASE plate.

**CAUTION**

To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (Figure 92, page 212). If you suspect the tips are contaminated with the contents of the well, discard the tips and use new tips.



Figure 92 Avoid Tip Contamination

7. Seal the ASE plate with microplate clear adhesive film.

8. Vortex the ASE plate at 1600 rpm for 20 seconds or until all beads are resuspended.
9. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
10. Remove the seal from the ASE plate, taking care to avoid splashing from the wells.
11. Using the same 8-channel pipette with the same tips, remove all AM1 reagent from each well. Leave the beads in the wells.
It is not necessary to change pipette tips until liquid has been removed from all 12 columns.
12. Repeat steps 6 through 11 once.
13. Remove the ASE plate from the raised-bar magnetic plate.
14. Using an 8-channel pipette with new tips, add 50 μ l UB1 to each well of the ASE plate.
15. Place the ASE plate onto the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
16. Using the same 8-channel pipette with the same tips, remove all UB1 reagent from each well. Leave the beads in the wells.
It is not necessary to change pipette tips until liquid has been removed from all 12 columns.
17. Repeat steps 13 through 16 once.
18. Using an 8-channel pipette with new tips, add 37 μ l MEL to each well of the ASE plate.
19. Seal the plate with microplate clear adhesive film.
20. Vortex the plate at 1600–1700 rpm for 1 minute or until the beads are resuspended.
21. Incubate the ASE plate on the preheated 45°C heat block for exactly 15 minutes.

**CAUTION**

Do not allow the ASE plate to incubate at 45°C longer than 15 minutes.

22. During the incubation, perform the *Make PCR Plate* procedure.
23. On the lab tracking form, record the start and stop times.
24. Proceed to *Inoculate PCR Plate* on page 216. Leave the ASE plate at room temperature if you proceed immediately, or store it at 4°C for up to 1 hour.

Make PCR Plate

This process adds the Illumina-recommended DNA Polymerase and optional Uracil DNA Glycosylase (UDG) to the master mix for PCR (MMP reagent) and creates a 96-well plate for the Inoc PCR process.

Estimated Time Hands-on: ~15 minutes

Consumables

Item	Quantity	Storage	Supplied By
MMP reagent	1 tube per plate	-20°C	Illumina
Titanium Taq DNA Polymerase	64 µl	-20°C	User
Uracil DNA Glycosylase (UDG, Optional)	50 µl	-20°C	User
96-well 0.2 ml skirted microplate	1 per ASE plate		User

Preparation

- ▶ Thaw the MMP tube to room temperature.
- ▶ Apply a PCR barcode label to a new 96-well microplate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - PCR plate barcode
 - MMP reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Add 64 µl DNA Polymerase to the MMP tube. Check off this action in the lab tracking form.
2. [Optional] Add 50 µl Uracil DNA glycosylase to the MMP tube. Check off this action in the lab tracking form.
3. Invert the tube several times to mix the contents.
4. Pour the contents of the tube into a reagent reservoir.
5. Using an 8-channel pipette, add 30 µl of the mixture into each well of the PCR plate.
6. Seal the PCR plate with microplate clear adhesive film.
7. Pulse centrifuge to 250 xg.

8. Proceed to *Inoculate PCR Plate* on page 216.

Inoculate PCR Plate

This process uses the template formed in the extension and ligation process in a PCR reaction. This PCR reaction uses three universal primers (MMP reagent): two are labeled with fluorescent dyes and the third is biotinylated. The biotinylated primer allows capture of the PCR product and elution of the strand containing the fluorescent signal. The eluted samples are transferred from the ASE plate to the PCR plate.

Estimated Time	Hands-on: ~30 minutes
-----------------------	-----------------------

Consumables

Item	Quantity	Storage	Supplied By
UB1 reagent	Bottle	4°C	Illumina
IP1 reagent	1 tube per plate	-20°C	Illumina

Preparation

- ▶ Remove the ASE plate from the heat block.
- ▶ Reset the heat block to 95°C.
- ▶ Pour 6 ml UB1 into a reagent reservoir.
- ▶ Thaw the IP1 reagent to room temperature. Pour the contents of the tube into a reagent reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - ASE plate barcode
 - PCR plate barcode
 - IP1 reagent barcode
 - UB1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
2. Remove the microplate clear adhesive film from the ASE plate.
3. Using an 8-channel precision pipette, remove and discard the supernatant (~50 µl) from all wells of the ASE plate. Leave the beads in the wells.

It is not necessary to change pipette tips until liquid has been removed from all 12 columns.

4. Visually inspect pipette tips after removing solution from each column to ensure no beads have been removed. If beads are visible in pipette tips, return the solution to the same wells, allow magnet to re-collect beads, and change the pipette tips.
5. Leaving the plate on the magnet and using an 8-channel precision pipette with new tips, add 50 µl UB1 to each well of the ASE plate.



To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (Figure 92). If you suspect the tips are contaminated with the contents of the well, discard the tips and use new tips.

6. Leave the ASE plate on the raised-bar magnetic plate for approximately 2 minutes, or until the beads are completely captured.
7. Remove and discard the supernatant (~50 µl) from all wells of the ASE plate. Leave the beads in the wells.
It is not necessary to change pipette tips until liquid has been removed from all 12 columns.
8. Remove the plate from the magnet.
9. Using an 8-channel precision pipette with new tips, add 35 µl IP1 to each column of the ASE plate.
10. Seal the plate with microplate clear adhesive film.
11. Vortex at 1800 rpm for 1 minute, or until all the beads are resuspended.
12. Place the plate on the 95°C heat block for 1 minute.
13. Place the ASE plate back onto the raised-bar magnetic plate for 2 minutes or until the beads have been completely captured.
14. Using an 8-channel pipette with new tips, transfer 30 µl supernatant from each well in the first column of the ASE plate to the first column of the PCR plate.
15. Repeat for each column of the ASE plate. Change tips between column dispenses.



Take special care not to disturb or transfer the beads when aspirating eluted product.

16. Discard the ASE plate.
17. Seal the PCR plate with the appropriate PCR plate-sealing film for your thermocycler.
18. Proceed immediately to *Thermal Cycle PCR Plate* on page 218.

Thermal Cycle PCR Plate

This process thermal cycles the PCR plate to fluorescently label and amplify the templates generated in the pre-PCR process.

Estimated Time Thermal Cycle: ~2 hours 45 minutes

Preparation

- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - PCR plate barcode
 - Thermocycler ID
 - Thermocycler program

Steps

1. Place the sealed plate into the thermocycler and run the thermocycler program shown in this table.

Table 47 Thermocycler Program

	Temperature	Time
	37°C	10 minutes
	95°C	3 minutes
X 34 {	95°C	35 seconds
	56°C	35 seconds
	72°C	2 minutes
	72°C	10 minutes
	4°C	5 minutes

2. Do one of the following:

- Proceed immediately to *Bind PCR Products* on page 219. Store the PCR plate at room temperature (22°C) in a light-protected drawer.
- Seal and store the PCR plate at -20°C overnight.

Bind PCR Products

In this process, MPB reagent is added to the PCR plate and the solution is transferred to a filter plate. The filter plate is incubated at room temperature to bind the biotinylated strand to paramagnetic particles, thus immobilizing the double-stranded PCR products.

Estimated Time	Hands-on: ~30 minutes Incubation: 1 hour
-----------------------	---

Consumables

Item	Quantity	Storage	Supplied By
MPB reagent	1 tube per PCR plate	4°C	Illumina
0.45 µM clear Styrene filter plate with lid	1 per PCR plate		User

Preparation

- ▶ Vortex the MPB tube several times until the beads are well resuspended. Pour the contents of the tube into a non-sterile reagent reservoir.
- ▶ Write the PCR plate barcode number in the space provided on a "Filter Plate: GS _____ -PCR" label. Apply the label to the top surface of the filter plate, adjacent to column 12 (Figure 93).



This ensures that the filter plate is associated with the correct PCR plate. Do not attach the label to the lid of the filter plate, as the lid could get separated from the plate.

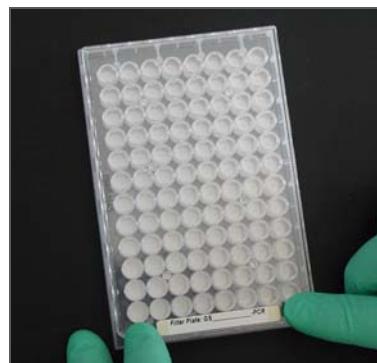


Figure 93 Apply Label to Filter Plate

- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - MPB reagent barcode
 - Filter plate barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Pulse centrifuge the PCR plate to 250 xg.
2. Using an 8-channel pipette with new tips, add 20 μ l resuspended MPB into each well of the PCR plate.

It is not necessary to change pipette tips until liquid has been transferred to all 12 columns.



CAUTION

To avoid contaminating the tips, place the tips against the top edge of the wells (see Figure 92). If you suspect the tips are contaminated with the contents of the well, discard the tips and use new tips.

3. Set an 8-channel pipette to 85 μ l to allow space for bubbles, and attach new tips.
4. Pipette the solution in the PCR plate up and down several times to mix the beads with the PCR product
5. Transfer the mixed solution into the first column of the filter plate. There should be about 70 μ l fluid in each well.
6. Repeat step 5 for each column of the PCR plate. Change tips between column dispenses.
7. Discard the empty PCR plate.
8. Cover the filter plate with its lid and store it at room temperature, protected from light, for 60 minutes.
9. On the lab tracking form, record the start and stop times.
10. Proceed to *Make Intermediate Plate for VeraCode Bead Plate* on page 221.

Make Intermediate Plate for VeraCode Bead Plate

In this process, the PCR product is washed in the filter plate with UB2 and NaOH. The single-stranded, fluor-labeled material is then eluted into an INT plate containing MH2 reagent.

Estimated Time Hands-on: ~30 minutes

Consumables

Item	Quantity	Storage	Supplied By
MH2 reagent	1 tube per plate	Room temperature	Illumina
UB2 reagent	Bottle	Room temperature	Illumina
0.1N NaOH	Bottle	4°C	User
96-well V-bottom plate	2 per PCR plate		User

Preparation

- ▶ Apply a INT barcode label to a new 96-well V-bottom plate.
- ▶ Using a serological pipette, transfer 6 ml UB2 into a sterile reservoir.
- ▶ Pour 4 ml 0.1N NaOH into a second sterile reservoir.
- ▶ Pour the contents of the MH2 tube into a third sterile reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - INT plate barcode
 - UB2 reagent barcode
 - MH2 reagent barcode
 - NaOH lot number



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Place the filter plate adapter on an empty 96-well V-bottom plate (waste plate) (Figure 94).
2. Place the filter plate containing the bound PCR products onto the filter-plate adapter (Figure 94).



Figure 94 Assemble Filter Plate

3. Centrifuge to 1000 xg for 5 minutes at 25°C.
4. Remove the filter plate lid.
5. Using an 8-channel pipette with new tips, add 50 µl UB2 to each well of the filter plate. Dispense slowly to avoid disturbing the beads.



CAUTION

To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (see Figure 92, page 212).

6. Replace the filter plate lid.
7. Centrifuge to 1000 xg for 5 minutes at 25°C.
8. Using an 8-channel pipette with new tips, add 30 µl MH2 to each well of the INT plate.
9. Replace the waste plate with the INT plate. Orient the INT plate so that well A1 of the filter plate matches well A1 of the INT plate.



WARNING

Be sure to replace the waste plate with the INT plate. Failure to replace the waste plate will result in loss of samples.

10. Discard the waste plate.
11. Using an 8-channel pipette with new tips, add 30 µl 0.1N NaOH to all wells of the filter plate.
12. Replace the filter plate lid.



CAUTION

Due to the sensitivity of the dyes to 0.1N NaOH, proceed quickly. Prolonged incubation with NaOH is unnecessary; less than 5 minutes is sufficient. The DNA is denatured almost instantly.

13. Centrifuge immediately at 1000 xg for 5 minutes at 25°C. At the end, no beads should be visible in the wells of the INT plate.
14. Discard the filter plate. Save the adapter for later use in other protocols.
15. Gently mix the contents of the INT plate by moving it from side to side without splashing.
16. Cover the INT plate with a clear adhesive seal.
17. Do one of the following:
 - Proceed to *Hybridize VeraCode Bead Plate* on page 224.
 - Seal the INT plate with a cap mat and store it at -20°C.

Hybridize VeraCode Bead Plate

This process uses the VeraCode Vortex Incubator, an incubating microplate shaker, to hybridize the VeraCode Bead Plate (VBP). Once the samples are transferred to the VBP, they are ready for hybridization at 45°C.

Estimated Time	Hands-on: ~20 minutes
	Incubation: 3 hours

Consumables

Item	Quantity	Storage	Supplied By
MH2 reagent	1 tube per plate	Room temperature	Illumina
0.1N NaOH	Bottle	4°C	User
VeraCode Bead Plate	1 per INT plate	4°C	Illumina

Preparation

- ▶ If the INT plate has been frozen, thaw to room temperature in a light-protected drawer, then pulse centrifuge to 250 xg.
- ▶ Preheat the VeraCode Vortex Incubator to 45°C and allow it to equilibrate.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - INT plate barcode
 - VBP plate barcode
 - MH2 reagent barcode
 - NaOH lot number



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps Add Neutralized MH2 to INT VBP

1. With a serological pipette, transfer 3 ml MH2 into a 15 ml conical tube.
2. With a different serological pipette, transfer 3 ml 0.1 N NaOH to the same 15 ml tube.
3. Vortex the tube until the contents are mixed.
4. Pour the mixture into a sterile reservoir.

5. Using an 8-channel pipette, add 50 µl of neutralized MH2 to each of the INT plate wells that contain sample. Be careful not to let the pipette tips touch the samples.

Hybridize VBP

1. Remove the VBP from the 4°C refrigerator and pulse centrifuge to 250 xg. If the beads are not at the bottoms of the wells, pulse centrifuge again.
2. Remove the cap mat from the VeraCode Bead Plate. Save the cap mat for subsequent use in hybridization.
3. Using an 8-channel pipette with new tips, pipette each column of sample in the INT plate up and down 4–5 times.
4. Using the same tips, transfer 100 µl of each assay product from the INT plate into the corresponding well of the VeraCode Bead Plate.
5. Place the cap mat back on the VeraCode Bead Plate.
6. Place the VeraCode Bead Plate Bead Plate, which now contains samples, into the VeraCode Vortex Incubator. You can load up to 2 VBP plates in the vortexer.



NOTE

If you load only one plate, load an empty 96-well plate in the opposite position as a balance.

7. Close the lid and make the following settings:
 - Push the **Encoder** knob until **RPM** is highlighted. Rotate the knob to 85 (850 rpm).
 - Push the **Encoder** knob until **Time** is highlighted. Rotate the knob below 0.30 or above 99.5 until HLD appears. This sets the Vortex Incubator to run continuously.
 - Push the **Encoder** knob until **Temperature** is highlighted. Rotate the knob to 45 (45°C).
8. Press **Start/Stop** and incubate for 3 hours.
9. Proceed to *Wash VeraCode Bead Plate* on page 226.

Wash VeraCode Bead Plate

In this process, the VeraCode Bead Plate is removed from the VeraCode Vortex Incubator and washed two times with the VW1 reagent.

Estimated Time Hands-on: ~15 minutes

Consumables

Item	Quantity	Storage	Supplied By
VW1 reagent	Bottle	Room temperature	Illumina

Preparation

- ▶ Pour 45 ml of VW1 into a nonsterile reservoir.
- ▶ On the lab tracking form, record:
 - Date and time
 - Operator
 - VW1 reagent barcode



NOTE

You can download copies of the lab tracking form from the documentation CD you received with your system. You can then edit the form on your computer and save and/or print copies for each run.

Steps

1. Stop the VeraCode Vortex Incubator. When the speed indicator reaches 0, open the lid and remove the VeraCode Bead Plate.
2. Pulse centrifuge the plate to 250 xg.
3. Remove the cap mat.
4. Using an 8-channel pipette, add 200 μ l VW1 buffer to each well, making sure to agitate the bead pellet.
5. Gently swirl the plate in a circular motion on the benchtop.
6. Wait 2 minutes for the beads to collect in the bottom of the well.
7. Aspirate the supernatant with the vacuum manifold at a pressure of 50 mbar.
8. Repeat steps 4 through 7 once.
9. Do one of the following:
 - a. Proceed to *Scan VeraCode Bead Plate* on page 227.
 - b. Seal the VBP plate with an adhesive seal and store it in the dark at room temperature for up to 24 hours.

Scan VeraCode Bead Plate

The BeadXpress Reader uses lasers to excite the Cy3 and Cy5 fluors of the single-stranded PCR products bound to the VeraCode beads. Light emissions from these fluors are then recorded in a data file. Fluorescence data are analyzed to derive assay results using Illumina's GenomeStudio software package.

Estimated Time	80 samples/hour at 96-plex 27 samples/hour at 384-plex
-----------------------	---

Preparation	<ul style="list-style-type: none">▶ Prepare a scan settings file containing information about your samples, the BeadXpress Reader settings, and VeraCode beads. If you intend to analyze GoldenGate methylation data in GenomeStudio, you should enter the VeraCode Bead Plate serial number (CK#-VBP) in the Plate_ID field.
--------------------	---

Steps	For instructions on scanning VeraCode Bead Plate, see the <i>BeadXpress Reader System Guide</i> .
--------------	---

Chapter 7

Bead Kitting

Topics

- 230 Introduction
- 230 Materials for this Assay
- 231 Kit VeraCode Beads
- 242 Store Kitted VeraCode Beads
- 242 Clean the VeraCode Bead Kitting System

Introduction

The VeraCode Bead Kitting System offers a unique approach to rapidly and accurately distribute VeraCode microbeads into standard 96-well microplates or strip well plates.

Using the VeraCode Bead Kitting System streamlines the workflow of custom multiplexed assays by virtually eliminating manual pipetting. This system is specifically designed for use with VeraCode universal oligo and carboxyl bead sets.

Follow the bead kitting procedures described in this chapter to kit VeraCode beads for the assays described in Chapter 8, *Universal Oligo Beads Example Protocol* and Chapter 9, *Carboxyl Beads Example Protocols*.

Materials for this Assay

Use the following materials and bead kitting procedure to kit VeraCode universal oligo or carboxyl beads.

Table 48 Materials for Kitting VeraCode Beads

Item	Source	Catalog Number
VeraCode Bead Kitting System	Illumina (included with the BeadXpress Reader System)	VC-501-1000
VeraCode universal oligo beads or carboxyl beads	Illumina	See Chapter 8, <i>Universal Oligo Beads Example Protocol</i> or Chapter 9, <i>Carboxyl Beads Example Protocols</i>
8-pin aspirator	Illumina	included with VC-501-1000
Polypropylene strip wells or 96-well polypropylene plate	VWR or Corning	14230-404 or 3371
Polypropylene cap mat	Corning	3080
Easy peel heat seals	ABgene	AB-0745
Storage plate cap strips	ABgene	AB-0981
EtOH 70%	N/A	N/A
EtOH 30% in 1X PBS, pH 7.4	N/A	N/A
Vacuum manifold (or house vacuum capability)	N/A	N/A

Kit VeraCode Beads

Universal VeraCode beads are shipped in vials that contain enough beads for one 96-well plate. The vials may contain either a single bead type or a pool of 48 bead types. Each Universal VeraCode bead type has a unique sequence attached to its surface. These sequences are capture sequences for the downstream hybridization reaction. Vials of Universal VeraCode beads can be used individually, or they can be multiplexed to increase the number of bead types for a reaction. Universal VeraCode beads are supplied by Illumina in an ethanol/water mixture, and are stable at -20°C.

Carboxyl VeraCode beads are shipped in an ethanol/water mixture in vials that contain enough beads for six 96-well plates and are stable at 4°C. After user-immobilization of the protein or nucleic acid, the immobilized Carboxyl VeraCode beads are quantitated and combined to form a multiplex bead pool. Protein-immobilized Carboxyl VeraCode beads are typically stored in a buffer containing protein (e.g., BSA), while nucleic acid-immobilized VeraCode Carboxyl beads are stored in a solvent (e.g., EtOH). Prior to kitting, protein-immobilized Carboxyl VeraCode beads stored in a BSA-containing buffer require a simple wash step to remove exogenous protein which interferes with the kitting process.

Table 49 Buffers for VeraCode Bead Types

VeraCode Bead	Immobilized Molecule	Storage Buffer	Kitting Buffer
Universal Oligo	Oligonucleotide	70% EtOH	70% EtOH
Carboxyl	N/A	70% EtOH	N/A
Carboxyl	Protein	1x PBS/1% BSA	30% EtOH in 1x PBS
Carboxyl	Oligonucleotide	70% EtOH	70% EtOH

The VeraCode Bead Kitting System (Figure 95) is used to kit VeraCode beads for use with VeraCode assays. The VeraCode Bead Kitting System consists of three parts:

- ▶ A deep reservoir (box)
- ▶ A funnel plate (funnel)
- ▶ A shallow reservoir (catch pan)

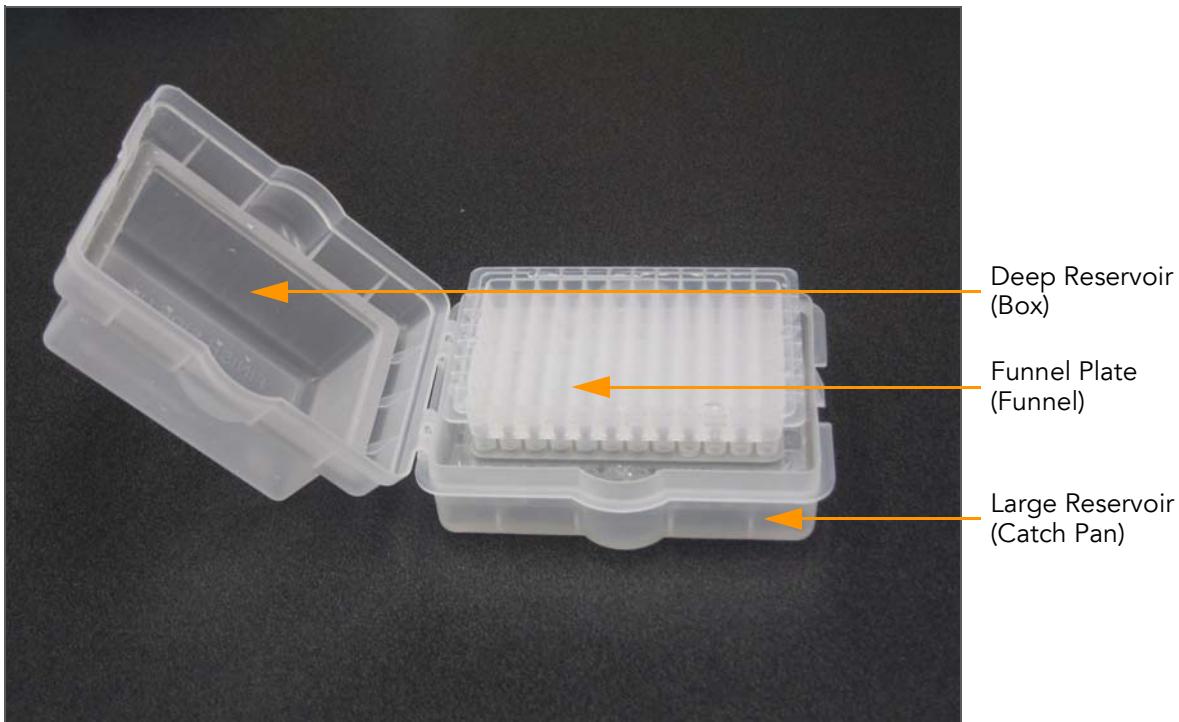


Figure 95 VeraCode Bead Kitting System

In addition, the bead kitting process requires either a strip well plate (VWR #14230-404) or a polypropylene plate (Corning #3371). Use strip well plates if you have fewer than 96 reactions in an experiment. The strips can be separated after kitting to enable you to perform multiples of eight reactions at a time.



CAUTION

Do not cut a single well. The BeadXpress Reader needs eight wells per scan, although all eight wells don't have to be populated with beads. *Do not try to kit a partial strip plate.*

1. Place the VeraCode Bead Kitting System on the lab bench, with the deep reservoir down (Figure 96).



Figure 96 VeraCode Bead Kitting System, Deep Reservoir Down

2. Press the rectangular gasket into place around the rim of the deep reservoir (Figure 97).



Figure 97 Place Rectangular Gasket into Deep Reservoir

3. Add 160 ml kitting buffer to the deep reservoir (Figure 98).
 - **For Universal Oligo Beads:** Use 70% EtOH as the buffer.
 - **For Carboxyl Beads:** Use 30% EtOH in 1X PBS, pH 7.4 as the buffer.

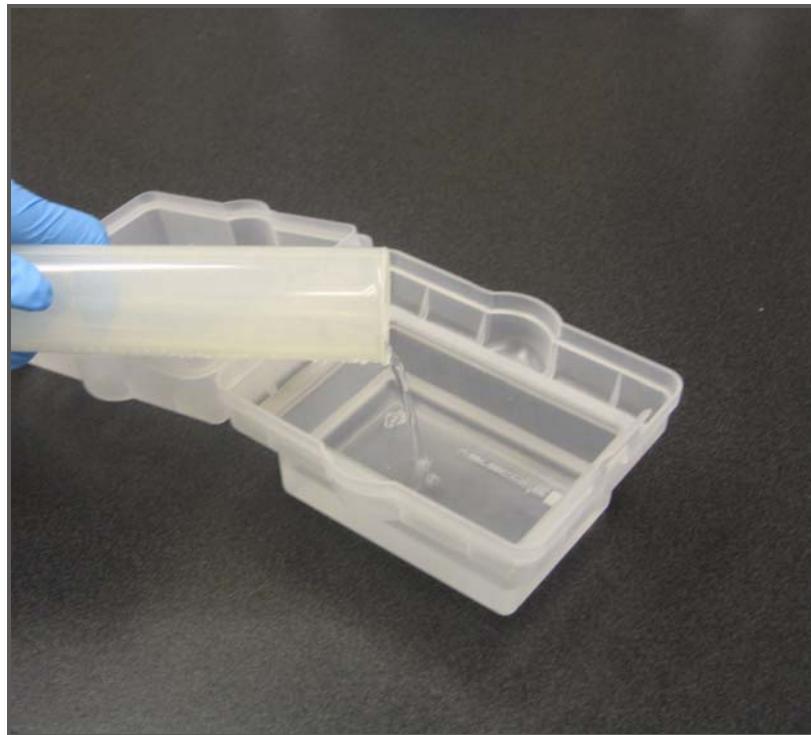


Figure 98 Add Kitting Buffer

4. Perform the following steps to transfer the beads from the bead tube to the deep reservoir.



NOTE

If you are using universal oligo beads and you need multiple codes in a plate, you must use multiple vials of beads.

If you are using stored protein-immobilized beads, remove enough beads from multiplex stock to deliver 30 beads per type per well. Wash 2x with 1x PBS, pH 7.4 to remove residual BSA from the storage buffer prior to kitting.

- a. Vortex and pulse centrifuge prior to transferring the beads. This helps dislodge beads from the walls and cap of the tube.
- b. Using a 1000 μ l pipette, transfer all of the bead solution to the deep reservoir (Figure 99).



Figure 99 Transfer Beads

- c. To rinse the tube, pipette 500 μ l of buffer from the deep reservoir to the tube and transfer all of the rinse solution back into the deep reservoir.



Make sure to rinse the walls and cap of the tube during the rinse steps.

- d. Repeat the rinse 6x to ensure complete bead transfer.
5. Add the funnel plate to the deep reservoir (Figure 100).
The funnel plate follows the guiding ribs to settle into the correct position.

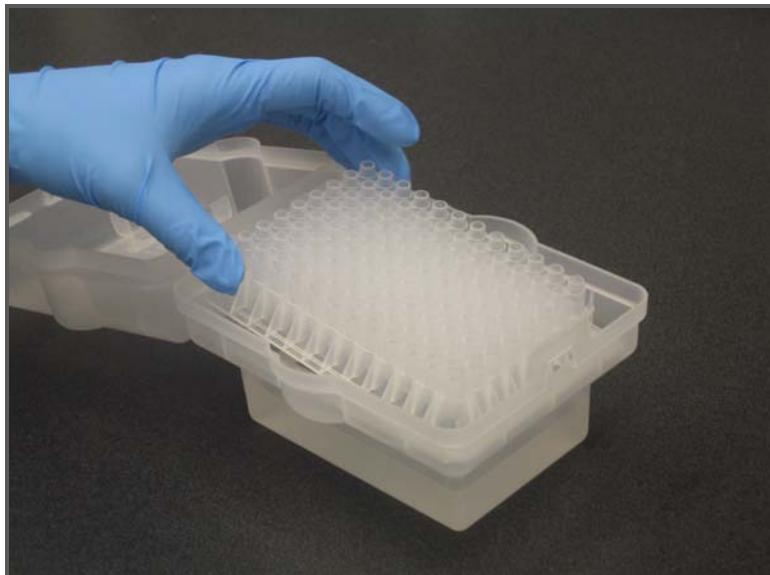


Figure 100 Add Funnel Plate

6. Press the 96-hole gasket onto the funnel plate (Figure 101).

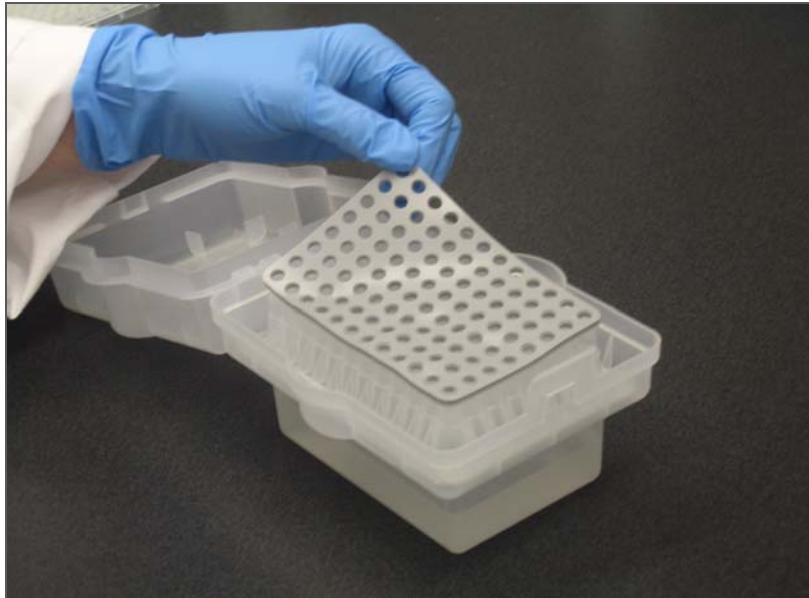


Figure 101 Press Gasket onto Funnel Plate

7. Put the plate upside-down on the gasket to match up with the funnel plate holes and press it down to seat it (Figure 102).



Figure 102 Put Plate on Gasket

8. Close the VeraCode Bead Kitting System and secure it with the latch (Figure 103).



Figure 103 Close and Latch VeraCode Bead Kitting System

9. Holding the VeraCode Bead Kitting System with both hands, shake it with some force approximately 6 inches in every direction, as described in the following steps.



Shake the VeraCode Bead Kitting System as fast as you can to ensure homogeneous bead distribution. If you shake in a slow, circular motion with little force, the beads will not mix properly.

Do not pause between shaking and flipping as this will allow the beads to settle.

- a. Rapidly shake the VeraCode Bead Kitting System 4x front to back, then 4x left to right.



Step a. constitutes one shaking cycle and should take no more than 2-3 seconds.

- b. Without pausing, repeat the shaking cycle for a total of 15–20 seconds (Figure 104).



Figure 104 Shake VeraCode Bead Kitting System

- c. After the last shaking cycle, immediately flip the VeraCode Bead Kitting System upside-down onto the bench.
- d. Tap the VeraCode Bead Kitting System firmly on the bench 5x to remove bubbles from the plate wells and funnel plate (Figure 105).

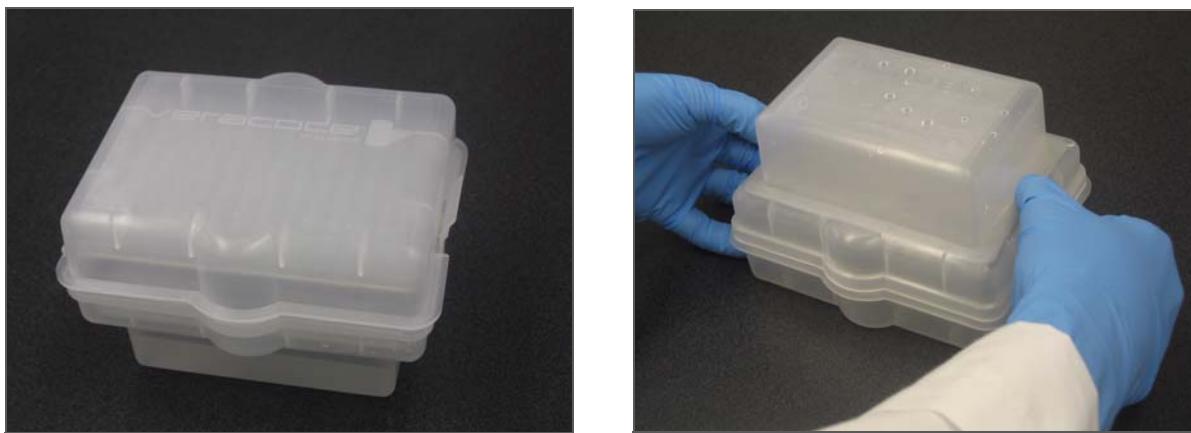


Figure 105 Flip VeraCode Bead Kitting System

10. After tapping, place the VeraCode Bead Kitting System on the bench.

Let it rest for at least 1 minute to allow the beads to settle.

- 11.** Perform the following steps to dislodge any beads that may be stuck on the funnel plate and walls of the VeraCode Bead Kitting System.



CAUTION

When performing the following steps, do not tilt the VeraCode Bead Kitting System at an angle greater than 30°.

- a.** Firmly tap each of the four bottom edges of the VeraCode Bead Kitting System on the lab bench 5x (Figure 106).



Figure 106 Tap VeraCode Bead Kitting System

- b.** Keeping the VeraCode Bead Kitting System level, firmly tap the bottom on the lab bench 5x.
c. Wait at least 1 minute to allow the beads to settle to the bottom of the wells.
d. Keeping the VeraCode Bead Kitting System level, firmly tap the bottom an additional 5x.
e. Wait at least 1 minute to let the beads settle.
- 12.** Unlatch the VeraCode Bead Kitting System, wait 10–15 seconds to allow most of the excess liquid to seep out, then slowly open it (Figure 107).



Figure 107 Open VeraCode Bead Kitting System

13. Lifting straight up, remove the funnel plate and the attached gasket (Figure 108).

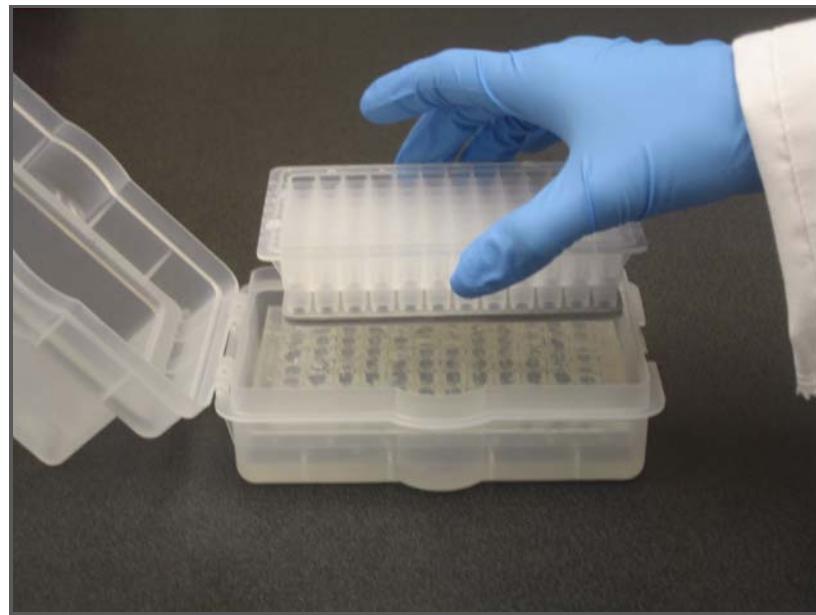


Figure 108 Remove Funnel Plate and Gasket from Deep Reservoir

14. Decant the excess liquid by angling the plate approximately 30 degrees against the side of the catch tray, and remove the plate from the catch tray (Figure 109).



Figure 109 Remove Plate from Deep Reservoir

15. Carefully aspirate off the kitting buffer using the 8-pin aspirator manifold. This leaves some EtOH in the bottom of each well. Do not try to remove all of the EtOH.

For carboxyl beads only:

- a. Add 150 µl PBST (1 X Phosphate Buffered Saline + 0.05% Tween 20 (0.05%)) to the wells.
- b. Centrifuge at 1500 rpm for 5 seconds.
- c. Aspirate the wells with the 8-pin aspirator.
- d. Repeat steps a through c 2x.
- e. Do one of the following:
 - If you are using the plate immediately:
Proceed to the Multiplex Cytokine Protein Assay on page 289.
 - If you are not using the plate immediately:
Continue to Step 16.

16. Seal the plate by doing one of the following:

For universal oligo beads:

- If you are using Corning 96-well plates, seal with the cap mat (Corning 3080).
- If you are using the strip well plate, seal with strip caps (ABgene AB-0981).

For carboxyl beads:

Add 150 µl PBS-BSA (1%) and seal the plate with the ABgene easy peel heat seal (AB-0745).

Store Kitted VeraCode Beads

Store kitted VeraCode beads as described below.

- ▶ Store universal oligo beads at -20°C until you are ready to use them.
- ▶ Store carboxyl beads at 4°C until you are ready to use them.

Clean the VeraCode Bead Kitting System

To clean the VeraCode Bead Kitting System, perform the following steps:

1. Add about 100 ml of deionized water into the deep reservoir (box).
2. Place both gaskets into the deep reservoir.
3. Place the funnel plate into the deep reservoir.
4. Close the system with the latch.
5. Shake vigorously to clean the whole system.
6. Decant any liquid.
7. Repeat steps **1** through **6** 2x.
8. Rinse the entire VeraCode Bead Kitting System (box, funnel, catch pan, and gaskets) under running deionized water to ensure that it is completely clean.
9. Air-dry the VeraCode Bead Kitting System, gaskets, and funnels.

Chapter 8

Universal Oligo Beads

Example Protocol

Topics

- 244 Introduction
- 246 Materials and Reagents for this Assay
- 256 Design PCR and ASPE Primers
- 258 Match ASPE Primers to VeraCode Capture Sequences
- 260 Contamination and Controls
- 261 Two-Plate Protocol for Low-Plex Genotyping
- 266 Single-Plate Protocol for Low-Plex Genotyping
- 271 Troubleshooting

Introduction

ASPE (Allele-Specific Primer Extension) is a method of SNP detection that uses two primers: one for the wildtype allele and one for the variant allele. Each ASPE primer is composed of two distinct regions. The 5' end contains the capture sequence which is used in subsequent hybridization reactions. The 3' end is the genomic region with the SNP nucleotide at the extreme 3' end.

The region of genomic DNA containing the SNP of interest is amplified by PCR. Both wildtype and variant ASPE primers are then annealed to the PCR product and undergo multiple rounds of primer extension incorporating biotin. In the case of a wildtype genotype, the wildtype primer extends preferentially over the variant primer because of the mismatch between the primer and the target DNA at the variant primer's 3' end. Likewise, in the case of a variant genotype, the variant primer extends preferentially over the wildtype primer. Only in the case of a heterozygote will both primers extend.

After the primer extension, the products are mixed with VeraCode beads. The capture sequence on the primers hybridizes to the capture sequence on the VeraCode beads. Wildtype and variant primers and products each hybridize to a unique bead type. Labeling is then performed with a streptavidin-fluorophore conjugate. Only biotinylated extension products will be labeled and subsequently produce a fluorescent signal during the scan.

The genotype of the SNP is determined by the ratio of the relative fluorescent levels (RFU) of the two bead types.



This protocol is an example of a low-plex genotyping assay protocol developed specifically for a thrombosis multiplexed panel of four SNPs. Other assays will require specific optimization.

References

- S Bortolin, M Black H Modi, I Boszko, D Kobler, D Fieldhouse, E Lopes, J Lacroix, R Grimwood, P Wells, R Janeczko and R Zastawny, *Validation of the Tag-It High-Throughput Microsphere-Based Universal Array Genotyping Platform: Application to the Multiplex Detection of a Panel of Thrombophilia-Associated Single-Nucleotide Polymorphisms*, 2004, Clin Chem 50:11 2028-2036.
- S Johnson, D Marshall, G Harms, C Miller, C Sherrill, E Beaty, S Ledered, E Roesch, G Madsen, G Hoffman, R Haessig, G Kipish, M Baker, S Benner, P Marrell, J Prudent, *Multiplexed Genetic Analysis Using an Expanded Genetic Alphabet*, 2004, Clin Chem 50:11 2019-2027.

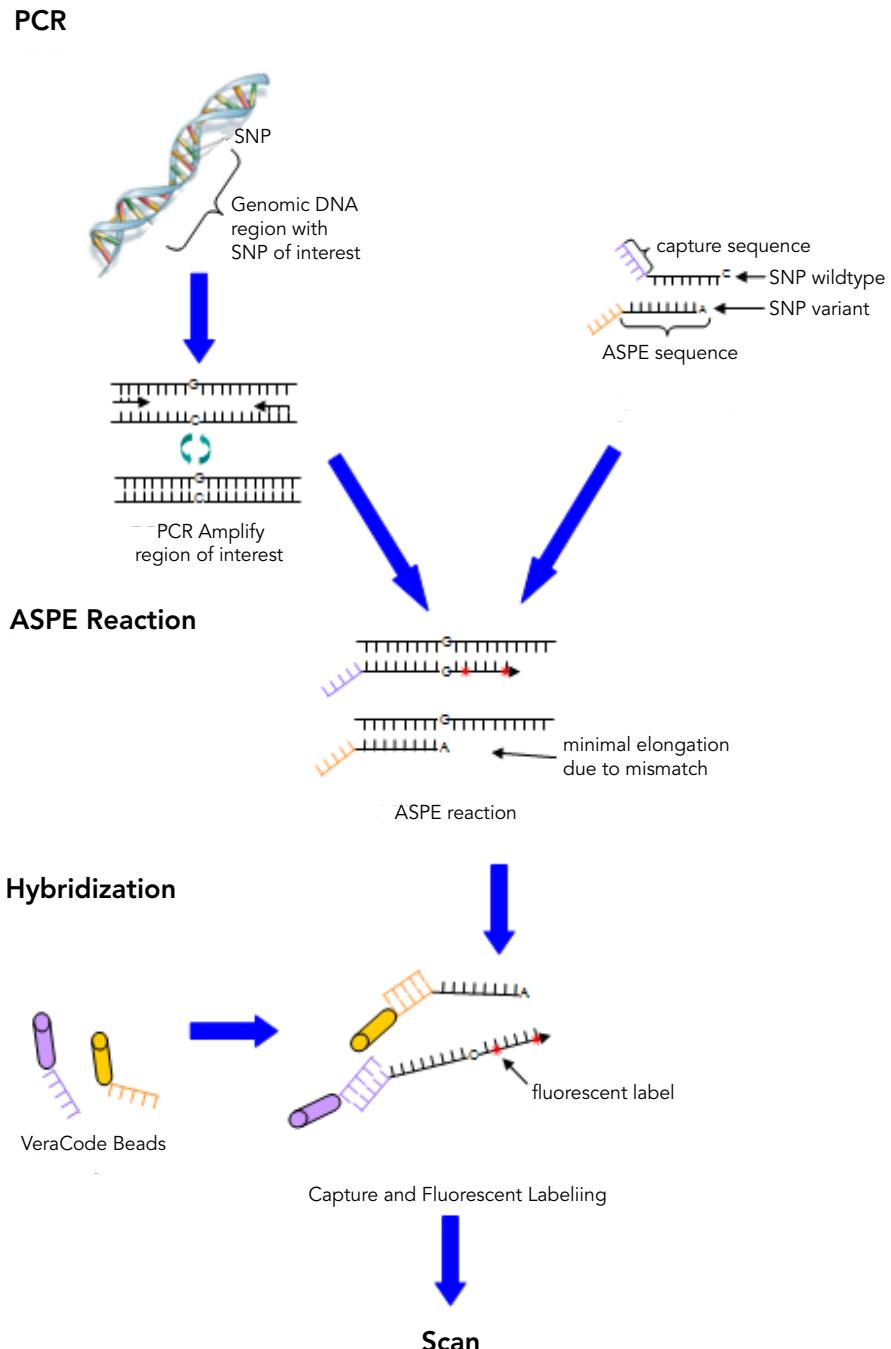


Figure 110 PCR, ASPE Reaction, Hybridization

Materials and Reagents for this Assay

These items are specifically required for performing VeraCode Assays using Universal Oligo Bead Sets on the BeadXpress Reader. For a list of equipment, materials, and reagents required for all assays in a BeadXpress Reader lab, see *Standard Equipment, Materials, and Reagents* on page 17.

Materials User-Supplied

Table 50 User-Supplied Materials

Item	Source
PCR plates	Fisher Scientific, 08-408-225
Strip caps	ABgene, AB0602
PCR quick tubes with caps	Phenix Research, MPC-425

Reagents User-Supplied

To perform VeraCode Assays using Universal Oligo Bead Sets on the BeadXpress Reader, you must obtain primers for each SNP:

- ▶ PCR primers (forward and reverse) for each target DNA
- ▶ ASPE primers (wildtype and variant) for each SNP

Table 51 User-Supplied Reagents

Item	Source
Platinum Taq Polymerase	Invitrogen, 10966-034
dNTPs	Invitrogen, 10297-018
Biotin dCTP	Invitrogen, 19518-018
Streptavidin-Alexa 647*	Invitrogen, S-32357
20X SSC	Sigma, S6639
Tween 20 (10% solution)	Sigma, P8942
Shrimp Alkaline Phosphatase	USB, 70092Y
Exonuclease I	USB, 70073Z



NOTE

Other fluorescent labels can be attached to the streptavidin. There are two lasers in the BeadXpress Reader:

- ▶ One excites at 532 nm and has an optical filter that picks up 550–610 nm transmitted light.
- ▶ One excites at 635 nm and has an optical filter that picks up 670–770 nm transmitted light.

Illumina-Supplied

Multiplexed assays can be performed by combining individual Universal Oligo Bead Sets, by using a pre-pooled Universal Oligo Bead Set, or by using a combination of the two. For information about combining VeraCode Universal Oligo Bead Sets, see *Kit VeraCode Beads* on page 231.

Illumina supplies 48 distinct VeraCode Universal Bead Sets (individual bead codes), and two VeraCode Universal Oligo Pooled Bead Sets (48-plex pooled bead codes).

Table 52 Illumina-Supplied Reagents

Item	Catalog #
VeraCode Universal Bead-XXXX (6 tubes), where XXXX is the code for one of the 48 distinct VeraCode Universal Bead Sets	VC-301-XXXX
VeraCode Universal Bead Set, Code 0481 (includes 6 tubes of 48-plex VeraCode Universal Bead Pool 0481)	VC-301-0481
VeraCode Universal Bead Set, Code 0482 (includes 6 tubes of 48-plex VeraCode Universal Bead Pool 0482)	VC-301-0482



NOTE

For more information about individual and pooled Universal Oligo Bead Sets, see *Individual Bead Sets* on page 248 and *Pooled Bead Sets* on page 251.

Universal Oligo Bead Sets

Individual Bead Sets

Each VeraCode Universal Bead Set contains one type of VeraCode bead split into six tubes, suitable for 6 x 96 samples. Every set is unique, as each set contains VeraCode beads with different VeraCode Bead Codes and unique IllumiCodes (DNA oligo capture sequences). You can pool each tube of VeraCode Universal Oligo Beads with other VeraCode Universal Oligo Beads (with different VeraCode Bead Codes and IllumiCodes) to create a multiplexed assay. Alternatively, you can combine VeraCode Universal Bead Sets with a VeraCode Universal Bead Set Pools, which come as pre-pooled 48-plex bead sets.

Table 53 lists catalog numbers, descriptions, bead codes, IllumiCodes, and probe sequences for Illumina's Individual Universal Oligo Bead Sets.

Table 53 VeraCode Bead Codes for Individual Universal Oligo Bead Sets

Illumina Catalog Number	Description	VeraCode BeadCode	IllumiCode	Probe Sequence
VC-301-5440	VeraCode Universal Bead Set, Code 5440	5440	42	TTCGTAACCGTGCAGTGCC
VC-301-5632	VeraCode Universal Bead Set, Code 5632	5632	103	ACGATGGTACGGTCGCTGTGA
VC-301-5634	VeraCode Universal Bead Set, Code 5634	5634	208	GGTTAGCGATCATACCGGCACT
VC-301-5640	VeraCode Universal Bead Set, Code 5640	5640	620	CCCGGTTGTCAGTCCGAAAGGG
VC-301-5664	VeraCode Universal Bead Set, Code 5664	5664	648	ACCTGAGTTACCGGCGTTACGT
VC-301-5760	VeraCode Universal Bead Set, Code 5760	5760	691	GCTGGATTGTCCGCACTCAAGT
VC-301-6144	VeraCode Universal Bead Set, Code 6144	6144	692	TATGCTTCGCCGCAGGACCACT
VC-301-6145	VeraCode Universal Bead Set, Code 6145	6145	751	GCAACGTGTCATTGCATCCTC
VC-301-6146	VeraCode Universal Bead Set, Code 6146	6146	844	AGGAGTCCAACCGCATCTTGCA
VC-301-6147	VeraCode Universal Bead Set, Code 6147	6147	974	CTCGAACCTACTGCCGGATCA

Table 53 VeraCode Bead Codes for Individual Universal Oligo Bead Sets (Continued)

Illumina Catalog Number	Description	VeraCode BeadCode	IllumiCode	Probe Sequence
VC-301-6148	VeraCode Universal Bead Set, Code 6148	6148	1041	GTTGCCGACGGTTAACCGAGT
VC-301-6150	VeraCode Universal Bead Set, Code 6150	6150	1051	CGGTTAGCGAGTAATAGTGCC
VC-301-6152	VeraCode Universal Bead Set, Code 6152	6152	1093	ACACTGGCAACGGTTCTGCGT
VC-301-6153	VeraCode Universal Bead Set, Code 6153	6153	1170	ACCGAAAGTCCCGGCTGTGGAT
VC-301-6156	VeraCode Universal Bead Set, Code 6156	6156	1191	CTATCAGGGTCGCCATGTGTCA
VC-301-6160	VeraCode Universal Bead Set, Code 6160	6160	1219	CCTCTTGTGGAAAGTCCACACG
VC-301-6162	VeraCode Universal Bead Set, Code 6162	6162	1307	ACGCCAGACTCCGGTCCAAGTT
VC-301-6168	VeraCode Universal Bead Set, Code 6168	6168	1432	TAGGC GTGGACCCTACCATCA
VC-301-6176	VeraCode Universal Bead Set, Code 6176	6176	1700	CACCGAACGGCAATGATCTGGT
VC-301-6177	VeraCode Universal Bead Set, Code 6177	6177	1761	TGGCCGTACATCACTAACCGAC
VC-301-6180	VeraCode Universal Bead Set, Code 6180	6180	2022	GACTGCAACCCGGCTCTGTCTA
VC-301-6192	VeraCode Universal Bead Set, Code 6192	6192	2174	GCGAACGGTCCTGTATTGCAGT
VC-301-6208	VeraCode Universal Bead Set, Code 6208	6208	2296	GGTCAACCAGCTTGATACGCC
VC-301-6210	VeraCode Universal Bead Set, Code 6210	6210	2321	CTTGTAGGAGCTCGGAAAGACT

Table 53 VeraCode Bead Codes for Individual Universal Oligo Bead Sets (Continued)

Illumina Catalog Number	Description	VeraCode BeadCode	IllumiCode	Probe Sequence
VC-301-6216	VeraCode Universal Bead Set, Code 6216	6216	2326	CCACATGCTCTCGGTGTCGAAT
VC-301-6240	VeraCode Universal Bead Set, Code 6240	6240	2717	ATTGGATGCCCTTCCTGCAA
VC-301-6272	VeraCode Universal Bead Set, Code 6272	6272	3041	GCGACGTGGACTGCTCAAACG
VC-301-6273	VeraCode Universal Bead Set, Code 6273	6273	3155	GAGGGAACGTGAATGCTGCTCT
VC-301-6276	VeraCode Universal Bead Set, Code 6276	6276	3285	GTCGGAGTAATTGTGCCACCA
VC-301-6288	VeraCode Universal Bead Set, Code 6288	6288	3293	GTACTCGCAGTCCCAGTGGCAT
VC-301-6336	VeraCode Universal Bead Set, Code 6336	6336	3375	TTCGTGCTGGCTGAGAGCGTAA
VC-301-6400	VeraCode Universal Bead Set, Code 6400	6400	3465	TAGCGCCTATCTGCCAGGGACT
VC-301-6402	VeraCode Universal Bead Set, Code 6402	6402	3518	TCTGACTGGGAGATTCCGATGC
VC-301-6408	VeraCode Universal Bead Set, Code 6408	6408	3534	TGAGCGCCTTCCCAACTGAGGA
VC-301-6432	VeraCode Universal Bead Set, Code 6432	6432	3569	AACCGGAGGCCAAGTTGCTGTC
VC-301-6528	VeraCode Universal Bead Set, Code 6528	6528	3592	TCCGGTCTGCATGAAGAGGAG
VC-301-6656	VeraCode Universal Bead Set, Code 6656	6656	3871	GATGCGACGACGACTATTCTGT
VC-301-6657	VeraCode Universal Bead Set, Code 6657	6657	4068	GAGACGACAACCTTCTCGCAACC

Table 53 VeraCode Bead Codes for Individual Universal Oligo Bead Sets (Continued)

Illumina Catalog Number	Description	VeraCode BeadCode	IllumiCode	Probe Sequence
VC-301-6660	VeraCode Universal Bead Set, Code 6660	6660	4229	CAAGTGATTGCCCGGTTAAC
VC-301-6672	VeraCode Universal Bead Set, Code 6672	6672	4247	GTGCGAAATTCAATTCCGACCGCT
VC-301-6720	VeraCode Universal Bead Set, Code 6720	6720	5211	TTACGAACCGATGAGCACCTAGTA
VC-301-6912	VeraCode Universal Bead Set, Code 6912	6912	5384	AATCCGTACTTGGTGCATCCGTA
VC-301-7168	VeraCode Universal Bead Set, Code 7168	7168	5389	GCCCCATCCACTATTCGGAGGTAA
VC-301-7170	VeraCode Universal Bead Set, Code 7170	7170	5537	TAATACGCCAGATGGTTGGTGCAT
VC-301-7176	VeraCode Universal Bead Set, Code 7176	7176	5650	TATTGCACCACCGCTACTGAGAAT
VC-301-7200	VeraCode Universal Bead Set, Code 7200	7200	5801	GGATATGTCACCTACTGCAACGGA
VC-301-7296	VeraCode Universal Bead Set, Code 7296	7296	5915	GTGGCATCATACCATAAACGCTCG
VC-301-7680	VeraCode Universal Bead Set, Code 7680	7680	6136	GTTACAATCCCTGGTCCGTATGC

Pooled Bead Sets

Each VeraCode Universal Bead Set Pool contains a pool of 48 types of VeraCode Beads split into six tubes, suitable for 6 x 96 samples. The 48 types of VeraCode Beads in each VeraCode Universal Bead Set Pool are unique, as each bead has a different VeraCode Bead Code and unique IllumiCode (DNA oligo capture sequence). As such, each VeraCode Universal Bead Set Pool is suitable for conducting 48-plex assays. You can pool each tube of VeraCode Universal Oligo Bead Set Pool with additional VeraCode Universal Oligo Beads (with different VeraCode Bead Codes and IllumiCodes) to create higher multiplexed assays.

Table 53 lists catalog numbers, descriptions, bead codes, IllumiCodes, and probe sequences for Illumina's Pooled Universal Oligo Bead Sets.

Table 54 VeraCode Bead Codes for Pooled Universal Oligo Bead Sets

Illumina Catalog Number	Description	VeraCode BeadCode	IllumiCode	Probe Sequence
VC-301-0481	VeraCode Universal Bead Set, Pool 0481	3072	140	CGGATGCAATCGGTATCGGGAA
		3073	205	CATGGACGAACTCACGCCGGCTT
		3074	453	GCGATTGAAGTGCACGGACCAATG
		3075	623	GTCGCGCTTATGAATCGGATGC
		3076	636	AGCCGTATCGGTTACCATGCCG
		3078	1020	GTACGACCTTATTGCCAGGC
		3080	1055	GAGGACGATCTACCTCCGCCG
		3081	1182	TTGCCAGTACCCGGACTAGCT
		3084	1229	CCCACCGGAATTGTAGTGCGGT
		3088	1321	ACGTCGTACAGGGATTCCGTCA
		3090	1337	AGCACTGGAACCGCATTCTGGG
		3096	1339	TCTGCTAACCGCCAAAGTGC
		3104	1343	CCAGAAGGCTCGACATGGTTGA
		3105	1678	GAATCGTGGTACTGGTCAACCG
		3108	1823	AAACTACCCGTGCTCTGGTCCA
		3120	1984	AGCTTGGCCTCGGTACATCAGCA
		3136	2027	TCGTTGACCACCCACTCGTAGG
		3138	2099	GTGCCCTTACCGTACGTGAATA
		3144	2149	TAGCCCACCGAAGAGTTGATGC
		3168	2425	GTCACCCGGATTAAGACAGGCT
		3200	2536	ACGTCGCTCTAGGTGGACAGT
		3201	2952	ATAAGCACGTTCCCAGTGAGCC
		3204	3042	GCGGCGATCTAAGGAGAGTTCC
		3216	3043	GTCGTTCCGCACAGCCCAGTAG
		3264	3069	ACGCCTCGTGGTGTGGAGATAA
		3328	3075	TGGATCACCCATCTGTCGCGTA

Table 54 VeraCode Bead Codes for Pooled Universal Oligo Bead Sets (Continued)

Illumina Catalog Number	Description	VeraCode BeadCode	IllumiCode	Probe Sequence
		3330	3085	GCAACTGGTCCTTCAGGCGAGA
		3336	3136	CTAGGCTTCACAGATCGGCACG
		3360	3348	AAGGACCTCAGTGGATAGCGTG
		3456	3479	CACGCACTGGAGAGTATATGGC
		3584	3523	CACAGCGGCTTGGCTTCAACAT
		3585	3648	GTCGTTCCACTGGCTGGCAAAC
		3588	3697	CATGTGACCGTACTAACCGCTGA
		3600	3736	GCCGACAATTACCCGTTGCTAGA
		3648	3922	CAGTTGCCGTCGTGTCATTGAGA
		3840	3931	GATGCTCGTTCGTTGAAGTCCAG
		4098	4010	GCCATTCCAACGGTGAAAGGTT
		4099	4104	GCATGGTCTTACAATCGGTAGGC
		4101	4144	CCATAGAGCTTAGACCCGATCCA
		4102	4211	CGACTAACGGCATCTGACATCA
		4104	4268	GGATTACCATGTACGTGTGGAGC
		4105	4307	GGTGAGGTCCATACTCTTCGCAT
		4106	4334	CGCAGATGAATCACGGAGGCTTT
		4107	5669	CCAGACGGACCAGGGTGAATAAT
		4108	5683	TTGACCCCTAACAAATTCTGTGCCTG
		4110	5715	CTAAGCGGATATGTTGGAAGCACG
		4113	5716	ATATCTGTCGGTAGAAAGCCTGGA
		4114	5910	GGCCCTTGAGTAGTATGAGCGTA
VC-301-0482	VeraCode Universal Bead Set, Pool 0482	1	44	ATCTGTACGAACGTAGCCGCAG
		2	137	CTACCGAATCTACGGATGCCA
		4	237	CCTGGTAACGAGACGACTGGGT
		5	278	TTCTCGAATCTAGCGCCCTAGC

Table 54 VeraCode Bead Codes for Pooled Universal Oligo Bead Sets (Continued)

Illumina Catalog Number	Description	VeraCode BeadCode	IllumiCode	Probe Sequence
		8	279	GTTGCACCGCAGATCGTAGGCT
		10	329	GGCTAACGTTACGGGCTACGCAT
		16	501	CGTAACGTCTCCGATCCCAGG
		17	526	TTGCCCTACGCTAAAGGGTCCG
		20	590	CTTACACCAACGAAGCCGTCGT
		32	592	GCACTCTCGCGCTGACAGTAA
		34	658	GTCACTTTGGGCTAGGAACGT
		40	662	CCAAGAGAGCGACGGGCTGTTT
		64	737	GAGCACTCTAACGCGCGGTCAT
		65	858	AACGGTGCTTGTCGGGTCAAC
		68	910	CAAGACCCGTCGTTCTGTGGAC
		80	912	CCCTCTCACGAAGATTGAGCGC
		128	962	GTTTGGAGGCCGGTCCGCAAG
		130	975	CTACGCCGTTGTAACACCTCGG
		136	1003	CCTCGAACTGTTGAGCGCGGAG
		160	1070	GAGCTTGGTGCCTCCGACAA
		256	1112	AGACTCTCACGAATGTCCGC
		257	1142	GCGTCTATTGGATGCGGCACAT
		260	1209	TGGCGAGCTAGTTGCTAACGTC
		272	1235	CGCACGGATAGCTCTGACGGTT
		320	1251	CCTGTTGGGCACTAAGACTCC
		512	1306	CGATATTGCTAGCCGGGATCAC
		514	1365	AATCGCGTCCAGATACTGCCCC
		520	1611	CAATTGCGACCCCTGAACAGC
		544	1632	TAGACAGACCCGGCACTGTGTA
		640	1696	CTTGTACGGCTCAGTTACAGCG
		1024	1716	GCGAACTTCGAGGAATCATGCC

Table 54 VeraCode Bead Codes for Pooled Universal Oligo Bead Sets (Continued)

Illumina Catalog Number	Description	VeraCode BeadCode	IllumiCode	Probe Sequence
		1025	1742	AGGCTTTAGGGTGCAGTCACAT
		1028	1878	TGGTAGGACGCAGAGCTATGCC
		1040	1968	TGACGAAGACTAGGGTCCCTCG
		1088	1992	GCGAAACTCGGACTCCTGAAC
		1280	2832	GCTCGACAATGAGTGGTGACCT
		2048	2911	CTCCTTACCTGGCGAGGACAC
		2050	2908	AGCAACGACTGGCCTCTGACC
		2056	3008	GGCGCTTCGATAAATGAGGCTC
		2080	3137	TCTTCCACGACACCTGGATGGA
		2176	3433	TTACGGCCCAAGTGTCAAGGAGA
		2560	3511	CCCCGGATCACAACTGCATGTT
		4096	3802	CCCAACGACACGGCTAAGTATGT
		4097	3845	CTATCTCACCGACCAAATAGGCG
		4100	3864	GGTTTGTGTGCGGATCTAACGA
		4112	3885	CGAGATCGCTGTACTCCGTTA
		4160	4254	GAGCTAATTGCCCCACACTGA
		4352	4824	CTTACACACGAACGTATCGGAATC

Design PCR and ASPE Primers

The ASPE method of genotyping requires two sets of primers.

The first set of primers consists of forward and reverse primers to amplify each genomic region containing SNPs of interest in the multiplexed PCR reaction.

The second set of primers consists of the wildtype and variant primers that define each SNP. This second set of primers is used in the multiplexed primer extension reaction.

PCR Primers

To design PCR primers:

1. Identify SNPs of interest.
2. Design PCR primer pairs that encompass the SNP's using a multiplex PCR program such as:
 - The PCR Suite
(Klinische Genetica, Erasmus MC Rotterdam, Netherlands)
 - Primer3
http://www2.eur.nl/fgg/kgen/primer/Genomic_Primers.html
3. Target a temperature of 60°C using the nearest neighbor algorithm and a 50% GC content.
4. Keep PCR products as small as possible, ideally 100-200 bp. Try to design PCR products so each product can be differentiated on a gel when multiplexed.
5. Confirm the oligos don't exhibit self-annealing or primer-dimer formation.
6. Confirm there is no homology between any two primers in the multiplex mix at the hybridization temperature.
7. Redesign any primers that don't meet the criteria.

ASPE Primers

ASPE primers are designed around each SNP in the panel. For each SNP, there is one primer which matches the wildtype sequence, and one primer for each variant of interest. The 3' end of each primer is the nucleotide location of the SNP. This differs from other genotyping methods in which the primer ends just before the SNP location.

- ▶ For each SNP site there are two possible primer orientations: primers from the sense strand, or primers from the antisense strand.
- ▶ For each SNP, two primers need to be designed: wildtype and variant. These differ from each other by only a single base at the 3' end.
- ▶ If there are multiple SNPs within one PCR product, make sure all ASPE primers are from the same strand to keep from making unwanted PCR products (see Figure 111).



Figure 111 Unwanted PCR Products from Poorly-Designed ASPE Primers

- Otherwise, the strand used depends only on which one makes a better primer.
 - Keep the temperature of the primers close to 50°C.
 - Design both wildtype and variant primers from the same strand.
 - Use mFold or a similar product to ensure that there is no secondary structure that could inhibit the ASPE reaction or hybridization.

Match ASPE Primers to VeraCode Capture Sequences

Once you've designed the ASPE primers, a capture sequence needs to be added to the 5' end of each primer. The capture sequence is the inverse complement of the capture sequence on the VeraCode bead.

Up to 144 VeraCode bead types are available as part of the Universal Capture Bead product. Each VeraCode bead type has a different capture sequence on its surface. Each of the ASPE primers must be paired to a unique VeraCode bead. For each SNP there will be one bead type for the wildtype allele, and one bead type for each of the variant alleles.

Each complete ASPE primer is between 40 and 50 bases long and consists of three parts:

5' (capture sequence) - (genomic sequence) - SNP 3'

For an example of the final ASPE primer construct, see *Primers Used for Thrombosis Panel* on page 259.

To match ASPE primers to VeraCode capture sequences:

1. Assign each primer to a unique VeraCode.
 - Use each VeraCode only once. The wildtype and variant primers must have different VeraCodes.
2. Each ASPE primer is made by combining the inverse complement of the VeraCode to the 5' end of the ASPE primer.
 - Make sure the SNP nucleotide is at the very 3' end of the combined primer.
3. Check each combined primer for secondary structure using a program such as mFold: <http://www.bioinfo.rpi.edu/applications/mfold/>.
 - If there is structure that would persist at the hybridization temperature, pick another VeraCode for that ASPE oligo.
 - It may take five or six iterations to get a complete set of ASPE oligos matched with unique VeraCodes.
4. Check each combined primer against all other primers to confirm there is no homology (except for the wildtype/variant pairs).
 - If the SNP portion of a primer is homologous to a different SNP, try creating the SNP oligo from the other strand. Remember to change both the wildtype and the variant oligo.
 - If the VeraCode portion is creating the homology, pick another VeraCode.

At the end of this process there will be:

- A forward and reverse PCR primer for each gene section containing a SNP of interest.
- A wildtype and variant SNP attached to the inverse complement of a unique VeraCode.

Primer and Oligo Guidelines

All primers can be ordered desalted.

Primers Used for Thrombosis Panel

The Thrombosis Panel SNPs used in this protocol are: Factor V G1691A, Factor II G20210A, MTHFR 667 C667T, and MTHFR 1298 A1298C.

The VeraCode is the identifier for the oligo on the bead. It is the inverse complement of the capture sequence. The sequence in *blue* is the capture sequence for the VeraCode used. The nucleotide in *orange* is the SNP nucleotide.

Table 55 Factor V

PCR forward	CGCCTCTGGGCTAATAGGAC
PCR reverse	GCCCCATTATTAGCCAGGA
ASPE Wt VeraCode 5632	TACACAGCGACCGTACCATCGTA GGACAAAATACCTGTATTCCCTC
ASPE Var VeraCode 6153	ATCCACAGCCGGACTTCGGTA GGACAAAATACCTGTATTCCCTT

Table 56 Factor II

PCR forward	GAACCAATCCCGTGAAAGAA
PCR reverse	CCAGAGAGCTGCCCATGA
ASPE Wt VeraCode 5640	CCCTTCGGACTGACAACCGGG ACAATAAAAGTGACTCTCAGCG
ASPE Var VeraCode 5634	AGTGCCGGTATGATCGCTAACCA ACAATAAAAGTGACTCTCAGCA

Table 57 MTHFR 667

PCR forward	CTTGAGGCTGACCTGAAGC
PCR reverse	CAAAGCGGAAGAATGTGTCA
ASPE Wt VeraCode 6146	TGCAAGATGCGGTTGGACTCCT AGAGAAGGTGTCTGCAGGGAGC
ASPE Var VeraCode 6148	ACCTGGTTAACCGTCGGCAAC AGAGAAGGTGTCTGCAGGGAGT

Table 58 MTHFR 1298

PCR forward	AGGAGCTGCTGAAGATGTGG
PCR reverse	CTTGTGACCATTCCGGTT
ASPE Wt VeraCode 5664	ACGTAACGCCGTAACTCAGGT AACAAAGACTTCAAAGACACTT
ASPE Var VeraCode 6145	GAGGATGCGAATGACACGTTGC AACAAAGACTTCAAAGACACTT

Contamination and Controls

The following sections address contamination containment and controls.

Contain Contamination

As the number of PCR reactions increases, the likelihood of PCR cross-contamination also increases. To avoid this, follow the guidelines described in *Prevent PCR Product Contamination* on page 14. In addition:

- ▶ Perform regular bleaching and UV light treatment to reduce the risk of contamination.



NOTE

Autoclaving is not a reliable method of reducing the contamination of DNA.

- ▶ Be careful when pulling the strip caps and strip mats off the plates, as this is another potential source of cross-contamination. It is critical that there is no splashing between the wells. Less than 1 μ l of a PCR or ASPE product splashing into a neighboring well can affect your data quality.

Controls

You should include a set of controls in all PCR experiments in order to help identify problems when they occur.

- ▶ Negative controls should include non-DNA wells randomly included in PCR plates. For non-DNA wells, use water in place of a genomic sample. If carried through the hybridization, these samples should have very low fluorescent counts. If any of the samples has a high count, the data may be unreliable.
- ▶ Positive controls should include a subset of samples of known genotypes. These controls confirm that the PCR and ASPE reactions are working.
- ▶ A region of the genome with no known SNPs can be amplified in the multiplexed reaction. The ASPE primers would be the wildtype and a SNP that is known to be absent in the population. If this SNP gives any call other than wildtype, the data from the other SNPs in the well may be unreliable.
- ▶ Include replicates in the plate. All replicates should give the same call. The CVs of the technical replicates should be low.

Two-Plate Protocol for Low-Plex Genotyping

In this protocol, the ASPE and PCR reactions are performed in separate plates. One-fifth of the PCR reaction is used in the ASPE reaction. It is a robust protocol and is useful when new SNP panels are being designed. It is also the better protocol to use if either the ASPE primers or the PCR primers are not high quality.



This protocol is specifically optimized for four SNPs related to thrombosis. The primer concentrations and PCR program times and temperatures may need to be adjusted for a different SNP panel.

The optimum concentration of primers for other SNP panels needs to be empirically determined. One way to optimize is to matrix multiple dNTP concentrations and primer concentrations. The optimum primer concentrations may be different for each SNP, but the optimal dNTP concentration must work for all primers.

PCR

1. Confirm concentrations of individual primers using a spectrophotometer and make adjustments as necessary.



The concentrations of the PCR primers are critical to the success of this assay.

2. Make a 10 µM stock solution of all Thrombosis PCR primers. Aliquot as single use and store unused primers at -20°C.
3. Make a stock solution of the nucleotides.
The four deoxynucleotides are each at 5 mM in the stock solution. Aliquot as single use and store unused dNTPs at -20°C.
4. Genomic DNA should be between 10–100 ng/µl.
5. Program the thermocycler with the PCR program as follows:
 - 5 minutes at 95°C
 - 30 cycles
 - 30 seconds at 95°C
 - 30 seconds at 58°C
 - 30 seconds at 72°C
 - Forever at 4°C
6. Make enough master mix for the number of reactions in the experiment plus one or two extra reactions.

Table 59 Two-Plate Protocol, PCR Master Mix

Reagent	Initial Concentration	Volume $\mu\text{l}/\text{Reaction}$	Concentration in Reaction
TAQ Buffer	10X	1.5	1X
MgCl	50 mM	0.5	1.67 mM
dNTPs	5 mM	0.6	200 μM
PCR primer mix	10 μM	1	10 pmol each
Water	N/A	10.25	N/A
Platinum TAQ	5 units/ μl	0.15	0.75 units

7. Transfer 14 μl of the master mix to each well or PCR tube.
8. Add 1 μl DNA (10–100 ng).
9. Place the PCR reactions in the thermocycler and run the PCR program.

Two-Plate Protocol SAP/ EXO

SAP/EXO is a mixture of Shrimp Alkaline Phosphatase and Exonuclease I. The Shrimp Alkaline Phosphatase is used to inactivate any remaining nucleotides from the PCR reaction. The Exonuclease I inactivates any remaining PCR primers.

1. Program the thermocycler with the SAP/EXO program as follows:
 - 45 minutes at 37°C
 - 15 minutes at 99°C
 - Forever at 4°C
2. Make enough master mix for the number of wells of PCR plus one or two extra reactions.

Table 60 Two-Plate Protocol, SAP/EXO Master Mix

Reagent	Initial Concentration	Volume $\mu\text{l}/\text{Reaction}$	Concentration in Reaction
Shrimp Alkaline Phosphatase	1 unit/ μl	2	2 units
Exonuclease I	10 units/ μl	0.5	5 units

3. Centrifuge the PCR reactions for 1 minute.



CAUTION

Use caution when removing strip caps. Contamination between wells will negatively impact your results.

4. Add 2.5 μ l of the SAP/EXO master mix to each PCR reaction.
5. Run the SAP/EXO program.

Two-Plate Protocol ASPE

During the ASPE reaction, multiple rounds of primer extension are performed, during which biotinylated dCTPs are incorporated into extension products. Primers with a match at the 3' terminus are preferentially extended.



NOTE

This protocol is specifically optimized for the Thrombosis SNP Panel. The primer concentrations and PCR program times and temperatures may need to be adjusted for a different SNP panel.

1. Add TE (10 mM Tris pH 7 1 mM EDTA) to each ASPE primer to approximately 100 μ M.
2. Confirm the concentrations of the individual primers with a spectrophotometer and adjust as necessary.
3. Make a working stock solution of all the ASPE primers (5 μ M). Aliquot as single use and store unused primers at -20°C.
4. Make a 100 μ M stock solution of the unlabeled nucleotides (dATP, dGTP, dTTP). Aliquot as single use and store unused primers at -20°C. The biotin dCTP is used neat.
5. Program the thermocycler with the ASPE program as follows:
 - 120 seconds at 96°C
 - 40 cycles
 - 30 seconds at 94°C
 - 30 seconds at 54°C
 - 60 seconds at 74°C
 - Forever at 4°C

Table 61 Two-Plate Protocol, ASPE Master Mix

Reagent	Initial Concentration	Volume μ l/Reaction	Concentration in Reaction
TAQ Buffer	10X	2	1X
MgCl	50 mM	0.5	1.58 mM
dATP, dGTP, dTTP	100 μ M	1.0	5 μ M
Biotin 14-dCTP	400 μ M	0.25	5 μ M
ASPE primer mix	5 μ M	1.0	5 pmol
Water	N/A	12.05	N/A
Platinum TAQ	5 units/ μ l	0.2	1 unit

This procedure can be stopped and stored at the end of the PCR program, the end of the SAP/EXO cycle, or the end of the ASPE program. If the procedure is not completed in one day, store the products at 4°C until used. The ASPE product can be stored for at least 1 week without degradation.

6. Put 17 µl ASPE premix into each well of the new PCR plate.
7. Centrifuge the PCR/SAP/EXO reactions for 1 minute.

**CAUTION**

Use caution when removing strip caps.
Contamination between wells will negatively impact your results.

8. Add 3 µl of the PCR/SAP/EXO reaction to the ASPE premix.
9. Run the ASPE program.

Hybridization

The ASPE primer extension products are hybridized to the VeraCode beads that have been kitted into 96-well polypropylene plates or strip well plates. If there are fewer than 96 reactions, use a subset of strip wells. Do not cut the strip wells.

The first step is to exchange the EtOH in the wells with hybridization buffer. The ASPE reaction is then added to the beads and hybridized for 1 hour. The hybridization reaction is then fluorescently labeled.

1. Use streptavidin coupled to a fluorescent label that excites at 532 nm or 635 nm and emits between 550 nm–610 nm or 670 nm–770 nm.
2. Make the hybridization buffer as follows:
 - 3X SSC
 - 0.1% TWEEN
 - Sterile filter
3. Remove the kitted VeraCode Beads (in 70% EtOH) from the freezer and thaw them to room temperature.
4. Add 150 µl of the hybridization buffer to the kitted VeraCode beads and mix briefly.
5. Let the plate sit for 1 minute to allow the beads to settle.
6. Use the 8-channel aspiration manifold to carefully aspirate off the buffer/EtOH mix.

Be sure to aspirate thoroughly. You may need to perform several aspirations until there is no more liquid.

**NOTE**

If there are extra wells that have beads but will not have a hybridization reaction, the beads can be pipetted out for later use, or you can add the SA-hybridization buffer and treat the data as blanks.

7. Centrifuge the ASPE reaction for 1 minute.

**CAUTION**

Use caution when removing PCR strip caps.
Contamination between wells will negatively impact your results.

- 8.** Add 5 μ l of an ASPE reaction to a well of beads with hybridization buffer.
- 9.** Incubate for 1 hour at 45°C with agitation (1000 rpm/setting of 100) in the VeraCode Vortex Incubator.
- 10.** Make enough streptavidin mix for each well. Assume 50 μ l per well.
 - 3.75 μ l Streptavidin-Alexa-647
 - 1 ml Hybridization buffer
- 11.** Centrifuge the plate of hybridized beads for 2 minutes.
- 12.** Add 50 μ l streptavidin mix to each well.
- 13.** Cover the plate with foil, to minimize exposure to light.
- 14.** Incubate for 15 minutes at room temperature while shaking.
- 15.** Uncover and scan the VeraCode Bead Plate.

See the *BeadXpress Reader System Manual* for instructions.

Single-Plate Protocol for Low-Plex Genotyping

The single-plate protocol is faster than the two-plate protocol, uses fewer reagents, and eliminates transfer from one plate to another. However, this method may require extensive optimization to achieve robust performance.

PCR

1. Confirm concentrations of individual primers using a spectrophotometer and make adjustments as necessary.



The concentrations of the PCR primers are critical to the success of this assay.

2. Make a 2 µM stock solution of all Thrombosis PCR primers. Aliquot as single use and store unused primers at -20°C.



The optimum concentration of primers for other SNP panels must be empirically determined. One way to optimize is to matrix multiple dNTP concentrations and primer concentrations.

The optimum primer concentrations may be different for each SNP but the optimal dNTP concentration must work for all primers. For more information, see the *Troubleshooting* section in this chapter.



Use caution when removing strip caps. Contamination between wells will negatively impact your results.

3. Make a stock solution of all four deoxynucleotides (1 mM each). Aliquot as single use and store unused dNTPs at -20°C.



It is important to limit the amount of PCR product made. This is accomplished by limiting both the primers and the nucleotides in the PCR reaction.

Genomic DNA should be between 10–100 ng/µl.

4. Program the thermocycler with the PCR program as follows:
 - 5 minutes at 95°C
 - 30 cycles
 - 30 seconds at 95°C
 - 30 seconds at 58°C
 - 30 seconds at 72°C
 - Forever at 4°C
5. Make enough master mix for the number of reactions in the experiment plus one or two extra reactions.

Table 62 Single-Plate Protocol, PCR Master Mix

Reagent	Initial Concentration	Volume μ /Reaction	Concentration in Reaction
TAQ Buffer	10 X	1.5	1X
MgCl	50 mM	0.5	1.6 mM
dNTPs	1 mM	0.6	40 μ M
Primer mix	2 μ M	1	2 pmol
Water	N/A	10.25	N/A
Platinum TAQ	5 units/ μ l	0.15	0.75 units

6. Transfer 14 μ l of the master mix to each well or PCR tube.
7. Add 1 μ l DNA (10–100 ng).
8. Place the PCR reactions in the thermocycler and run the PCR program.

Single-Plate Protocol SAP/ EXO

SAP/EXO is a mixture of Shrimp Alkaline Phosphatase and Exonuclease I. The Shrimp Alkaline Phosphatase is used to inactivate any remaining nucleotides from the PCR reaction. The Exonuclease I inactivates any remaining PCR primers.

1. Program the thermocycler with the SAP/EXO program as follows:
 - 25 minutes at 37°C
 - 15 minutes at 99°C
 - Forever at 4°C
2. Make enough master mix for the number of wells of PCR plus one or two extra reactions.

Table 63 Single-Plate Protocol, SAP/EXO Master Mix

Reagent	Initial Concentration	Volume μ l/Reaction	Concentration in Reaction
Shrimp Alkaline Phosphatase	1 unit/ μ l	1.6	1.6 units
Exonuclease I	10 unit/ μ l	0.4	4 units

3. Centrifuge the PCR reaction for 1 minute.
4. Add 2 μ l of the SAP/EXO master mix to each PCR reaction.
5. Run the SAP/EXO program.

Single-Plate Protocol ASPE

During the ASPE reaction, multiple rounds of primer extension are performed, during which biotinylated dCTPs are incorporated into extension products. If a SNP is absent, that primer does not extend, and the fluorescent counts are low in the subsequent hybridization. In the case of a heterozygote, both primers extend. In the single-plate protocol, the ASPE primers are added directly to the PCR reaction following the SAP/EXO step.



NOTE

This protocol is specifically optimized for four SNPs related to thrombosis. The primer concentrations and PCR program times and temperatures may need to be adjusted for a different SNP panel.

1. Add TE (10 mM Tris pH 7.0 1 mM EDTA) to each ASPE primer to approximately 100 µM.
2. Confirm the concentrations of the individual primers with a spectrophotometer and adjust as necessary.
3. Make a 5 µM working stock solution of all of the ASPE primers.
4. Aliquot as single use and store unused primers at -20°C.
5. Make a 100 µM stock solution of the unlabeled nucleotides (dATP, dGTP, dTTP).
6. Aliquot as single use and store unused dNTPs at -20°C.
The biotin is used neat.
7. Program the thermocycler with the ASPE program as follows:
 - 120 seconds at 96°C
 - 40 cycles
 - 30 seconds at 94°C
 - 30 seconds at 54°C
 - 60 seconds at 74°C
 - Forever at 4°C
8. Make enough master mix for the number of reactions in the experiment plus one or two extra reactions.

Table 64 Single-Plate Protocol, ASPE Master Mix

Reagent	Initial Concentration	Volume µl/Reaction	Concentration in Reaction
TAQ Buffer	10X	0.7	1X
MgCl	50 mM	0.125	1.58 mM
dATP, dGTP, dTTP	100 µM	1.0	4.5 µM
Biotin 14-cDTP	400 µM	0.25	4.5 µM
ASPE primer mix	5 µM	1.0	5 pmol
Water	N/A	1.73	N/A

Table 64 Single-Plate Protocol, ASPE Master Mix (Continued)

Reagent	Initial Concentration	Volume $\mu\text{l}/\text{Reaction}$	Concentration in Reaction
Platinum TAQ	5 units/ μl	0.2	1 unit

9. Centrifuge the PCR/SAP/EXO reactions for 1 minute.

**CAUTION**

Use caution when removing strip caps. Contamination between wells will negatively impact your results.

10. Add 5 μl of the ASPE premix to each PCR reaction.

11. Run the ASPE program.

Hybridization

The ASPE primer extension products are hybridized to the VeraCode beads that have been kitted into 96-well polypropylene plates or strip well plates. If there are not 96 reactions, a subset of strip wells can be used. Do not cut the strip wells.

The first step is to exchange most of the EtOH in the wells with hybridization buffer. The ASPE reaction is then added to the beads and hybridized for 30 minutes. Following the hybridization, the reaction is fluorescently labeled.

1. Use streptavidin coupled to a fluorescent label that excites at 532 nm or 635 nm and emits between 550 nm–610 nm, or 670 nm–770 nm.
2. Make hybridization buffer as follows:
 - 3X SSC
 - 0.1% TWEEN
 - sterile filter
3. Remove the kitted VeraCode Beads (in 70% EtOH) from the freezer and allow to warm up to room temperature.
4. Add 150 μl of the hybridization buffer to the kitted VeraCode beads and mix briefly.
5. Let with plate sit for 1 minute to allow the beads to settle.
6. Use the 8-pin aspiration manifold to carefully aspirate off the buffer/EtOH mix.

Be sure to aspirate thoroughly. You may need to perform several aspirations until there is no more liquid.

**NOTE**

If there are extra wells that have beads but will not have a hybridization reaction, the beads can be pipetted out for later use, or you can add the SA-hybridization buffer and treat the data as blanks.

7. Centrifuge the ASPE reaction for 1 minute.



CAUTION

Use caution when removing strip caps. Contamination between wells will negatively impact your results.

8. Add 10 µl of an ASPE reaction to a well of beads.
9. Incubate for 30 minutes at 45°C with agitation (1000 rpm/setting 100) in the VeraCode Vortex Incubator.
10. Make enough streptavidin mix for each well. Assume 50 µl per well.
 - 3.75 µl Streptavidin-Alexa-647
 - 1 ml Hybridization buffer
11. Centrifuge the plate of hybridized beads for 2 minutes.
12. Add 50 µl streptavidin mix to each well.
13. Incubate at room temperature 15 minutes.
14. Scan the VeraCode Bead Plate using the BeadXpress Reader.

For instructions about scanning VeraCode Bead Plates using the BeadXpress Reader, see the *BeadXpress Reader System Manual*.

Troubleshooting

Use the information in the following sections to troubleshoot this assay.

Optimization Reaction

Optimization of the primers is necessary when a new SNP panel is being developed or a new lot of primers is used. The quality of primers can vary tremendously from lot to lot depending on the supplier. Poor quality or quantitation will result in low fluorescent counts and miscalls in the data. To avoid this, Illumina recommends that you set up a small matrixed experiment whenever new lots of primers are used. It is important to use at least 5–10 samples of known genotypes for this experiment in order to generate enough data.

1. Determine the concentration of the individual PCR primers.
2. Make a solution with each PCR primer at 10 µM.
3. For each sample, perform three 2-plate PCR reactions using 1.25 µl primer (12.5 pmoles), 1 µl primer (10 pmoles), and 0.75 µl primer (7.5 pmoles).
You must adjust the water for the different volumes.
4. For each PCR reaction, perform a normal SAP/EXO two-plate reaction following the PCR.
5. Determine the concentration of the individual ASPE primers.
6. Make a solution with each ASPE primer at 10 µM.
7. Make dilutions of the ASPE primers to 5 µM, 2.5 µM, 1 µM, and 0.5 µM.
8. Each PCR reaction can be used in four ASPE reactions. For each PCR reaction, perform a two-plate ASPE reaction at each of the ASPE primer concentrations.
9. Perform a normal two-plate protocol hybridization and labeling.
At the end of this experiment, there are 12 data points for each SNP in each sample.

Table 65 Optimization Reaction

ASPE Primer Concentrations	PCR Primer Concentrations		
	12.5 µM PCR 5 µM ASPE	10 µM PCR 5 µM ASPE	7.5 µM PCR 5 µM ASPE
	12.5 µM PCR 2.5 µM ASPE	10 µM PCR 2.5 µM ASPE	7.5 µM PCR 2.5 µM ASPE
	7.5 µM PCR 1 µM ASPE	7.5 µM PCR 1 µM ASPE	7.5 µM PCR 1 µM ASPE
	7.5 µM PCR 0.5 µM ASPE	7.5 µM PCR 0.5 µM ASPE	7.5 µM PCR 0.5 µM ASPE

The best concentrations for each SNP is the set that gives the largest separation of genotypes, the tightest cluster within a genotype, and the highest fluorescent counts. This is easily visualized using the genoplots in GenomeStudio.

Additional Information

- ▶ The single-plate protocol can be used when the oligos are of high quality; i.e., when most of them are full-length. If a substantial portion of the oligos are less than full-length, Illumina recommends using the two-plate protocol, or using purified oligos, especially in the ASPE reaction. The two-plate protocol is advantageous for development because it yields multiple replicates from each ASPE and PCR reaction.
- ▶ PCR reactions can be tested by running an aliquot on an agarose gel. ASPE reactions can also be run on a gel. The results of an ASPE gel is the loss of the PCR bands and the addition of multiple bands between the primers and the upper PCR product. The ASPE reaction does not yield one discrete band for each SNP.

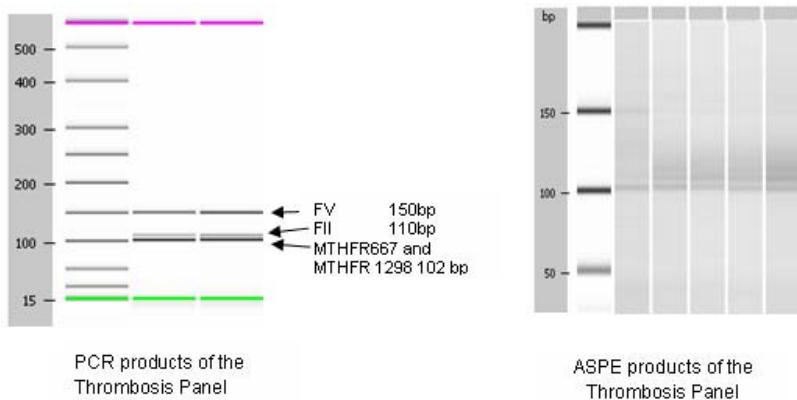


Figure 112 PCR Gel, ASPE Gel

- ▶ Make sure the concentrations of primers and dNTPs are correct. Do not rely on the data from the oligo manufacturer.



CAUTION

When you order new oligos, Illumina recommends that you run tests to confirm that they behave similarly to existing oligo stocks. Incorrect concentrations (too high or too low) will affect the efficiency of the PCR and ASPE reactions.

- ▶ Evaporation in the PCR and ASPE reactions can lead to poor results. Strip well caps appear to prevent evaporation better than the foil seal.
- ▶ Omitting the SAP/EXO step reduces the yield of ASPE products and leads to poor results.
- ▶ High concentrations of ASPE products in the hybridization inhibit the hybridization and result in low RFUs.
- ▶ Decreasing the hybridization time can lead to low signal and poor results.
- ▶ You can add the streptavidin directly to the hybridization, thereby eliminating one step. However, when you do this, the background RFUs tend to rise slightly. This should only be done when the PCR and ASPE reactions are of the highest quality.

- ▶ Contamination can be a major source of error. Take great care when removing the caps from the PCR and ASPE reactions. Even 1 μ l of sample splashing into another well can cause degradation of the calls. Contamination tends to move all calls in a well towards heterozygotes while the fluorescent values remain in the normal range.

Chapter 9

Carboxyl Beads Example Protocols

Topics

- 276 Introduction
- 277 Equipment, Materials, and Reagents for this Assay
- 280 One-Step Carbodiimide Coupling of Amine-Terminated Oligos to Carboxyl VeraCode Beads
- 282 Two-Step Protein Immobilization to Carboxyl VeraCode Beads
- 285 Quantitation and Manual Bead Kitting
- 288 Multiplex Cytokine Reagent Preparation
- 289 Multiplex Cytokine Protein Assay
- 292 Troubleshooting

Introduction

Cytokines are low-molecular-weight, hormone-like polypeptides which are secreted in the course of immunologic and inflammatory responses. They are important regulators of cell-mediated and humoral immune responses, and their expression has been associated with a wide variety of immune disorders. Cytokines function on a variety of cell types, having both stimulatory and inhibitory effects on proliferation, differentiation, and maturation.

The enzyme-linked immunosorbent assay (ELISA) is the most commonly reported method for the quantitation of secreted cytokines. However, the ELISA assay only measures a single cytokine level in any biological system. It requires more reagents, technician time, and larger sample volumes. In addition, it provides only partial information relevant to the response on a systematic level.

The use of multiplexed technology over conventional assay methods has advantages including:

- ▶ Simultaneous analyte detection
- ▶ Reduction of sample and reagent volumes
- ▶ High throughput of test results

This chapter describes a multiplexed cytokine assay using VeraCode beads based on the sandwich format. This assay provides detection of multiple cytokines from a single sample. A capture antibody is covalently bound to the carboxyl VeraCode bead surface. Samples are then incubated, and the antibody-bound VeraCode beads capture the analyte from solution. A conjugated secondary antibody (i.e., biotin) is added. This biotinylated detection antibody binds to the analyte and completes the “sandwich.” The complex is then incubated with a Streptavidin:Phycoerythrin label. The VeraCode beads are then ready to be scanned in the BeadXpress Reader.



NOTE

These protocols are example protocols using carboxyl beads. Other assays will require specific optimization.

Equipment, Materials, and Reagents for this Assay

These items are specifically required to perform VeraCode Assays using Carboxyl Beads on the BeadXpress Reader. For a list of equipment, materials, and reagents required for all assays in a BeadXpress Reader lab, see *Standard Equipment, Materials, and Reagents* on page 17.

Equipment User-Supplied

Table 66 User-Supplied Equipment

Item	Source
Plate shaker	Labline Instruments, Melrose Park, Illinois

Materials and Reagents User-Supplied Quantitation and Kitting

Table 67 Quantitation and Kitting

Item	Source
1 X Phosphate Buffered Saline (1X PBS)	
1 X Phosphate Buffered Saline + 1% Bovine Serum Albumin (PBS/BSA)	
1 X Phosphate Buffered Saline + 0.05% Tween 20 (PBST)	
Wide-orifice pipette tips for Rainin Multichannel LTS	Rainin HR-250W
Reagent reservoir (25 ml divided)	Matrix #8096
Reagent reservoir (50 ml)	Corning #4870

One-Step Carbodiimide Coupling of Amine-Terminated Oligos to Carboxyl VeraCode Beads

Table 68 One-Step Carbodiimide Coupling

Item	Source
EDC (freshly-made solution; 50 mg/ml in 0.1 M MES, pH 4.5)	Pierce catalog # 22980
5' Amine-terminated oligonucleotides (1 uM synthesis is recommended)	
0.1 M MES, pH 4.5	

Two-Step Protein Immobilization to Carboxyl VeraCode Beads**Table 69** Two-Step Protein Immobilization

Item	Source
0.1 M MES, pH 4.5	
Sulfo-NHS (freshly-made solution; 50 mg/ml in 0.1 M MES, pH 4.5)	Pierce catalog # 24510
EDC (freshly-made solution; 50 mg/ml in 0.1 M MES, pH 4.5)	Pierce catalog # 22980
PBS-Tween 20 (0.2%)	
PBS-BSA (1%) (stored at 4°C)	
PBS-BSA (1%)-Proclin 300 (stored at 4°C)	
BSA (stock solution used to make 1% PBS-BSA)	Sigma BSA 98% (# A7030-10g)
1 M NaCl in ultra-pure water	

Multiplex Cytokine Protein Assay**Table 70** Multiplex Cytokine Protein Assay

Item	Source
10x biotinylated detection antibody pool (stored at 4°C)	
Multiplex high standard pool (stored at -80°C)	
Multiplex cytokine standard beads (stored at 4°C)	
Streptavidin Phycoerythrin (stored at 4°C; 1 mg/ml)	
Cytokine Standards Diluent (CSD)	
Cytokine Reagent Diluent (CRD)	
PBS/T/BSA (PBS pH 7.4, 0.1% BSA-Fraction V, 0.05% Tween 20, Proclin 300)	
Wash Buffer	
PBS/T (PBS pH 7.4 and 0.05% Tween 20)	

Illumina-Supplied

Illumina supplies 10 distinct VeraCode Carboxyl Bead Sets. Sets A through I contain five tubes of Carboxyl Beads, each with its own distinct BeadCodes. The exception is set J, which contains only three tubes of Carboxyl Beads.

You can perform multiplexed assays by combining individual tubes of VeraCode Carboxyl Beads post-coupling to reach the desired level of multiplexing. Each tube of VeraCode Carboxyl Beads is sufficient to analyze 6 x 96 samples.

Table 71 lists catalog numbers, descriptions, and BeadCodes for Illumina's Carboxyl BeadSets.

Table 71 VeraCode Carboxyl Bead Sets

Illumina Catalog #	VeraCode Carboxyl Bead Set	BeadCodes
VC-311-8193	A	8193, 8195, 8196, 8197, 8198
VC-311-8199	B	8199, 8201, 8202, 8204, 8205
VC-311-8208	C	8208, 8209, 8210, 8211, 8212
VC-311-8214	D	8214, 8216, 8217, 8220, 8225
VC-311-8226	E	8226, 8228, 8229, 8232, 8234
VC-311-8240	F	8240, 8241, 8244, 8256, 8257
VC-311-8258	G	8258, 8259, 8260, 8262, 8264
VC-311-8265	H	8265, 8268, 8272, 8274, 8280
VC-311-8288	I	8288, 8289, 8292, 8304, 8321
VC-311-8322	J	8322, 8324, 8325

One-Step Carbodiimide Coupling of Amine-Terminated Oligos to Carboxyl VeraCode Beads

Materials and Reagents

- ▶ Carboxyl VeraCode beads
- ▶ EDC
- ▶ 5' Amine-terminated oligonucleotides
- ▶ 0.1M MES, pH 4.5

Additional Equipment

- ▶ Rocker
- ▶ Vortexer
- ▶ Microplate shaker
- ▶ Microcentrifuge

Steps

1. Bring the EDC to room temperature prior to use (15–30 minutes).



NOTE

EDC should be white in color. If it is not white, use new EDC. Purchase the smallest available quantity of EDC to avoid oxidation.

2. Resuspend the amine-terminated oligo to 1 mM in water.
3. Wash the carboxyl VeraCode beads (1 tube) 2x with 0.1 M MES, pH 4.5.
4. Remove the supernatant and resuspend in 50 µl 0.1M MES, pH 4.5.
5. Vortex to mix.
6. Prepare a dilution of 1 µM oligo in deionized water.



NOTE

Serial titration of 100 µM oligonucleotide is recommended to achieve optimal performance.

7. Add 20 µl of the diluted oligo to the microbeads and mix by vortexing.
8. Prepare a fresh solution of 50 mg/ml EDC in 0.1 M MES, pH 4.5.
9. Add 20 µl of fresh 50 mg/ml EDC solution to microbeads and mix by vortexing.
10. Incubate at room temperature for 30 minutes in the VeraCode Vortex Incubator with the speed set at 100 rpm.
11. Prepare a second fresh solution of 50 mg/ml EDC in 0.1 M MES, pH 4.5.
12. Add 20 µl of fresh 50 mg/ml EDC.
13. Incubate at room temperature for 30 minutes in the VeraCode Vortex Incubator with the speed set at 100 rpm.
14. Centrifuge the tube to pellet the beads, and remove the supernatant.

15. Add 1.0 ml of 0.02% Tween 20 in deionized water.
16. Vortex (or centrifuge) and let the microbeads settle.
17. Remove the supernatant.
18. Repeat the Tween wash 2x (3x total).
19. Add 1.0 ml of 0.1% SDS in deionized water.
20. Vortex, and then centrifuge or let the microbeads settle.
21. Remove the supernatant.
22. Repeat the SDS wash 2x (3x total).
23. [Optional] Incubate 1 hour with 1M NaCl in water.
24. Wash 3x with EtOH.
25. Store it at -20°C in EtOH.

Two-Step Protein Immobilization to Carboxyl VeraCode Beads

Materials and Reagents

- ▶ VeraCode Vortex Incubator
- ▶ Carboxyl VeraCode beads (1 tube)
- ▶ 0.1M MES, pH 4.5
- ▶ Sulfo-NHS (freshly-made solution; 50 mg/ml in 0.1 M MES, pH 4.5; Pierce Cat # 24510)
- ▶ EDC (freshly-made solution; 50 mg/ml in 0.1 M MES, pH 4.5; Pierce Cat # 22980)
- ▶ PBS-Tween 20 (0.2%)
- ▶ PBS-BSA (1%) (Stored at 4°C)
- ▶ PBS-BSA (1%) Proclin 300 (Stored at 4°C)
- ▶ BSA (Stock solution used to make 1% PBS-BSA) Sigma BSA 98% (# A7030-10g)
- ▶ 1M NaCl in ultra-pure water

Preparation

Perform the following steps to prepare for the antibody immobilization procedure.

1. Bring the carboxyl beads to room temperature (15–30 minutes).
2. Determine the batch size for immobilization.

See Table 72 to determine the total volume based on the amount of beads.

Table 72 Antibody Immobilization, Total Volume

# VeraCode Tubes	# VeraCode Beads/Tube	Capture Antibody (µg/ml)	Total Volume (µl)
1	24667	100	300
2	49334	100	300
3	74001	100	500
4	98668	100	500
5	123335	100	1000
6	148002	100	1000



NOTE

This concentration is used as a starting point. Illumina recommends that you optimize antibody concentration based on the requirements of your assay.

3. Wash the beads 3x with 1.0 ml of 0.1 M MES, pH 4.5.
A wash consists of the following steps:
 - a. Add 1.0 ml of buffer.
 - b. Allow beads to settle for approximately 30 seconds, or pulse centrifuge.
 - c. Gently remove the buffer with a 1.0 ml pipette without disturbing the beads.

**NOTE**

You can use vacuum aspiration wash instead of manual pipetting. Be careful not to disturb the bead pellets when aspirating.

4. Make Sulfo-NHS in 0.1 M MES pH 4.5. The final concentration is 50 mg/ml. Use Table 73 as a guide.

Table 73 Dilution of Sulfo-NHS

Number of Tubes	mg Sulfo-NHS	vol 0.1M MES
1	30	600
5	150	3000
10	300	6000

5. Mix briefly by vortexing.
6. Make EDC in 0.1 M MES, pH 4.5. The final concentration is 50 mg/ml. Use Table 74 as a guide.

Table 74 Dilution of EDC

Number of Tubes	mg EDC	vol 0.1M MES
1	30	600
5	150	3000
10	300	6000

7. Incubate at room temperature for 1 hour in the VeraCode Vortex Incubator with the speed set at 100 rpm to activate the beads.

**NOTE**

Make sure that the tubes are mixing properly, especially if you are using a VariMixer or a rocker mixer at this step. Many rocker mixers do not achieve a sufficient angle to allow mixing. Vortex briefly and invert manually to facilitate mixing.

8. Pulse centrifuge the beads to ensure that they are settled at the bottom and that none remain in the cap.
9. Remove supernatant.
10. Wash 2x with 1.0 ml 0.1 M MES, pH 4.5.

Antibody Immobilization Procedure

1. Add antibody solution (100 µg/ml in 0.1 M MES, pH 4.5) to the carboxyl beads (see Table 72).
2. Vortex briefly.
3. Incubate at room temperature for 1 hour in the VeraCode Vortex Incubator with the speed set at 100 rpm. Make sure all tubes are mixing well.
4. Pulse centrifuge the beads to ensure that they are settled at the bottom and that none remain in the cap.
5. Wash 2x with PBST (0.2%).
6. Wash 2x 1M NaCL in water.
7. Incubate at room temperature for 1 hour in the VeraCode Vortex Incubator with the speed set at 100 rpm.



NOTE

This incubation removes non-specifically bound capture antibody which may occur during the immobilization procedure. If you have tested sufficiently, you may eliminate this step.

8. Wash 2x PBS-BSA (1%).
9. Incubate PBS-BSA (1%) at room temperature for 1 hour in the VeraCode Vortex Incubator with the speed set at 100 rpm.
10. Wash 2x PBS-BSA (1%).
11. Store at 4°C in PBS-BSA (1%) with ProClin.
12. Store at 4°C in 1.0 ml PBS-BSA-Proclin 300 (1%)
13. Continue to *Pool Individual Immobilized VeraCode Beads*.

Quantitation and Manual Bead Kitting

Materials and Reagents	Buffers
	<ul style="list-style-type: none">▶ 1 X Phosphate Buffered Saline (1X PBS)▶ 1 X Phosphate Buffered Saline + 1% Bovine Serum Albumin (PBS-BSA)▶ 1 X Phosphate Buffered Saline + 0.05% Tween 20 (PBST)
Equipment	<ul style="list-style-type: none">▶ Wide-orifice pipette tips for Rainin Multichannel LTS Rainin HR-250W▶ Reagent reservoirs<ul style="list-style-type: none">• 25 ml divided Matrix #8096• 50 ml Corning #4870
Manual Quantitation Procedure for Carboxyl Beads	<p>Use the following protocol for manual quantitation of carboxyl beads after immobilization of protein. The beads are counted using a conventional light or inverted microscope (<10X objective).</p> <ol style="list-style-type: none">1. Vortex bead stock(s) in 2.0 ml screw-cap tubes for 30 seconds.2. Place 1.0 ml of 1X PBST (0.05%) in duplicate tube(s).3. Using a P200 pipette with a wide orifice tip set to deliver 50 µl, place 1.0 ml of buffer in duplicate tube(s) (PBS-BSA or PBST).4. Hold the stock bead tube on an angle (~45 degrees).5. Place the pipette tip about halfway inside stock tube.6. Aspirate up and down 10x. Look for good mixing of beads.7. On the tenth time, remove 50 µl of stock beads.8. Transfer beads and buffer to 1.0 ml of buffer (PBS-1% BSA or PBST). The dilution factor is now 1:20.9. Vortex diluted beads for 30 seconds.10. Set the P200 pipette to 50 µl (200 µl wide-bore tips).11. Repeat aspiration steps 5 and 6.12. Place 50 µl on microscope slide; repeat three times (3 x 50 µl spots per slide).13. Count the beads per 50 µl spot using a light microscope.14. Calculate the average number of beads per 50 µl spot.15. To make a multiplex beadpool, pool individual beads to equal numbers per ml (i.e., approximately 20,000 of each bead type per ml).16. Calculate the total number of beads, assuming a dilution factor of 400.

Manual Kitting Procedures for Carboxyl Beads

There are two manual kitting protocols for carboxyl beads:

- ▶ *Individual Microwell Kitting Protocol*
- ▶ *Multichannel Kitting Protocol*



These methods are not as accurate as using the VeraCode Bead Kitting System.

Individual Microwell Kitting Protocol

Use this individual microwell kitting protocol to deliver beads to individual microwells using a standard pipette.

1. Place 1.2 ml of PBS-BSA in 2.0 ml screw-cap tube.



This will kit two columns or 16 wells. Repeat this process for each two columns needed.

2. Using a 200 µl wide-bore tip, add the desired volume of stock beads (calculated above) to yield 30 of each bead type per 50 µl of final volume.
3. Hold tube on an angle (~45 degrees).
4. Place pipette tip (wide-bore) about halfway inside tube.
5. Aspirate up and down 10x (look for good resuspension of beads).
6. On the tenth time, remove 50 µl of stock beads.
7. Place 50 µl of beads in a well.
8. Repeat steps **5** through **7** for each well.



Mixing is important to ensure that an equal number of beads are delivered to each well.

Multichannel Kitting Protocol

Use this multichannel kitting protocol to deliver beads to individual microwells using an 8-channel pipette.

1. Add desired volume of stock beads to yield 30 beads per 50 µl volume in 6.0 ml buffer.



This will kit six columns. Repeat this process for each six columns needed.

2. Vortex the diluted beads for 15 seconds.

3. Place the beads in a reservoir.
4. Aspirate up and down 10x.
5. On the tenth time, remove 50 µl of stock beads.
6. Place 50 µl of beads in the first column of a microtiter plate.
7. Repeat steps **4** through **6** for each column.

**NOTE**

Mixing is important to ensure that an equal number of beads are delivered to each well.

Pool Individual Immobilized VeraCode Beads

1. Spin individual tubes of immobilized VeraCode beads.
2. Set a P200 to deliver 150 µl with a wide-bore tip.
3. Remove the pellet of beads and combine it to a single tube by doing the following:
 - a. Place the tip of the pipette just above the pellet.
 - b. Aspirate up into the pipette tip so that you can see beads in the tip.
 - c. Quickly remove the tip from the tube.
 - d. Pipette beads into a multiplex tube.
 - e. Repeat 2x (or as needed to remove beads) per tube of VeraCode beads.
 - f. Visually inspect the tube to ensure that all beads have been removed.
 - g. Remove the excess liquid from the multiplex pool tube as needed to end up with 1 ml of volume.

**NOTE**

Remember to record the VeraCodes of the beads used to make up the multiplex pool.

Multiplex Cytokine Reagent Preparation

Use this protocol as a guide. However, make sure to optimize your reagents (detection antibody, streptavidin, etc.) for your specific assay requirements.

Prepare Multiplex Detection Antibody

Follow the manufacturer's recommendations to determine the initial detection antibody concentration. Once concentrations are achieved, you can prepare a 10x detection pool.

Dilute 10x detection antibody in CRD based on the table below.

Total # Wells*	μl 10x Detection Antibody	μl Reagent Diluent
96	600.0	5400.0
48	300.0	2700.0
24	150.0	1350.0
12	75.0	675.0

*Final volumes assume a 10% overage.

Prepare Streptavidin Phycoerythrin Conjugate



NOTE

Dilute Streptavidin Phycoerythrin in label buffer to a final concentration of 6.4 $\mu\text{g}/\text{ml}$. Shield the solution from light.

This is a starting concentration. You may need to optimize the concentration for your particular assay.

Total # Wells*	μl Streptavidin: Phycoerythrin (1mg/ml)	μl 1X Wash Buffer
96	38.4	5961.6
48	19.2	2980.8
24	9.6	1490.4
12	4.8	745.2

*Final volumes assume a 10% overage.

Prepare PBS

To prepare 1 L 1X PBS, pH 7.4 from 10x concentrate:

- ▶ Add 100 ml 10x PBS to 900 mls deionized water.

Multiplex Cytokine Protein Assay

Materials and Reagents

Reagents

- ▶ 10x biotinylated detection antibody pool (stored at 4°C)
- ▶ Multiplex high standard pool (stored at -80°C)
- ▶ Multiplex cytokine standard beads (stored at 4°C)
- ▶ Streptavidin Phycoerythrin (stored at 4°C; 1 mg/ml)

Buffers

- ▶ Cytokine Standards Diluent (CSD)
- ▶ Cytokine Reagent Diluent (CRD)
PBS-T-BSA (PBS pH 7.4, 0.1% BSA-Fraction V, 0.05% Tween 20, Pro-clin 300)
- ▶ Wash Buffer
PBST (PBS pH 7.4 and 0.05% Tween 20)

Preparation

- ▶ Allow CSD, CRD, and Multiplex Bead Pool to warm to room temperature (at least 15 minutes).

Prepare the Cytokine Standard Curve in Cytokine Standards Diluent

1. Thaw high multiplex standard (100 µg/ml) on ice.
2. Thaw controls on ice.
3. Prepare diluted standard curve in CSD (Figure 113).



CAUTION

Be careful not to use CRD in this step.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10,000	1111	41	Control 1								
B	10,000	370	41	Control 1								
C	10,000	370	14	Control 2								
D	3333	370	14	Control 2								
E	3333	123	14	Control 3								
F	3333	123	0	Control 3								
G	1111	123	0	0								
H	1111	41	0	0								
Samples												
Standard Curve												
	pg/ml	ul Std	ul CSD	Replication								
	10000.0	30	270	3								
	3333.3	100	200	3								
	1111.1	100	200	3								
	370.4	100	200	3								
	123.5	100	200	3								
	41.2	100	200	3								
	13.7	100	200	3								
	0.0	0	200	5								
Control 1	10000.0	50 No Seal		2								
Control 2	1000.0	50 No Seal		2								
Control 3	100.0	50 No Seal		2								

Figure 113 Multiplex Cytokine Protein Assay

4. Kit the beads. See Chapter 4 for bead kitting procedures.
5. Allow multiplex standards to equilibrate on ice until kitting is complete.

Steps

1. Add 50 μ l of diluted cytokine standards in CSD to the wells.
2. Add 50 μ l of controls to the wells.
3. Add 50 μ l CSD to the wells designated "0" controls.
4. Seal the plate with a mylar seal.
5. Incubate the plate on the plate rocker for 1 hour at room temperature with the speed set at 600 rpm.
6. Prepare the multiplex detection antibody pool according to the instructions in *Prepare Multiplex Detection Antibody* on page 288.
7. Mix by rocking on the VariMixer at medium speed at room temperature.
8. Remove the cytokine plate from the plate rocker.
9. Centrifuge the plate at 1500 rpm for 5 seconds.
10. Remove the mylar seal.



CAUTION

Be careful not to cross-contaminate the wells.

11. Add 150 μ l of wash buffer to the wells.
12. Centrifuge the plate at 1500 rpm for 5 seconds.
13. Aspirate the wells with the 8-channel aspirator.
14. Wash 3x with wash buffer (for a total of 4 additions of wash buffer).
15. After the washes, aspirate the wash buffer from the plate.
16. Add 50 μ l of 1X detection antibody in CRD to the wells.

**NOTE**

Note that the antibody is diluted in CRD, not CSD.

- 17.** Incubate the plate for 1 hour at room temperature on the plate rocker with the speed set at 600 rpm.
- 18.** Prepare the Streptavidin Phycoerythrin as described in *Prepare Streptavidin Phycoerythrin Conjugate* on page 288.
- 19.** Mix the Streptavidin Phycoerythrin by rocking it at room temperature until it is dissolved, shielding the solution from light.

**NOTE**

It is usually sufficient to rock the Streptavidin Phycoerythrin for the balance of the 60 minute incubation above.

- 20.** Add 150 µl of wash buffer to the wells.
- 21.** Centrifuge the plate at 1500 rpm for 5 seconds.
- 22.** Aspirate wells with 8-channel aspirator.
- 23.** Wash 3x with wash buffer (for a total of 4 additions of wash buffer).
- 24.** Add 50 µl of Streptavidin Phycoerythrin (6.4 µg/ml in PBST).
- 25.** Cover the plate with foil or otherwise shield it from light.
- 26.** Incubate the plate for 30 minutes at room temperature on the plate rocker with the speed set to 600 rpm.
- 27.** Add 150 µl of wash buffer to the wells.
- 28.** Centrifuge the plate at 1500 rpm for 5 seconds.
- 29.** Aspirate wells with 8-channel aspirator.
- 30.** Wash 3x with wash buffer (for a total of 4 additions of wash buffer).
- 31.** After the final wash, aspirate the wash buffer (PBST) from the beads.
- 32.** Resuspend the beads in 75 µl of wash buffer.
- 33.** Place the plate in the BeadXpress Reader.
- 34.** Scan the plate using the Scan Settings File supplied by Illumina (set at 0.75 green PMT, which is the recommended starting point).

For information about scanning VeraCode Bead Plates with the BeadXpress Reader, see the *BeadXpress Reader System Manual*.

Troubleshooting

Use the following troubleshooting guidelines to help you successfully resolve potential issues you may face when performing the assays in this chapter.

High Background

Table 75 High Background

Possible Cause	Solution
Insufficient washing	Increase number or volume of washes
Insufficient blocking	Increase blocking time during the immobilization step Recheck blocking buffer calculations
Incubation times too long	Reduce incubation times
Buffer contamination, interfering substances	Make fresh buffers Run additional controls
Label concentration too high	Check dilution; titrate label concentration
Detection antibody concentration too high	Check dilution; titrate detection antibody concentration

No Signal

Table 76 No Signal

Possible Cause	Solution
	Repeat assay
Reagent preparation incorrect or incorrect order	Review protocol Check calculations and make new reagents
Suspected performance issues	Run T&C beads
Standard has gone bad: signal seen in sample (unknown wells)	Use new standard Use proper handling of standard
Not enough secondary antibody used	Check calculations and titer concentration
Not enough label used	Check calculations and titer concentration
Capture antibody did not bind to VeraCode beads	Run labeled anti-species assay

Table 76 No Signal (Continued)

Possible Cause	Solution
Buffer contamination	Make fresh buffers

Too Much Signal

Table 77 Too Much Signal

Possible Cause	Solution
Insufficient washing or wash step skipped	Review protocol
	Repeat assay
Label concentration too high	Check dilution; titrate label concentration
Buffer contamination	Make fresh buffers

Low or Flat Standard Curve

Table 78 Low or Flat Standard Curve

Possible Cause	Solution
Label concentration limiting	Increase label concentration
Incorrect procedure	Review assay guide and repeat
Detection antibody limiting	Check dilution; titer concentration
Standards are bad	Insure proper handling; repeat assay with new standards
Capture antibody did not bind to VeraCode beads	Run labeled anti-species assay; titer antibody during immobilization

Poor Replicates

Table 79 Poor Replicates

Possible Cause	Solution
Insufficient washing	Review assay guide
	Increase number and volume of washes
Insufficient mixing	Vary mixing speeds
Buffer contamination	Make fresh buffers

Table 79 Poor Replicates (Continued)

Possible Cause	Solution
Capture antibody did not bind to VeraCode beads or uneven coating	Run labeled anti-species assay; titer antibody during immobilization
	Ensure proper mixing during immobilization
Reagent evaporation	Make sure plate is sealed properly during incubation

Poor Reproducibility (Assay-to-Assay)

Table 80 Poor Reproducibility

Possible Cause	Solution
Insufficient washing	Review assay guide
	Increase number and volume of washes
Variations in protocol	Use same protocol run to run
Improper dilutions	Check dilutions
Buffer contamination	Make new dilutions
	Make fresh buffers

No Signal in Samples, Standard Curve Fine

Table 81 No Signal in Samples, Standard Curve Fine

Possible Cause	Solution
No cytokine in sample	Use internal controls
Sample matrix interference	Repeat experiment
	Dilute samples (1:2) in appropriate diluent

Sample Values too High, Standard Curve Fine

Table 82 Sample Values too High, Standard Curve Fine

Possible Cause	Solution
Samples contain high levels of cytokine	Dilute samples and re-run

Appendix A

GoldenGate Genotyping Assay for VeraCode Controls

Topics

- 296 Introduction
- 296 View the Control Graphs
- 297 VeraCode Bead Types and IllumiCode Sequence IDs
- 298 Allele-Specific Extension Controls
- 299 PCR Uniformity
- 300 Gender Controls
- 302 Extension Gap Control (U3 & U5 Match)
- 303 Second Hybridization Controls
- 304 Contamination Detection Controls

Introduction

This appendix describes the GoldenGate control oligos, including the VeraCode Sequence IDs used, and their expected outcomes and how to view them. The control oligos include:

- ▶ Allele-specific extension controls
- ▶ PCR uniformity controls
- ▶ Extension gap controls
- ▶ Gender controls
- ▶ First hybridization controls
- ▶ Second hybridization controls
- ▶ Contamination detection controls

These control oligos (with the exception of second hybridization controls) are designed to human genomic DNA sequences.

View the Control Graphs

To view control graphs using the GenomeStudio Genotyping Module, select **Analysis | View Controls Dashboard**.

For more information about control graphs, see the *GenomeStudio Genotyping Module User Guide*.

VeraCode Bead Types and IllumiCode Sequence IDs

Table 83 lists the VeraCode Bead Types and IllumiCode Sequence IDs that were used as controls, along with a description and expected outcome for each.

Table 83 VeraCode Bead Types and IllumiCode Sequence IDs

VeraCode Bead Type	IllumiCode Sequence ID	Description	Expected Outcome
0010	329	AA mismatch	U3 match
0520	1611	CC mismatch	U5 match
0257	1142	GG mismatch	U3 match
0008	279	GT mismatch	U5 match
1025	1742	High AT (31% GC)	U3 match
4352	4824	High GC (62% GC)	U5 match
1028	1878	Gender control set 1	XX = U3 match XY = U3 and U5 match
2048	2911	Gender control set 2	XX = U3 match XY = U3 and U5 match
0034	658	15-base-pair gap	U3 and U5 match
0128	962	First hybridization controls, 42/57 T _m	U5 match
0260	1209	First hybridization controls, 57/72 T _m	U5 match
0001	44	Second hybridization controls	U3 match
0005	278	Second hybridization controls	U3 match
0256	1112	Second hybridization controls	U5 match
0544	1632	Second hybridization controls	U5 match
0016	501	Second hybridization controls	U3 and U5 match
0136	0003	Second hybridization controls	U3 and U5 match

Allele-Specific Extension Controls

The allele-specific extension controls test the extension efficiency of properly matched versus mismatched allele-specific oligos (ASO). These controls test for A-A, C-C, G-G, and G-T mismatches corresponding with VeraCode Sequence IDs 0010, 0520, 0257, and 0008, respectively. Sequence IDs 0010 and 0257 should be predominately Cy3, and Sequence IDs 0008 and 0520 should be predominately Cy5.

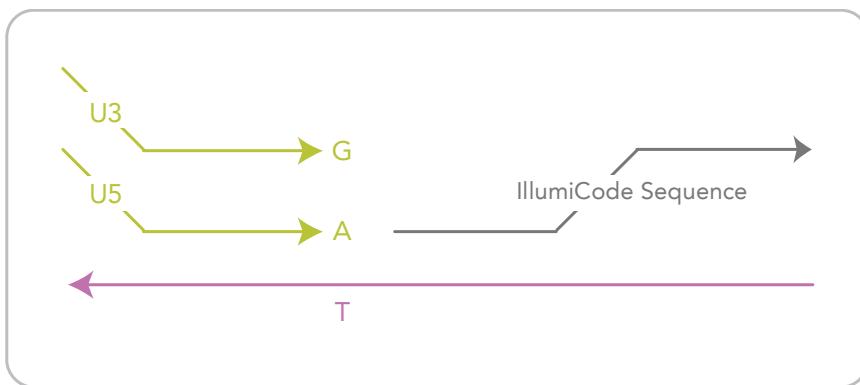


Figure 114 ASE Controls

PCR Uniformity

The PCR uniformity controls are used to test the PCR amplification efficiency for high AT and high GC regions of DNA. VeraCode Sequence ID 1025 checks amplification efficiency for high AT (31% GC) and should result in Cy3 signal. VeraCode Sequence ID 4352 amplifies over a high GC (62% GC) region and should result in Cy5 signal.

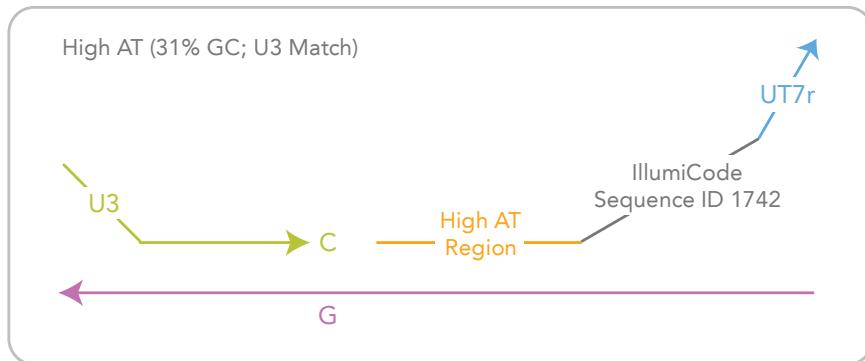


Figure 115 PCR Uniformity Controls (U3)

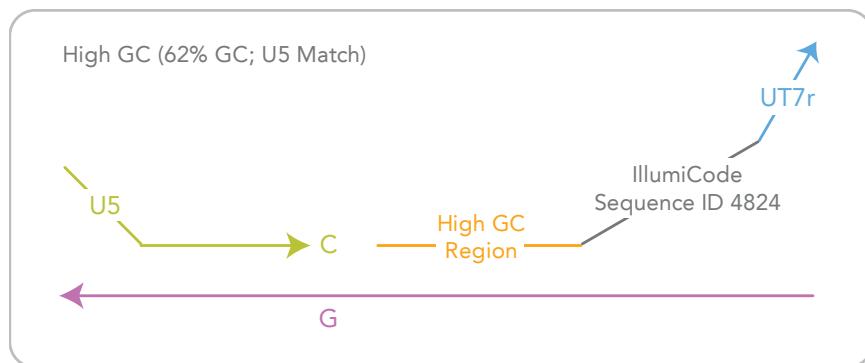


Figure 116 PCR Uniformity Controls (U5)

Gender Controls

VeraCode Sequence IDs 1028 and 2048 are used to verify the sex of DNA samples. For both VeraCode Sequence IDs, females are indicated by Cy3 homozygotes and males as heterozygotes.

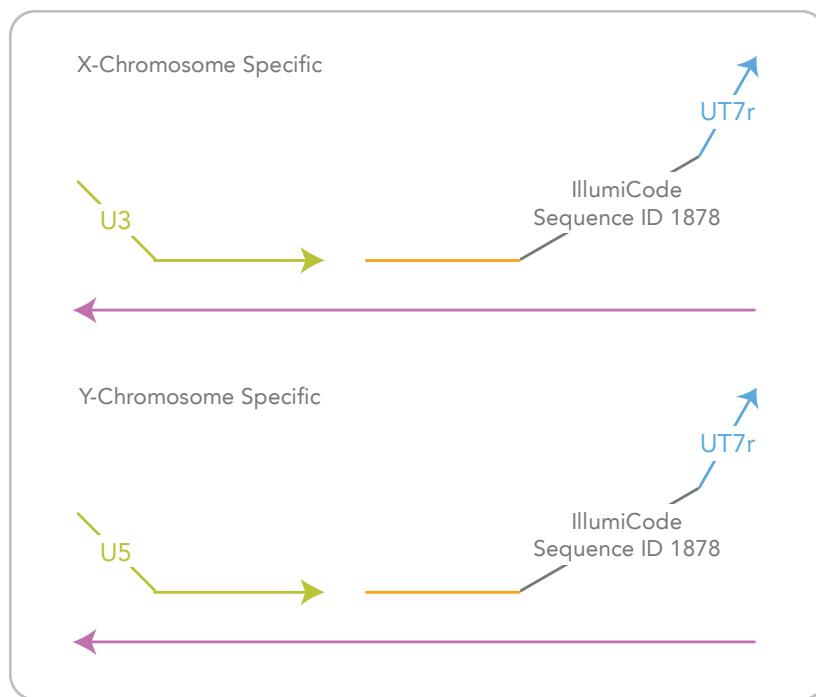


Figure 117 Gender Controls (Set 1)

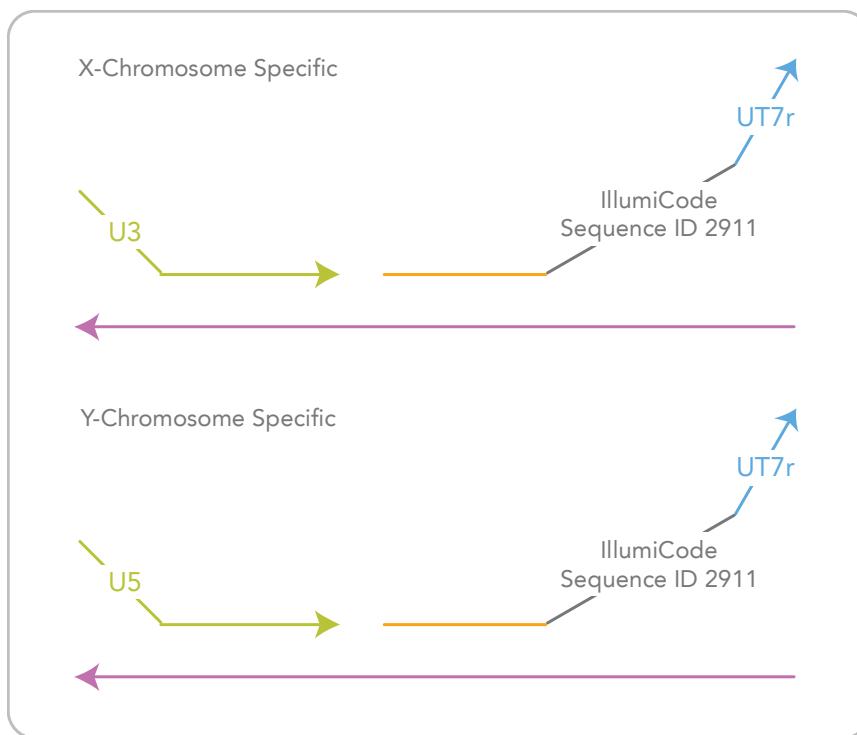


Figure 118 Gender Controls (Set 2)

Extension Gap Control (U3 & U5 Match)

The extension gap control (VeraCode Sequence ID 0034) tests the efficiency of extending 15 bases from the 3' end of the allele-specific oligo to the 5' end of the locus-specific oligo. Both Cy3 and Cy5 signal should be detected in this control.



Figure 119 Extension Gap Control (U3 & U5 Match)

First Hybridization Controls

The first hybridization controls test the specificity of annealing ASOs with different T_m to the same DNA locus. Both VeraCode Sequence ID 0128 and 0260 should result in a Cy5 match.

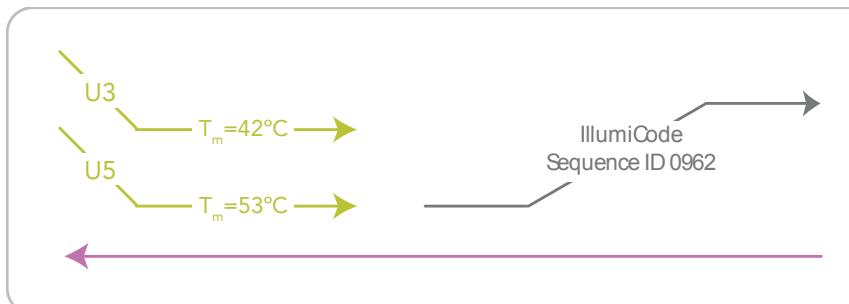


Figure 120 First Hybridization Controls

Second Hybridization Controls

The second hybridization controls test the hybridization of single-stranded assay products to VeraCode Sequences on the array beads. VeraCode Sequence IDs 0001 and 0005 should result in Cy3 signal only, Sequence IDs 0256 and 0544 should result in only Cy5 signal, and Sequence IDs 0016 and 0136 should not have signal contributed by either Cy3 or Cy5.

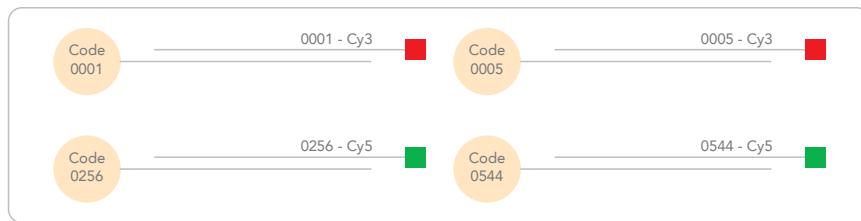


Figure 121 Second Hybridization Controls

Contamination Detection Controls

The PCR contamination detection controls are divided into four types; only a single type is added to each oligo pool (OPA) tube. When a single OPA is run, only one contamination control type should have high signal. If two or more contamination control types have high signal, then significant contamination may have occurred.

Figures 122 through 124 provide graphic representations of these controls under three BeadStation process conditions:

- ▶ Figure 122 represents a contamination-free environment
- ▶ Figure 123 represents a contaminated environment *without* UDG treatment
- ▶ Figure 124 represents a contaminated environment *with* UDG treatment

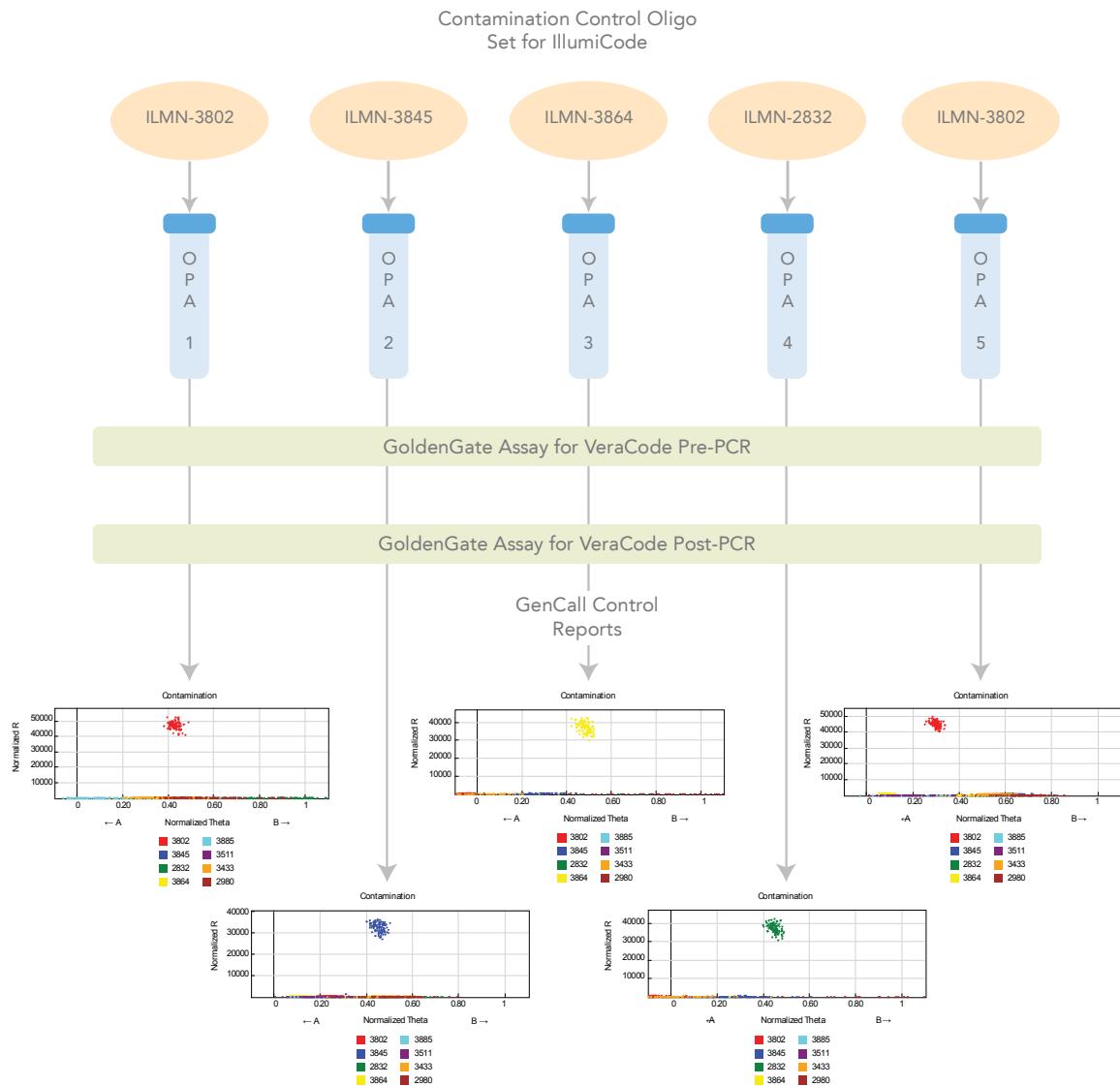


Figure 122 Contamination-Free Environment

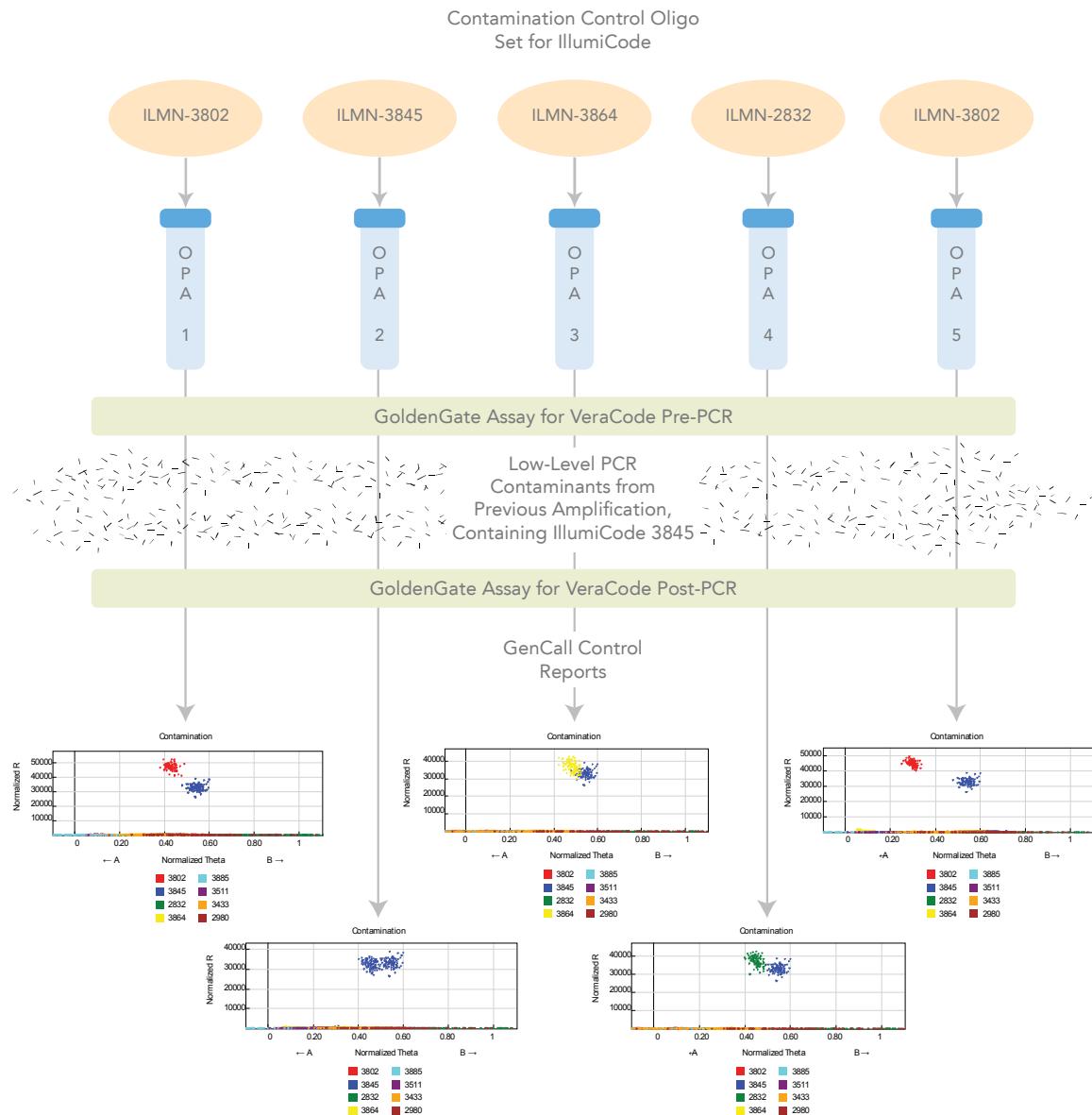


Figure 123 Contaminated Environment without UDG Treatment

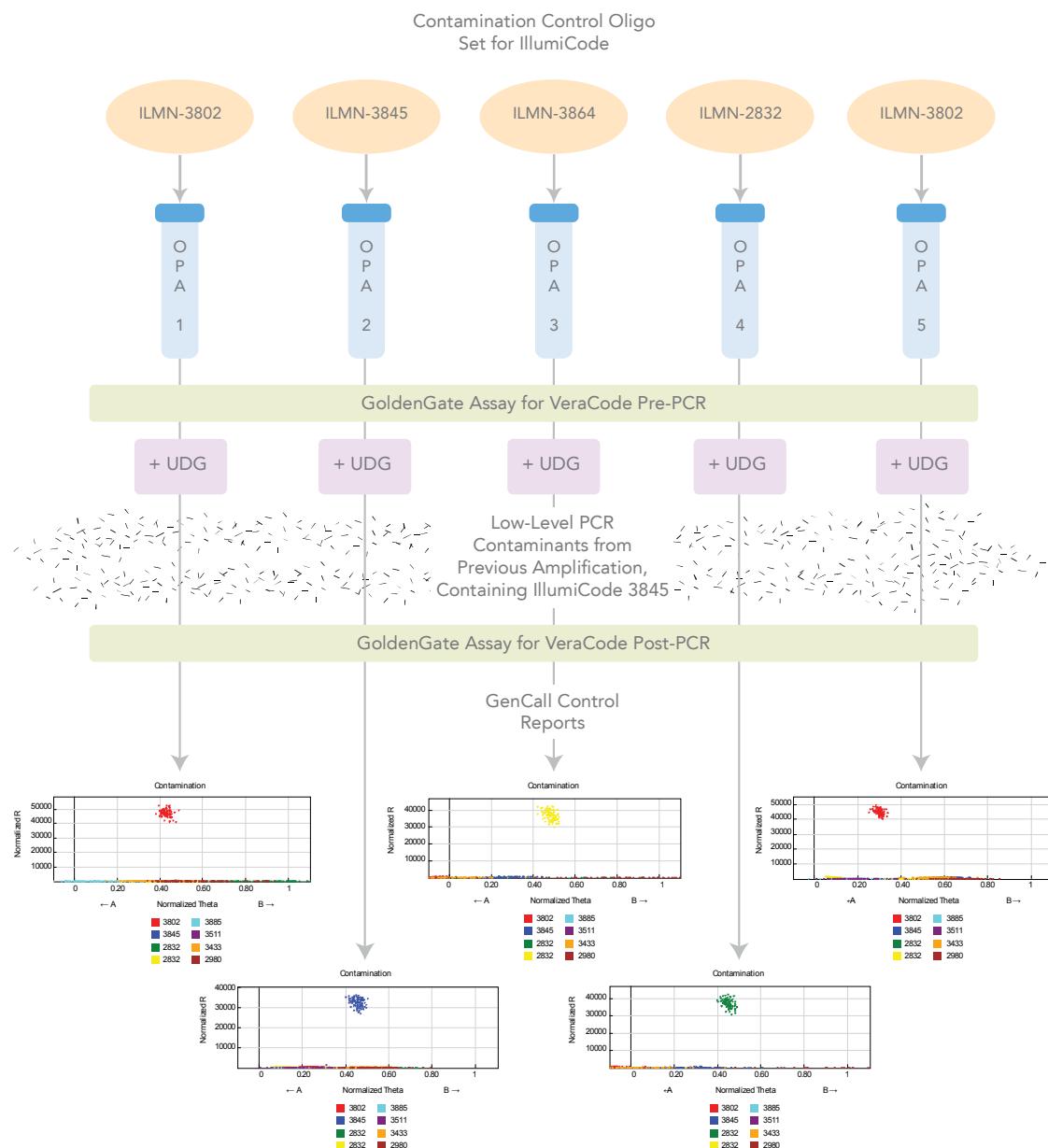


Figure 124 Contaminated Environment with UDG Treatment

Appendix B

DASL Gene Expression Assay for VeraCode Controls

Topics

- 308 Introduction
- 308 View the Control Graphs
- 309 Control IllumiCode Sequence IDs
- 310 Negative Controls
- 311 Extension Gap Control
- 311 First Hybridization Controls
- 312 Second Hybridization Controls
- 313 Contamination Detection Controls

Introduction

This appendix describes the control oligos, including the used IllumiCode Sequence IDs, their expected outcomes, and how to view them. The control oligos include:

- ▶ Negative controls
- ▶ Extension gap controls
- ▶ First hybridization controls
- ▶ Second hybridization controls
- ▶ Contamination detection controls

These control oligos (with the exception of negative controls and second hybridization controls) detect different target sites of the human QARS (glutaminyl-tRNA-synthetase) gene. The intensities on these IllumiCodes depend on the endogenous expression of the QARS gene.

View the Control Graphs

To view control graphs using the GenomeStudio Gene Expression Module, select **Analysis | View Controls Dashboard**.

For more information about control graphs, see the *GenomeStudio Gene Expression Module User Guide*.

Control IllumiCode Sequence IDs

Table 84 lists the IllumiCode Sequence IDs that were used as controls, along with a description and the expected outcome of each.

Table 84 Control IllumiCode Sequence IDs

IllumiCode Sequence ID	Description	Expected Outcome
IllumiCode0858	20-base gap control	Moderate signal
IllumiCode0962	First hybridization control, 42/53 T _m	Cy5 signal greater than Cy3 signal
IllumiCode1209	First hybridization control 58/72 T _m	Cy3 signal greater than Cy5 signal
IllumiCode0044	Second hybridization control	Signal in Cy3
IllumiCode0278	Second hybridization control	Signal in Cy3
IllumiCode0501	Second hybridization control	Signal in both Cy3 and Cy5
IllumiCode1003	Second hybridization control	Signal in both Cy3 and Cy5
IllumiCode1112	Second hybridization control	Signal in Cy5
IllumiCode1632	Second hybridization control	Signal in Cy5
IllumiCode1968	DASL contamination control	Signal in one of these
IllumiCode1992	DASL contamination control	
IllumiCode2980	DASL contamination control	
IllumiCode3433	DASL contamination control	
IllumiCode2832	GGGT contamination control	Very low signal
IllumiCode3802	GGGT contamination control	Very low signal
IllumiCode3845	GGGT contamination control	Very low signal
IllumiCode3864	GGGT contamination control	Very low signal

Negative Controls

This category consists of query oligos targeting 27 random sequences that do not appear in the human genome. The mean signal of these probes defines the system background. This is a comprehensive measurement of background, representing the imaging system background as well as any signal resulting from non-specific binding of dye or cross-hybridization. The GenomeStudio application uses the signals and signal standard deviation of these probes to establish gene expression detection limits.

Table 85 Negative Control IllumiCodes

IllumiCode Sequence ID	IllumiCode Sequence ID	IllumiCode Sequence ID
IllumiCode3008	IllumiCode0662	IllumiCode2911
IllumiCode1251	IllumiCode4842	IllumiCode3137
IllumiCode1878	IllumiCode1235	IllumiCode1611
IllumiCode3885	IllumiCode0590	IllumiCode0975
IllumiCode3511	IllumiCode1696	IllumiCode1365
IllumiCode1716	IllumiCode1306	IllumiCode0279
IllumiCode0526	IllumiCode0329	IllumiCode0137
IllumiCode1742	IllumiCode0910	IllumiCode1070
IllumiCode0658	IllumiCode4254	IllumiCode1142

Extension Gap Control

The extension gap controls test the efficiency of extension from the 3' end of the ASO to the 5' end of the LSO. This control should show moderate signal consistent with QARS gene expression.



Figure 125 Extension Gap Control

First Hybridization Controls

The first hybridization controls test the specificity of annealing ASOs with different T_m 's to the same cDNA target. In each case, the higher T_m ASO should give higher signals than the lower T_m ASO. For Illumicode 0962, the Cy5 signal should exceed the Cy3 signal. For Illumicode 1209, the Cy3 signal should be greater than the Cy5 signal.

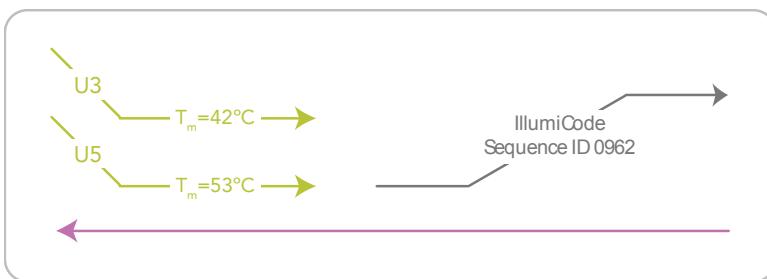


Figure 126 First Hybridization Controls (Low T_m)

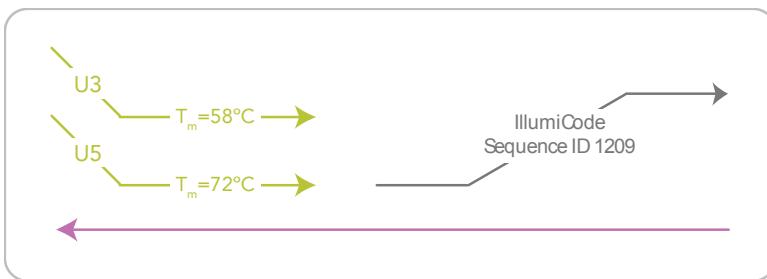


Figure 127 First Hybridization Controls (High T_m)

Second Hybridization Controls

The second hybridization controls test the hybridization of single-stranded assay products to IllumiCode Sequences on the array beads. IllumiCode Sequence IDs 44 and 278 should result in Cy3 signal only, Sequence IDs 1112 and 1632 should result in only Cy5 signal, and Sequence IDs 501 and 1003 should have signal contributed by both Cy3 and Cy5. The controls consist of 25-mer oligos labeled with the Cy3 or Cy5 dyes included in the MH2 reagent.

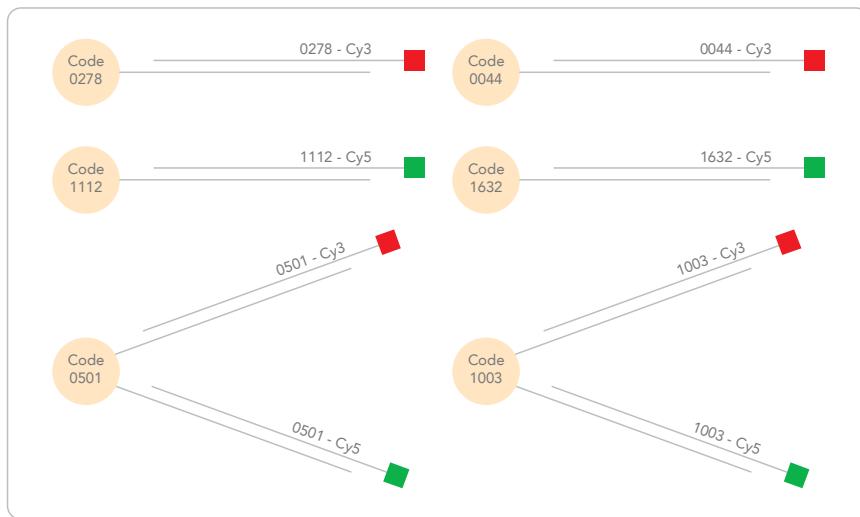


Figure 128 Second Hybridization Controls

Contamination Detection Controls

These PCR contamination detection controls are divided into four types, and only a single type is added to each oligo pool (DAP) tube. When a single DAP is run, only one contamination control type should have high signal. If two or more contamination control types have high signal, then significant contamination may have occurred.

Because the contamination control oligos target expression of the QARS gene, the signal from these oligos depends on QARS expression. If the samples show low expression of QARS, then the signal from these IllumiCodes may be low. In contrast, a genomic DNA sample typically yields moderate signals. Fluorescence signals that are high in all RNA samples suggest significant PCR amplicon contamination, unlike the variable pattern expected for QARS expression among diverse RNA samples.

Figures 129 through 131 provide graphic representations of these controls under three BeadStation process conditions:

- ▶ Figure 129 represents a contamination-free environment
- ▶ Figure 130 represents a contaminated environment *without* UDG treatment
- ▶ Figure 131 represents a contaminated environment *with* UDG treatment

The BeadStation Control Summary Graph for contamination controls includes intensities for the four DASL Assay contamination controls as well as the four GoldenGate Genotyping Assay contamination controls.

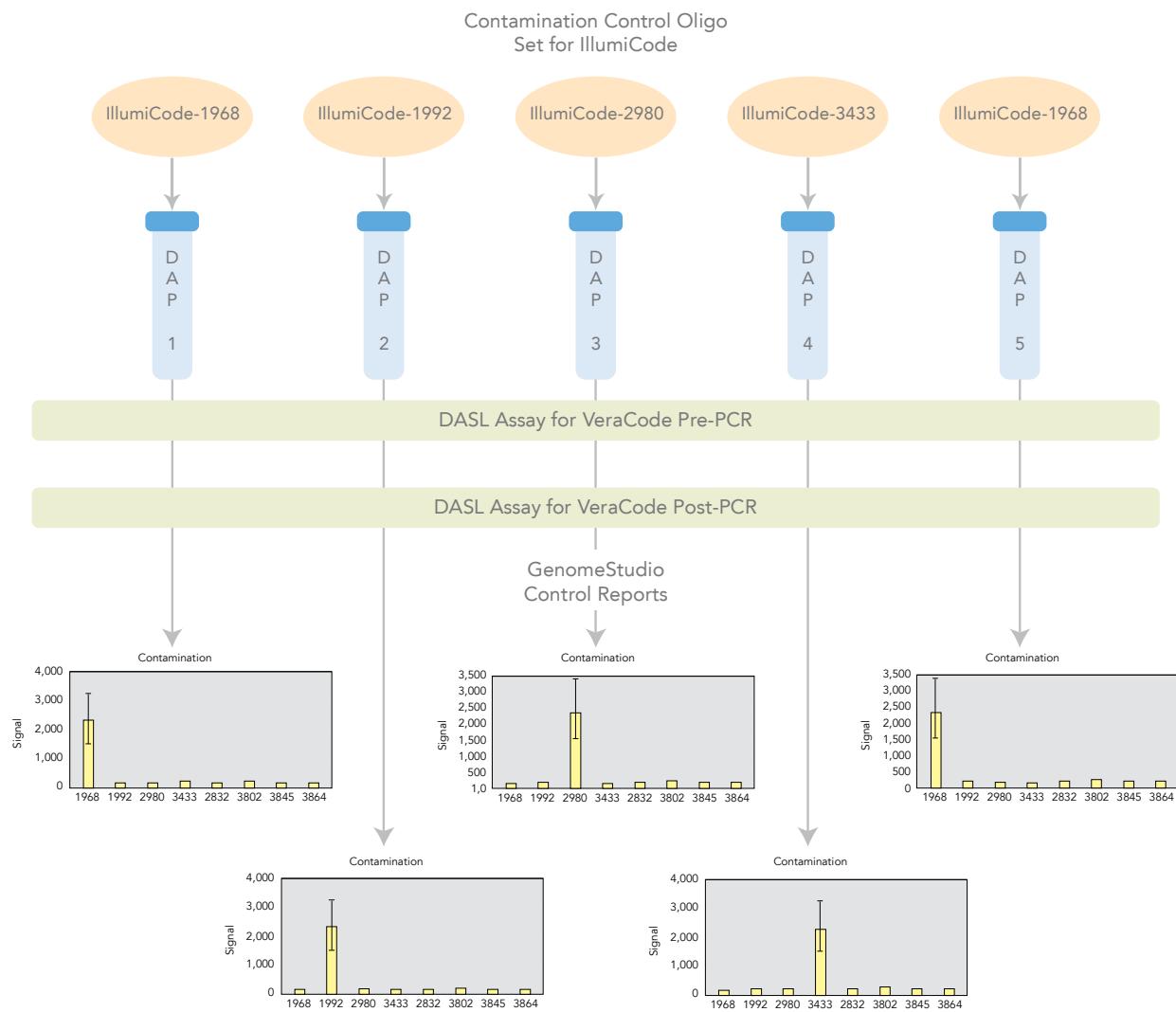


Figure 129 Contamination-Free Environment

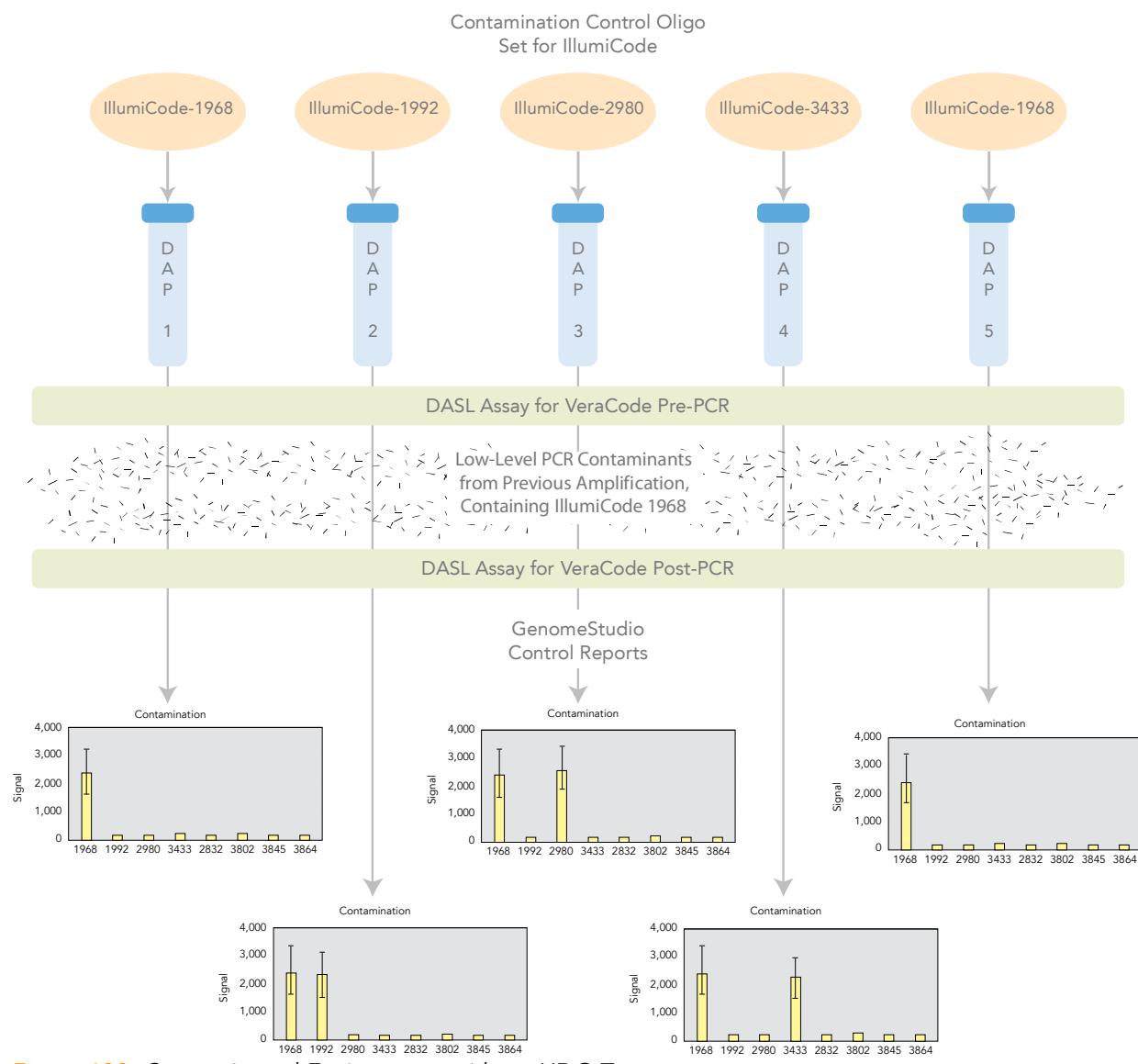


Figure 130 Contaminated Environment without UDG Treatment

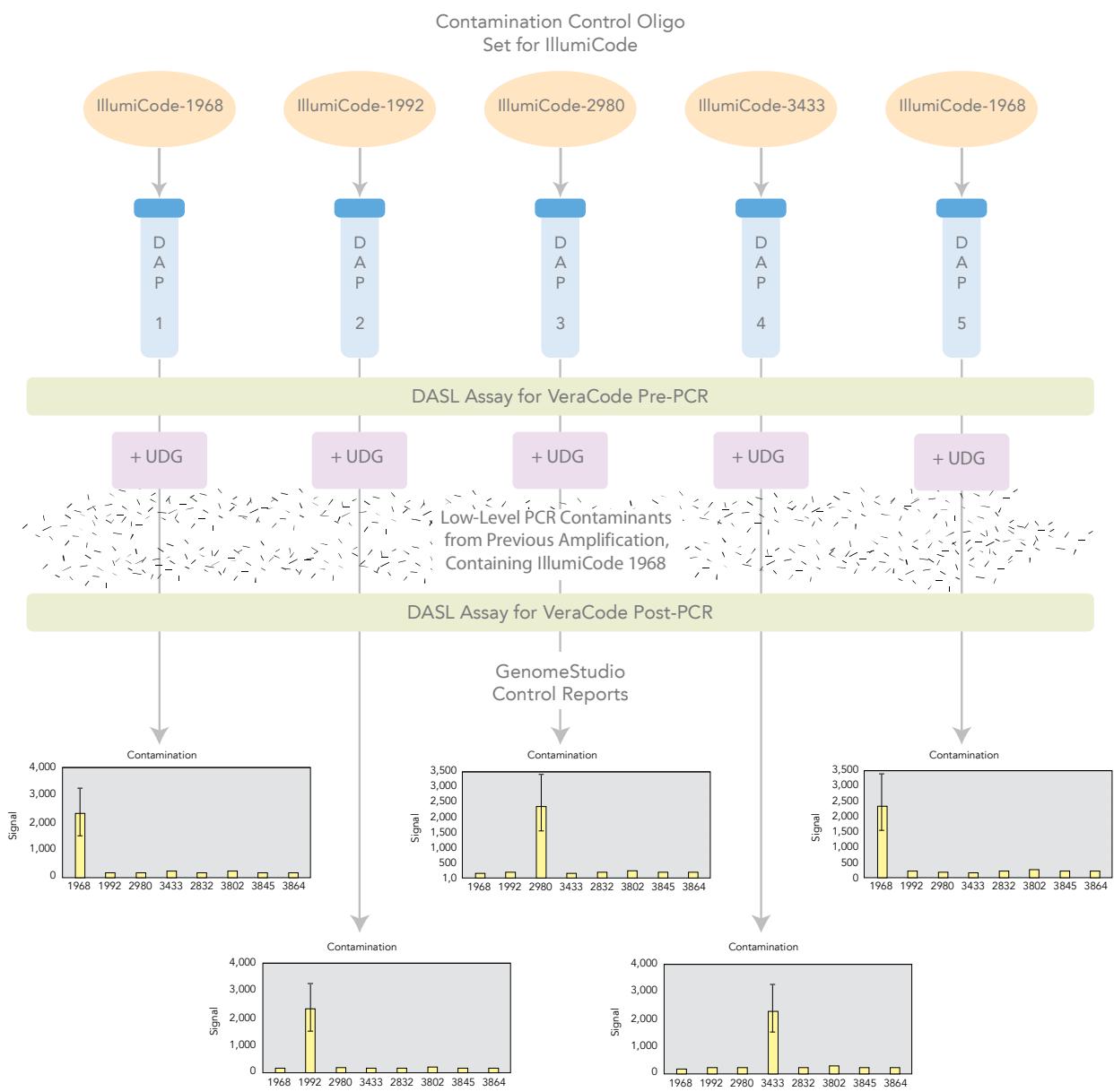


Figure 131 Contaminated Environment with UDG Treatment

Appendix C

GoldenGate Methylation Assay for VeraCode Controls

Topics

- 318 Introduction
- 318 View Control Oligos
- 319 Control IllumiCode Sequence IDs
- 320 Allele-Specific Extension Controls
- 321 Bisulfite-Conversion Controls
- 322 Gender Controls
- 323 Extension Gap Control (U3 and U5 Match)
- 324 First Hybridization Controls
- 325 Second Hybridization Controls
- 326 Negative Controls
- 327 Contamination Detection Controls

Introduction

This appendix explains how to view and interpret the control oligos for the assay for methylation, including the used IllumiCode Sequence IDs and their expected outcomes. The control oligos include:

- ▶ Allele-specific extension
- ▶ Bisulfite conversion controls
- ▶ Extension gap
- ▶ Gender
- ▶ First hybridization controls
- ▶ Second hybridization controls
- ▶ Negative controls
- ▶ Contamination detection controls

These control oligos, with the exception of second hybridization controls, are designed for bisulfite-converted human genomic DNA sequences. The performance of the control oligos (except for gender controls) does not depend on the methylation status of the template DNA, because target sequences do not contain CpG dinucleotides.

View Control Oligos

To view the control oligos, create a new project in the GenomeStudio Methylation module as described in its user guide. Load the intensity files, and then load an OMA manifest for the assays used on the SAM. You can see an overview of the control oligo performance in the Control Summary, or view details about the performance of the individual control oligos in the Control Probe Profile table.

Control IllumiCode Sequence IDs

Table 86 lists the IllumiCode Sequence IDs that were used as controls, along with a description and the expected outcome of each.

Table 86 Used IllumiCode Sequence IDs

IllumiCode Sequence ID	Description	Expected outcome
IllumiCode0279	GT mismatch	Signal in Cy3
IllumiCode0329	GT mismatch	Signal in Cy3
IllumiCode1142	GT mismatch	Signal in Cy3
IllumiCode0658	15 base-pair extension gap	Signal in both Cy3 and Cy5
IllumiCode0962	First hybridization 58/46 T _m	Signal in Cy3 greater than signal in Cy5
IllumiCode1209	First hybridization 58/71 T _m	Signal in Cy5 greater than signal in Cy3
IllumiCode1878	Gender control 1	Cy3 signal in Male, Cy3 and Cy5 signal in Female
IllumiCode2911	Gender control 2	Cy3 signal in Male, Cy3 and Cy5 signal in Female
IllumiCode1968	Contamination control	Signal in one of these
IllumiCode1992	Contamination control	
IllumiCode2980	Contamination control	
IllumiCode3433	Contamination control	
IllumiCode0044	Second hybridization	Signal in Cy3
IllumiCode0278	Second hybridization	Signal in Cy3
IllumiCode0501	Second hybridization	Signal in both Cy3 and Cy5
IllumiCode1003	Second hybridization	Signal in both Cy3 and Cy5
IllumiCode1112	Second hybridization	Signal in Cy5
IllumiCode1632	Second hybridization	Signal in Cy5
IllumiCode0858	Bisulfite conversion	Signal in Cy3
IllumiCode1742	Bisulfite conversion	Signal in Cy3

Allele-Specific Extension Controls

The allele-specific extension controls test the extension efficiency of properly matched versus mismatched allele-specific oligos (ASOs). These controls test for G-T mismatches that correspond with IllumiCode Sequence IDs 0279, 0329, and 1142. All three controls should have a predominantly Cy3 signal.

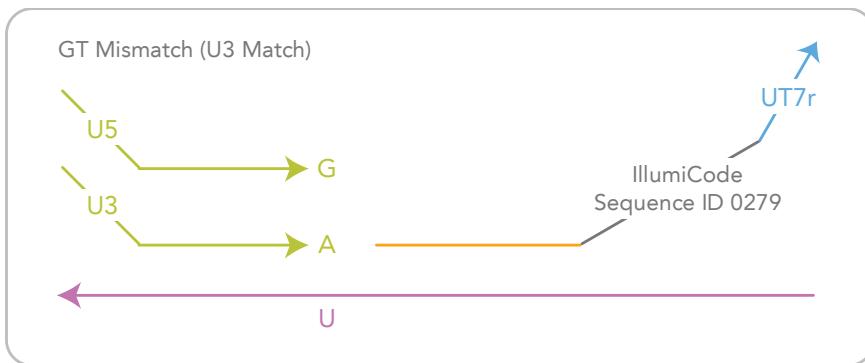


Figure 132 Allele-Specific Extension Controls

In the assay for methylation, the methylation status of a particular cytosine is carried out following bisulfite treatment of DNA by using a genotyping assay for a C/T polymorphism.

In assay oligo design, the A/T match corresponds to the unmethylated status of the interrogated C, and G/C match corresponds to the methylated status of C. G/T mismatch controls check for non-specific detection of methylation signal over unmethylated background. Controls 0279 and 0329 are designed against non-polymorphic T sites. Control 1142 is designed against a C, which should be converted into a U after bisulfite treatment.

Bisulfite-Conversion Controls

The bisulfite conversion controls test the efficiency of bisulfite conversion by checking for the presence of unconverted genomic DNA in the assay.

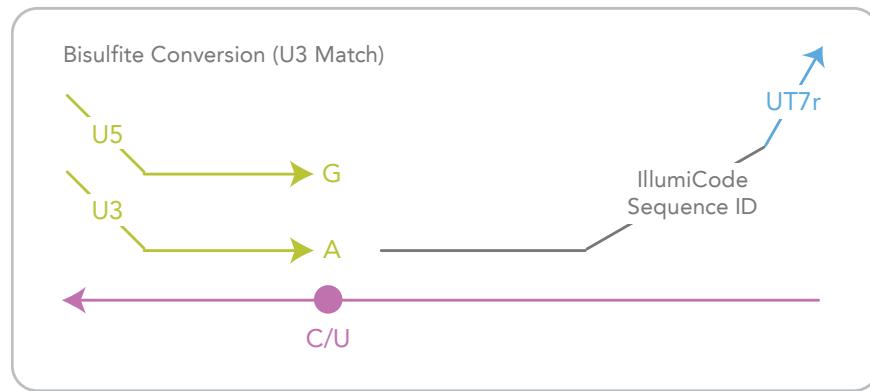


Figure 133 Bisulfite Conversion Controls

These controls correspond with IllumiCode sequence IDs 0858 and 1742. Primers are designed for the same DNA locus, with one pair targeting converted and other pair targeting unconverted DNA sequences. Both controls should have a predominantly Cy3 signal. Cy5 signal indicates the presence of unconverted DNA in the assay sample.

Gender Controls

IllumiCode Sequence IDs 1878 and 2911 verify the sex of DNA samples. DNA methylation is involved in dosage compensation of functional X-linked genes between male and female. This often results in semi-methylated status of CpG loci in the promoter regions of X-linked housekeeping genes in females, while in males these loci are unmethylated.

Methylation gender controls are designed against X-linked genes G6PD and ELK1. Cy3 and Cy5 signal should be detected in females, and only Cy3 signal should be detected in males.

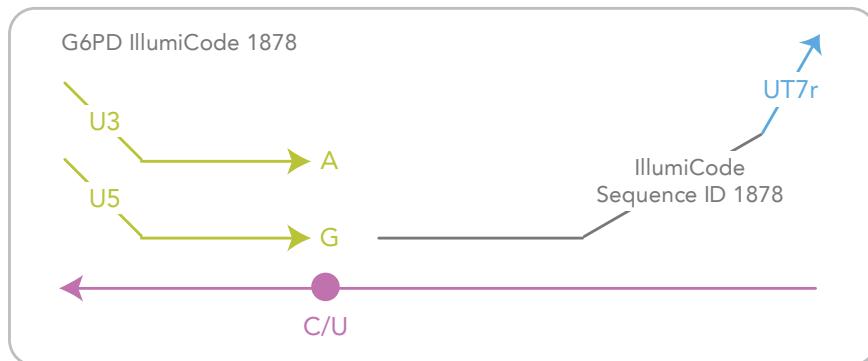


Figure 134 Gender Controls Oligo Configuration (G6PD)

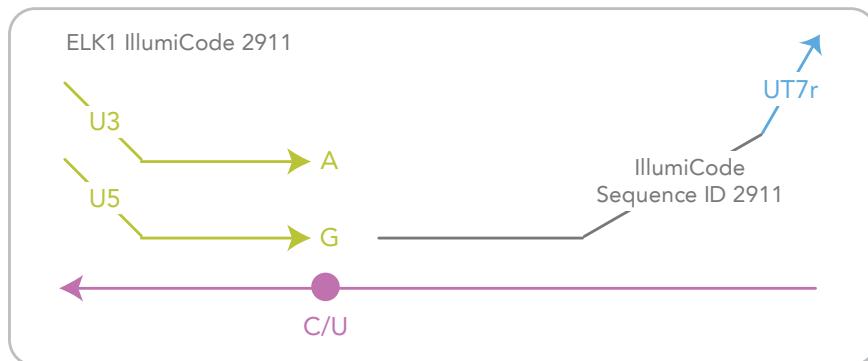


Figure 135 Gender Controls Oligo Configuration (ELK1)

In normal males, control loci should be unmethylated (Cy3 signal). In normal females, control loci should be semi-methylated (Cy3 and Cy5 signal).

Gender controls may not appear properly in samples with aberrant methylation, such as cancer cell lines.

Extension Gap Control (U3 and U5 Match)

The extension gap control (IllumiCode Sequence ID 0658) tests the efficiency of extending 15 bases from the 3' end of the allele-specific oligo to the 5' end of the locus-specific oligo. Both Cy3 and Cy5 signal should be detected in this control.

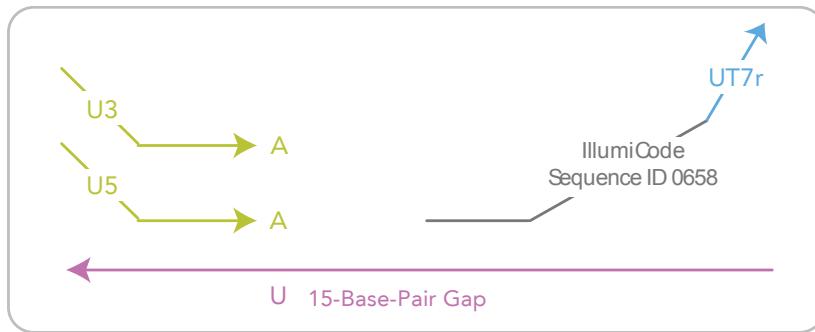


Figure 136 Extension Gap Control Oligo Configuration

First Hybridization Controls

The first hybridization controls test the specificity of annealing ASOs with different T_m to the same non-polymorphic DNA locus. IllumiCode Sequence ID 0962 should result in Cy3 signal, and 1209 should result in a Cy5 signal.

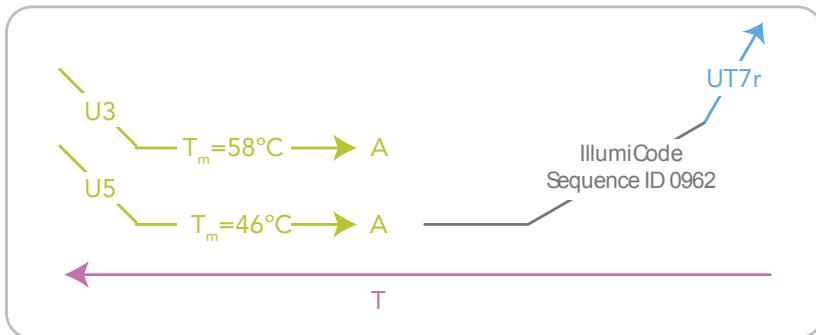


Figure 137 First Hybridization (T_m) Controls Oligo Configuration

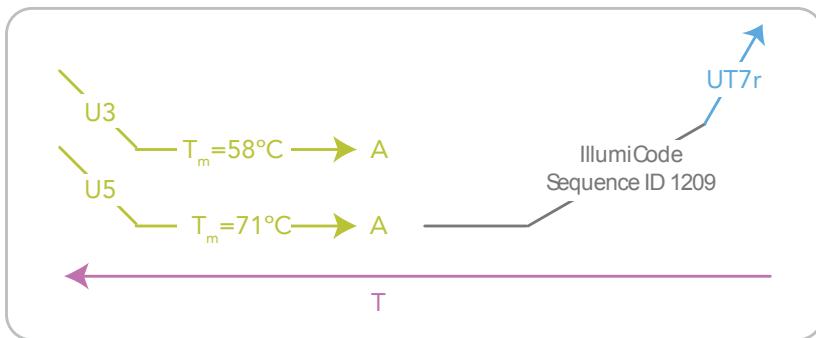


Figure 138 First Hybridization (T_m) Controls Oligo Configuration

Second Hybridization Controls

The second hybridization controls test the hybridization of single-stranded assay products to IllumiCode Sequences on the array beads. IllumiCode Sequence IDs 044 and 0278 should result in Cy3 signal only, Sequence IDs 1112 and 1632 should result in only Cy5 signal, and Sequence IDs 0501 and 1003 should have signal contributed by both Cy3 and Cy5.

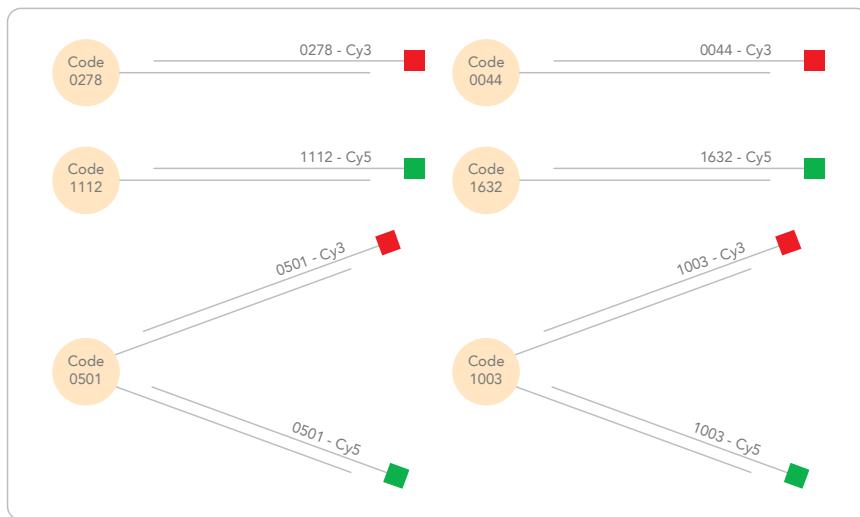


Figure 139 Second Hybridization Controls Oligo Configuration

Negative Controls

Negative controls target 22 bisulfite-converted sequences that do not contain CpG dinucleotides. LSO probes can hybridize to bisulfite-converted DNA. ASO sequences are randomly permuted and should not hybridize to the DNA template. As a result, an amplifiable target should not be formed, and the signal from these IllumiCodes should be low.

Negative controls are particularly important for methylation studies because of a decrease in sequence complexity after bisulfite treatment. The mean signal of these probes defines the system background. This is a comprehensive measurement of background, including signal resulting from cross-hybridization, as well as non-specific binding of dye and imaging system background. The GenomeStudio application uses the signals and signal standard deviation of these probes to establish detection limits for the methylation probes.

Table 87 Negative Controls IllumiCodes

IllumiCode Sequence ID	IllumiCode Sequence ID
IllumiCode0137	IllumiCode1251
IllumiCode0526	IllumiCode1306
IllumiCode0590	IllumiCode1365
IllumiCode0592	IllumiCode1611
IllumiCode0662	IllumiCode1696
IllumiCode0737	IllumiCode1716
IllumiCode0910	IllumiCode3008
IllumiCode0912	IllumiCode3137
IllumiCode0975	IllumiCode3511
IllumiCode1070	IllumiCode3885
IllumiCode1235	IllumiCode4254

Contamination Detection Controls

The PCR contamination detection controls are designed against bisulfite-converted human-genome DNA and are divided into four types. Only one type is added to each oligo pool for assay for methylation (OMA) tube. When a single OMA is run, only one contamination control type should have high signal. Should two or more contamination control types have high signal, then significant contamination may have occurred.

These figures provide graphic representations of these controls under three assay for methylation conditions:

- ▶ Figure 140 represents a contamination-free environment.
- ▶ Figure 141 represents a contaminated environment without UDG treatment.
- ▶ Figure 142 represents a contaminated environment with UDG treatment.

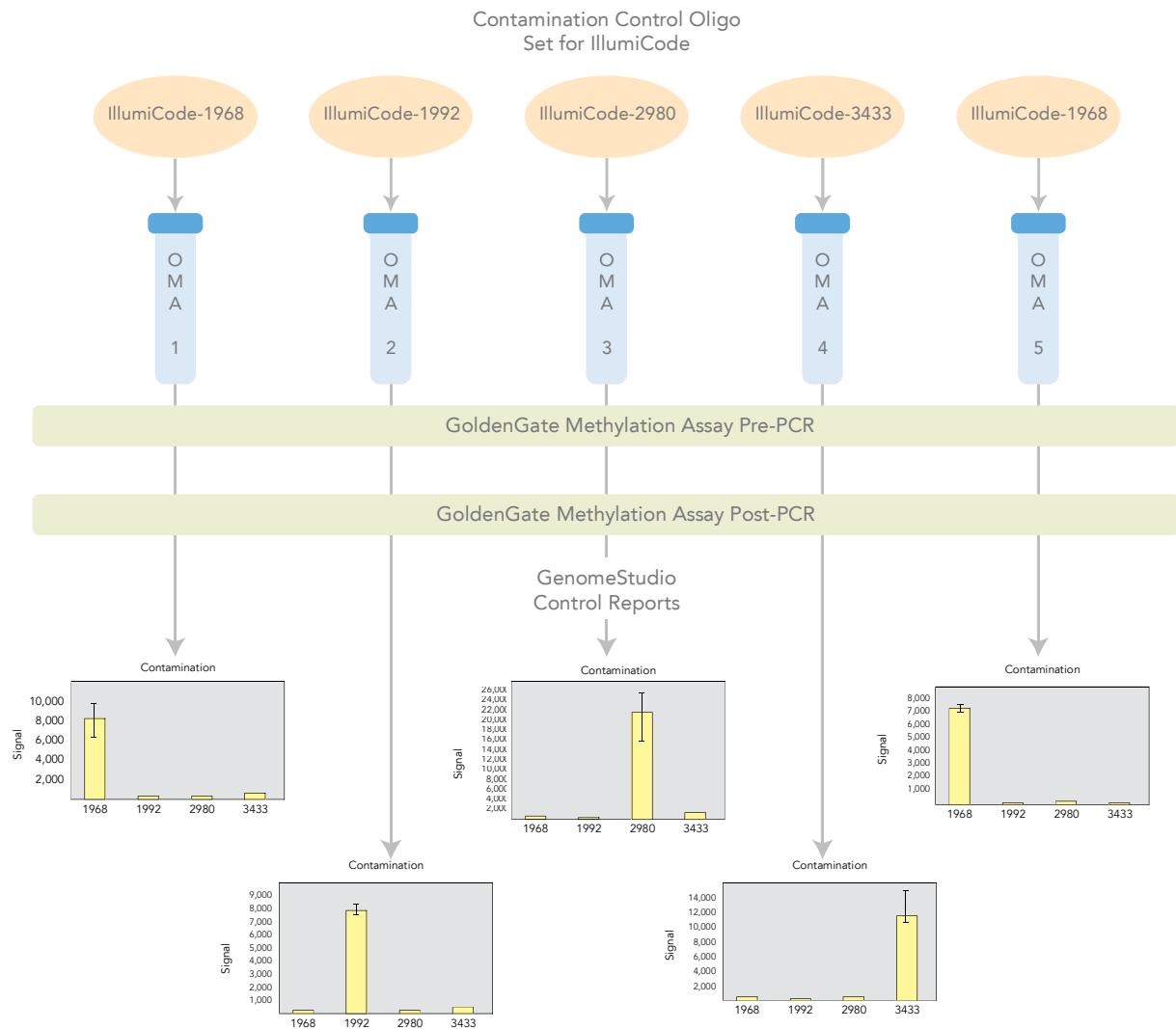


Figure 140 Contamination-Free Environment

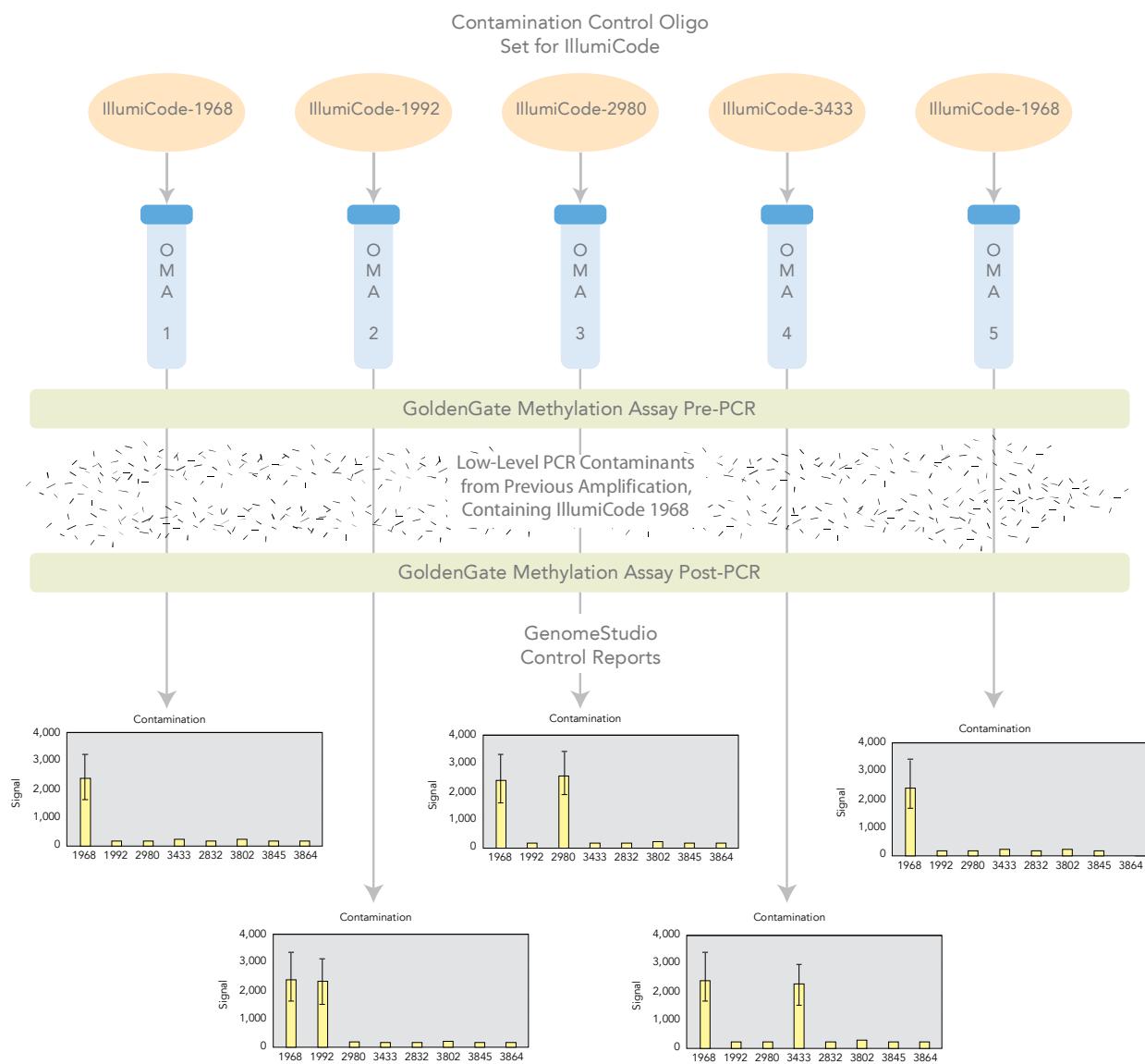


Figure 141 Contaminated Environment Without UDG Treatment

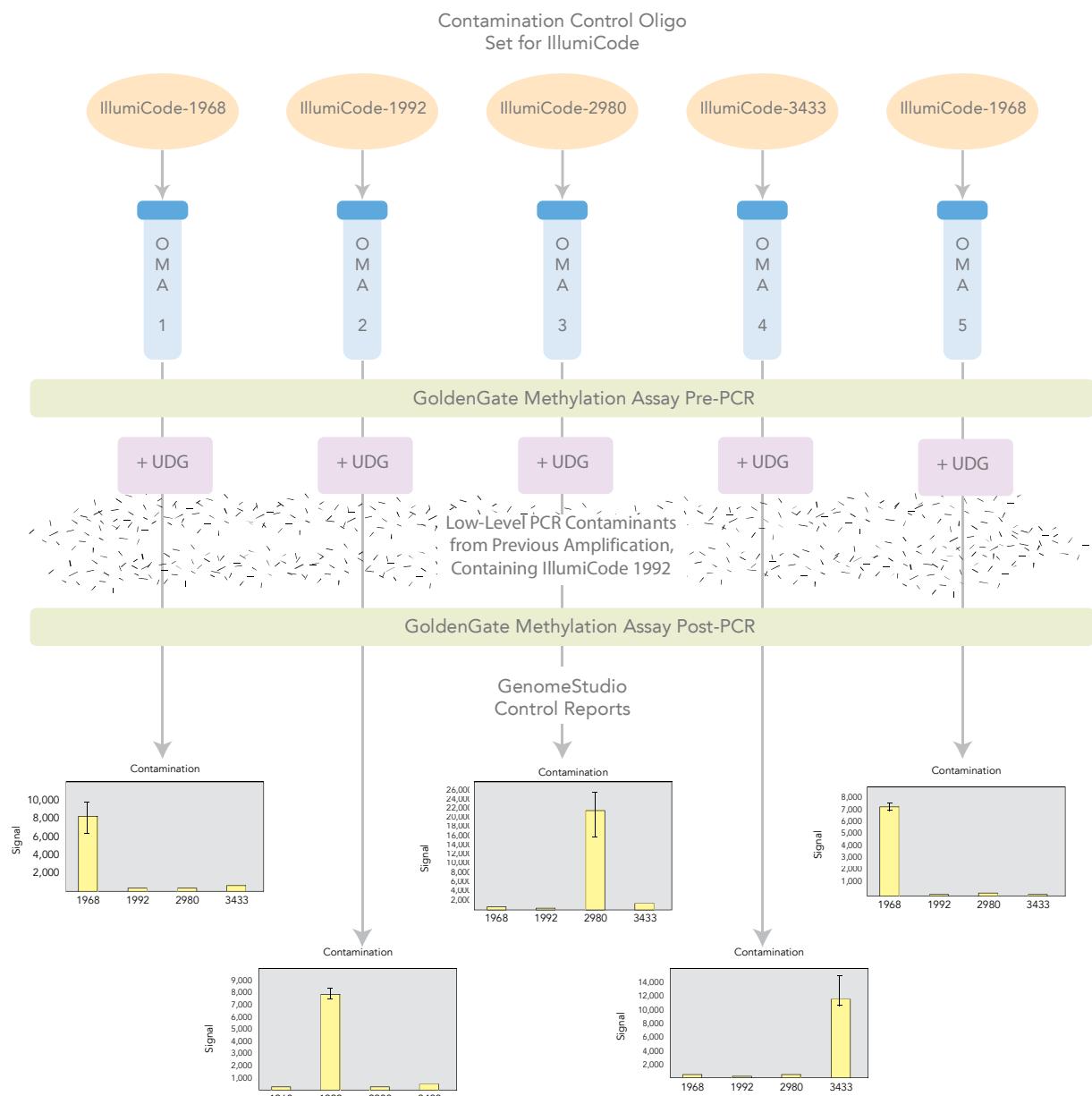


Figure 142 Contaminated Environment With UDG Treatment

Index

Numerics

8-channel pipettes. See multichannel pipettes

A

acronyms 10
allele-specific extension controls
 Genotyping 298
 Methylation 320
amine-terminated oligonucleotides 277
analysis tools 7
ASPE 2
 designing primers 256
 diagram 245
 overview 244
 testing reactions on gel 272
assay workflow
 Gene Expression 143
 Genotyping, automated 85
 Genotyping, manual 38
 Methylation 185
autoclaving 260
automated protocols 23–33

B

background, high 292
balancing centrifuge 22
barcode labels 22
Basic Run Parameters pane 25
bead kitting 229–242
 cleaning 242
 shaking directions 237
 storing kitted beads 242
 strip well plates, when to use 232
beads
 individual bead sets 248
 multiplex cytokine standard 278
 pooled bead sets 251
 resuspending, difficulty in 79
 See also carboxyl beads, universal
 oligo beads
BeadXpress Reader 5, 36
BeadXpress Reader workflow 5
bed map, robot 26

biotin dCTP 246
biotinylated detection antibody pool 278
biotinyling DNA 204
bisulfite conversion
 controls 321
 storing converted DNA 208
bleach
 bathing robot carriers 30
 cleaning lab regularly 14
 vapors in robot 27
 washing robot 26
BSA buffer 231

C

calibrating vortexer 21
carbodiimide coupling of amine-terminated oligos 280
carboxyl beads 275–294
 bead kitting 230
 multiplexing 4
 storage and use 231
 troubleshooting 292
centrifuge, balancing 22
cleaning
 lab with bleach 14
 pipettes 22
 robot system wash 24
 robot, conditioning dispense 33
 robot, schedule 30
 VeraCode Bead Kitting System 242
cluster locations 6
condensation 79
contamination
 bleach vapors in robot 27
 controls 16
 PCR product 14, 260
 pipette tips 62
 reagents splashing 32
 RNase 180
 UDG prevention 16
contamination detection controls
 Gene Expression 313
 Genotyping 304
 Methylation 327

strong signal 80
 control oligos
 Gene Expression 308–316
 Genotyping 296–306
 Methylation 318–329
 PCR contamination 16
 PCR, recommended 260
 cross-hybridization 326
 customer support 7
 Cy3/Cy5 ratio 81
 cytokines 276
 cytosines, unmethylated 200

D

DASL Gene Expression Assay for Vera-Code 141–182
 DB Access pane 25
 differential analysis 6
 DNA
 activating 204
 concentration, Genotyping assay 54
 concentration, Methylation assay 204
 Lambda DNA standard concentrations 46
 quantitation plate 191, 196
 storing bisulfite-converted 208
 dNTPs 246, 261
 documentation 8
 dye binding, non-specific 326

E

EDC 277, 280
 *.egt file (clusters) 6
 enzyme-linked immunosorbent assay (ELISA) 276
 equipment
 separate pre- and post-PCR 14
 evaporation 79, 272
 exonuclease I 246, 262
 extension gap control
 Gene Expression 311
 Genotyping 302
 Methylation 323

F

first hybridization controls
 Gene Expression 311
 Genotyping 302
 Methylation 324
 fluorescence testing the robot 33
 Flush W(ater) 24
 formalin-fixed, paraffin-embedded (FF-PE) samples 3, 158, 182

forms, lab tracking
 GoldenGate 39
 Methylation 186
 freeze-thaw cycle effect on RNA 158

G

gender controls
 Genotyping 300
 Methylation 322
 gene expression signal levels 6
 gene expression, DASL Assay for 3, 141–182
 GenomeStudio
 sample sheet, Gene Expression Module 144
 sample sheet, Genotyping Module 39
 sample sheet, Methylation Module 187
 software modules 6
 workflow 5
 GoldenGate Assay for BeadArray Reader vs. BeadXpress Reader 36
 GoldenGate Genotyping Assay for Vera-Code, automated protocols 83–139
 GoldenGate Genotyping Assay for Vera-Code, manual protocols 35–81
 GoldenGate Methylation Assay for Vera-Code 183–227
 GTS robot control software 23

H

help, technical 7
 high background 292
 homology 256
 hybridization
 ASPE, single-plate 269
 ASPE, two-plate 264
 extra wells 264
 streptavidin 272
 VeraCode Bead Plate 75
 See also first hybridization controls, second hybridization controls

I

*.idat files (intensity data) 6
 IllumiCode Sequence IDs
 Gene Expression 309
 Genotyping 297
 Methylation 319
 individual-column dispense test, robot 28
 initializing robot 24
 It 266

K

kitting, bead. See bead kitting

L

lab tracking forms 22
GoldenGate 39
Methylation 186
labels
barcode 22
filter plate 70
fluorophores 66
Gene Expression assay 148
Genotyping assay 43
Methylation assay 190
streptavidin-fluorophore 244
streptavidin-phycoerythrin 276
vortexer calibration 21
Lambda DNA standard concentrations 46
laser
overview 5
excitation wavelengths 247
two-color or single-color 2
LIMS 25
liquid handling (LiHa) 24, 32
low correlation between replicates 80
low genotyping call rate 81
low r^2 correlation 182
low signal 80, 272

M

MES 277
methylation
data analysis 6
GoldenGate Assay for VeraCode 183–227
mFold 257
microbeads, distribution into wells 230
MSDS (material safety data sheet) 10
multichannel pipettes 22
multi-column dispense test 28
multiplex cytokine standard beads 278
multiplexing 2
carboxyl beads 4
universal oligo beads 4

N

NaCl 278
negative controls
Gene Expression 310
Methylation 326
nucleic acid assays 4
number of samples, less than 96 22

P

PBS-BSA 278
PBS-Tween 278
PCR
controls, recommended 260
designing primers 256
forward and reverse primers 246
master mix components 262
product contamination 14, 260
single-plate protocol 266
two-plate protocol 261
uniformity controls, genotyping 299
phosphate buffered saline 277
PicoGreen dsDNA quantitation reagent 191
pipettes
aspirating from magnetized columns 62
bead kitting alternative 230
multichannel 22
robot tip height 32
tip contamination 62
vacuum aspiration 283
plates
Ind-Col dispense plate 28
Multi-Col dispense plate 28
robot bed map 26
where used 10
Platinum Taq Polymerase 246
PMT setting 80
pre- and post-PCR areas 14
primer-dimer formation 256
primers
ASPE, parts 258
designing PCR and ASPE 256
low quality 261
optimizing 271
universal oligo bead sets 246
VeraCode capture sequences 258
Proclin 300 278
protein immobilization 282
protein-immobilized beads, kitting 234

Q

quantitation
DNA 191
read QDNA plate 196
RNA 149

R

raised-bar magnetic plate 62
reagents
automated liquid handling 32
dispensing technique, robot 32
reusing 16

- robot bed map 26
where used 10
- replicates as controls 260
- replicates, poor 293
- reproducibility 294
- resuspending beads, troubleshooting 79
- RFU (relative fluorescent levels) 244, 272
- RiboGreen RNA Quantitation Kit 149
- RNA
 - concentration 159
 - degraded 3
 - freeze-thaw cycles 158
 - quantitation 149
- RNase contamination 180
- robot 23–33
 - bed map 26
 - bleach bathe carriers 30
 - bleach wash 26
 - cleaning 30
 - diluter syringes 24
 - first use of day 26
 - fluorescence test 33
 - individual column dispense test 28
 - initializing 24
 - liquid handling system 24
 - multi-column dispense test 28
 - required items 25
 - software interface 23
 - tip height, set 32
 - tips, check accuracy 28
 - tips, wash 27
 - washes 24
- S**
 - safety, government and facility standards 10
 - SA-hybridization buffer 264
 - sample sheet 22
 - Gene Expression 144
 - Genotyping 39
 - Methylation 187
 - samples 22
 - SAP/EXO 262, 272
 - second hybridization controls
 - Gene Expression 312
 - Genotyping 303
 - Methylation 325
 - self-annealing formation 256
 - shaker, high-speed 21
 - shaking directions for bead kitting 237
 - shrimp alkaline phosphatase 246, 262
 - signal, missing 292, 294
 - signal, too much 293
 - single-plate protocol, ASPE 266
 - SoftMax Pro 50, 196
- software, robot 23
- SSC 246
- standard curve, low or flat 293
- storing kitted VeraCode beads 242
- streptavidin phycoerythrin 276, 278
- streptavidin, effect on background 272
- streptavidin-alexa 647* 246
- strip well plates, when to use 232
- Sulfo-NHS 278
- System Liquid carboy 27
- T**
 - Tecan 8-tip robot 23–33
 - technical assistance 7
 - thrombosis multiplexed panel 244, 259
 - tip wash 27
 - tracking samples 22
 - troubleshooting and tips
 - analysis 80
 - antibody concentration 282
 - aspirating beads 62
 - autoclaving 260
 - bleach vapor 15
 - DNA sample preparation 79
 - EDC color 280
 - evaporation 272
 - extra wells in hybridization 264
 - freeze-thaw cycles for FFPE samples 158
 - heterozygotes, bias towards 273
 - high background 292
 - low r^2 correlation 182
 - low RFU 272
 - low signal 272
 - mixing 283
 - no signal 292, 294
 - oligo concentrations 272
 - optimization reaction 271
 - partial strip plates 232
 - PCR primer amount 266
 - pipette tip contamination 56
 - poor replicates 293
 - primer concentration 266, 272
 - primer concentrations 261
 - reproducibility 294
 - RNA sample preparation 179
 - SAP/EXO step 272
 - serial titration of 100 μ M oligo 280
 - shaking bead kitting system 237
 - standard curve 293
 - too much signal 293
 - vortex speed 22
 - Tween 20 246
 - two-plate protocol, ASPE 261

U

universal oligo beads
 bead kitting 230
 choosing protocol 272
 individual sets 248
 labeling 244
 multiplexing 4
 pooled sets 251
 single-plate protocol 266
 storage and use 231
 troubleshooting 271
 two-plate protocol 261
unmethylated cytosines 200

V

vacuum aspiration 283
variant allele detection 244
VeraCode

capture sequences 258
carboxyl beads 231
overview 2
universal beads 231
workflow 5
viscous solutions 33
vortexer, setting up 21

W

Wash S(hort) 24
washes, robot system 24
wildtype allele detection 244
workflow 185
 Gene Expression 143
 Genotyping, automated 85
 Genotyping, manual 38
 Methylation 185
 VeraCode Assays 5

Illumina, Inc.
9885 Towne Centre Drive
San Diego, CA 92121-1975
1.800.809.4566 (toll free)
1.858.202.4566 (outside the U.S.)
techsupport@illumina.com
www.illumina.com

illumina®