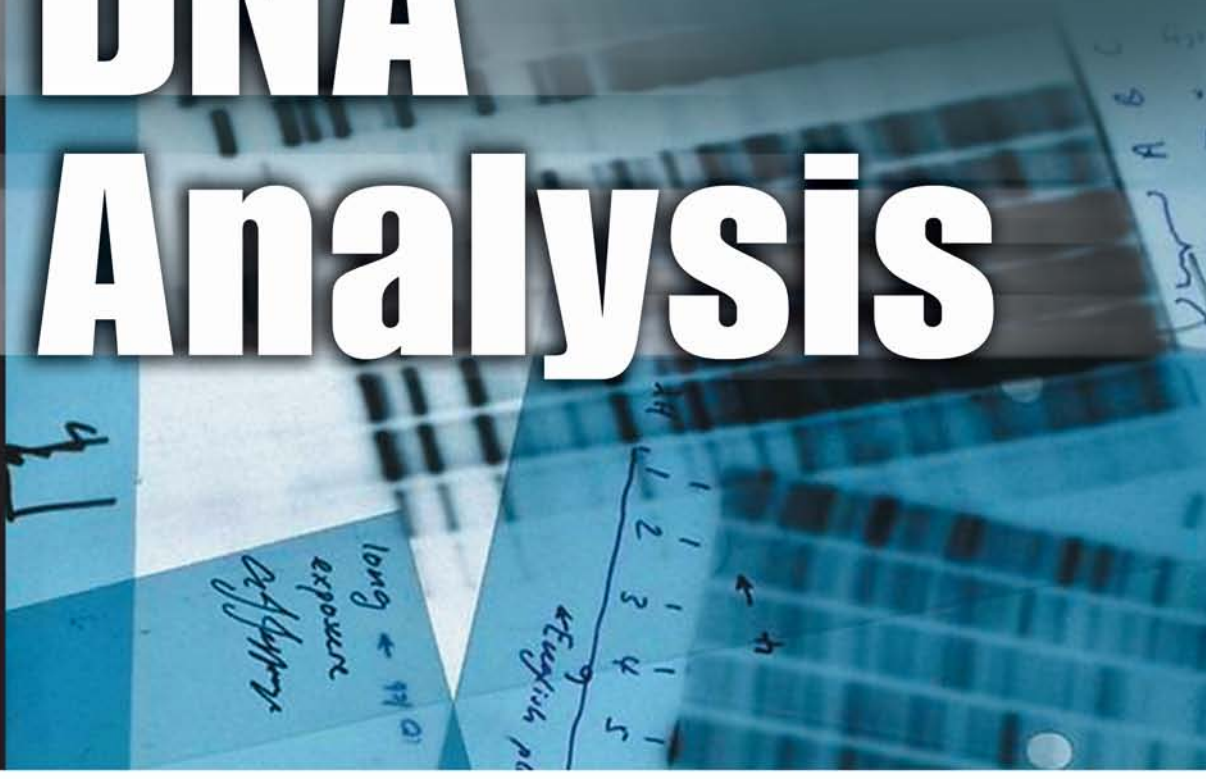
# Forensic DNA Analysis




## A Laboratory Manual

J. Thomas McClintock

CRC Press
Taylor & Francis Group

# Forensic DNA Analysis

## A Laboratory Manual

# Forensic DNA Analysis

## A Laboratory Manual

J. Thomas McClintock

CRC Press
Taylor & Francis Group
Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742


Printed in the United States of America on acid-free paper
10 9 8 7 6 5 4 3 2 1

International Standard Book Number-13: 978-1-4200-6329-5 (Softcover)



**Library of Congress Cataloging-in-Publication Data**

McClintock, J. Thomas.
Forensic DNA analysis : a laboratory manual / J. Thomas McClintock.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-1-4200-6329-5 (hardcover : alk. paper)
1. DNA--Analysis--Laboratory manuals. 2. DNA fingerprinting--Laboratory manuals. 3. Forensic genetics--Laboratory manuals. I. Title.
[DNLM: 1. DNA Fingerprinting--Laboratory Manuals. 2. Forensic Medicine--methods--Laboratory Manuals. W 625 M478f 2008]

RA1057.55.M33 2008
614'.1078--dc22 2007045327

**Visit the Taylor & Francis Web site at**
**http://www.taylorandfrancis.com**

**and the CRC Press Web site at**
**http://www.crcpress.com**

# Contents

# List of Figures

# List of Tables

# Welcome to the Forensic DNA Analysis Laboratory

DNA typing has revolutionized criminal investigations and has become a powerful tool in the identification of individuals in criminal and paternity cases. In the past few years, the general public has become familiar with forensic DNA typing based on exposure from media coverage (e.g., the O. J. Simpson trial, the President Bill Clinton and Monica Lewinsky scandal, and the identification of individuals killed in the September 11, 2001 attacks on the World Trade Center in New York City and the Pentagon in Arlington, Virginia) and television programs (e.g., *Forensic Files* and *CSI: Miami*). Although these cases have generated widespread media attention, they represent only a small fraction of the thousands of forensic DNA and paternity cases that are conducted by public and private laboratories in the United States and abroad.

The purpose of this *Forensic DNA Analysis Laboratory Manual* is to introduce the student to the science of DNA typing methods by focusing on basic techniques used in forensic DNA laboratories. This laboratory manual is designed to provide the student with a fundamental understanding of forensic DNA analysis as well as a thorough background of the molecular techniques used to determine an individual's identity or parental lineage. This manual is intended to challenge the student with the methodology of the investigation in DNA typing, help the student develop an understanding of the scientific principles involved in DNA analysis, and ensure the student is able to analyze and interpret the data that are generated in each exercise with clarity and confidence.

The exercises in this laboratory manual have been organized to first provide an overview of forensic DNA analysis, the sources or types of biological material used in DNA analysis, and then the background principles and practical methodology for a specific DNA typing technique. In some exercises, the protocols have been adapted from methods and protocols used in federal, state, and private forensic laboratories. Each exercise is designed to simulate human forensic testing but can also be used to simulate a wide range of applications for genetic analysis. The actual scenario employed in each exercise is up to the discretion of the course instructor. Lastly, an extensive glossary has been included to assist students with DNA typing terminology as well as basic terms used in molecular biology.

Compiled below is a brief history of forensic DNA typing. Since DNA testing was first introduced in the United States in 1986, it has been used in thousands of cases. However, the list below highlights specific events or developments in forensic DNA analysis as well as those cases brought to the attention of the general public by media exposure.

## Brief History of Forensic DNA Typing

*1980* Ray White describes first polymorphic RFLP marker.
*1985* Alec Jeffreys discovers multilocus VNTR probes.
*1985* First paper on Polymerase Chain Reaction (PCR)

*1986* DNA testing goes public (Cellmark and Lifecodes).
*1986* First RFLP case in the U.S. (Florida vs. Tommy Lee Andrews)
*1988* FBI starts DNA casework (RFLP).
*1989* The Technical Working Group on DNA Analysis Methods (TWGDAM) established
*1991* First Short Tandem Repeats (STR) paper
*1992* NRC I Report "DNA Technology in Forensic Science"
*1993* First STR kit available
*1995* Forensic Science Service (FSS) starts UK DNA database
*1995* O.J. Simpson trial; public becomes aware of DNA.
*1996* NRC II Report "The Evaluation of Forensic DNA Evidence"
*1996* First use of mitochondrial DNA test in a U.S. criminal trial (Tennessee v. Ware).
*1998* FBI launches Combined DNA Index System (CODIS) database.
*1998* Establishment of Quality Assurance standards for forensic DNA testing laboratories through the DNA Advisory Board.
*1998* Kenneth Starr investigates allegations of President Clinton's sexual relationship with White House intern Monica Lewinsky.
*1999* Multiplex STRs are validated.
*1999* The decision in State v. Ware (1996) was upheld by an appellate court.
*2002* Division of Forensic Science Laboratory in the Commonwealth of Virginia became the first state laboratory to mark 1,000 "cold hits" from its DNA database.
*2003* A field DNA test was completed to provide preliminary confirmation of the identification of Saddam Hussein less than 24 hrs after his capture. A full test performed in the laboratory, provided confirmation.
*2003* The National Institute of Standards and Technology develops a "mini-STR assay" to allow the remains from 16 additional victims from the September 11, 2001 attacks on the WTC to be positively identified.
*2006* Members of the Duke University men's lacrosse team arrested and accused of raping a female exotic dancer. Samples collected from the dancer and the men's lacrosse team for DNA analysis.
*2006* DNA testing failed to connect any members of the Duke University men's lacrosse team to the alleged sexual assault of an exotic dancer.
*2007* Prosecutor handling the Duke case is forced to recuse himself. North Carolina's attorney general declared three former Duke University lacrosse players who had been accused of gang-raping a stripper innocent of all charges, ending a prosecution that provoked bitter debate over race, class, and the tactics of the Durham County district attorney.
*2007* Applied Biosystems introduces the first DNA testing kit for analyzing degraded or limited DNA
*2007* FSS in the UK uses laser microdissection (LMD), which enables single cells to be extracted from a microscope slide, with fluorescence in situ hybridization (FISH), a method to highlight chromosomes, to distinguish between male (XY chromosomes) female (XX chromosomes) cells.

It is hoped that this manual will develop the curiosity and confidence of the student to further explore questions and issues involving forensic science investigations. I look forward to teaching you the techniques and applications in forensic DNA analysis.

# Laboratory Rules

1. No eating, drinking, smoking, applying cosmetics, or handling contacts in the laboratory at **ANY** time.
2. No pipetting by mouth. Use a pipettor at all times.
3. Minimize splashing and production of aerosols.
4. Store all books, backpacks, cell phones and other electronic devices, purses, coats, and so on in the cabinet of your laboratory bench (or designated area). Only your laboratory notebook should be on the bench.
5. Do not place pencils, pens, or any other object into your mouth while in the laboratory.
6. **NEVER** take any reagents, samples, or cell cultures out of the laboratory.
7. Notify the laboratory instructor immediately of any spills, of any accidents, or if you cut or injure yourself.
8. In most instances, you will be wearing disposable gloves. Be extra careful when handling reagents or chemicals to eliminate skin contact.
9. Wash your hands at the beginning and at the end of the laboratory exercises.
10. Clean your laboratory bench with dilute alcohol before you begin work and when you have completed the laboratory exercise.
11. Laboratory coats are not required. However, in forensic laboratories, laboratory coats and disposable gloves must be worn.
12. Children are not allowed in the laboratory.
13. Familiarize yourself with the location of the eye wash station, the fire extinguisher, and the fire blanket.

Chapter 1

# An Overview of Forensic DNA Analysis

## Introduction

Everyone has a unique set of fingerprints. As with a person's fingerprint, no two individuals share the same genetic makeup. This genetic makeup, which is the hereditary blueprint imparted to us by our parents, is stored in the chemical deoxyribonucleic acid (DNA), the basic molecule of life. Examination of DNA from individuals, other than identical twins, has shown that variations exist and that a specific DNA pattern or profile can be associated with an individual. These DNA profiles have revolutionized criminal investigations and have become powerful tools in the identification of individuals in criminal and paternity cases.

## Restriction Fragment Length Polymorphism

The first widespread use of DNA tests involved restriction fragment length polymorphism (RFLP) analysis, a test designed to detect variations in human DNA. In the RFLP method, DNA is isolated from a biological specimen (e.g., blood, semen, or vaginal swabs) and cut by an enzyme into pieces called restriction fragments. The DNA fragments are separated by size into discrete bands by gel electrophoresis, transferred onto a membrane, and identified using probes (known DNA sequences that are "tagged" with a chemical tracer). The resulting DNA profile is visualized by exposing the membrane to a piece of X-ray film allowing the scientist to determine which specific fragments the probe identified among the thousands in a sample of human DNA. A "match" is made when similar DNA profiles are observed between an evidentiary sample and those from a known sample (e.g., DNA from a victim or suspect). A determination is then made as to the probability that a person selected at random from a given population would match the evidence sample as well as the suspect. The entire analysis may require several weeks for completion.

## Polymerase Chain Reaction–Based Tests

In instances when the evidentiary sample contains an insufficient quantity of DNA or the DNA is degraded, a polymerase chain reaction (PCR)–based test is used to obtain a DNA profile. The PCR-based tests generally provide rapid results that can serve as an alternative or as a complement to other DNA tests. The first step in the PCR process involves the isolation of DNA from a biological specimen (e.g., blood, semen, saliva, or fingernail clippings). Next, the PCR amplification technique is used to produce millions of copies of a specific portion of a targeted DNA segment. The PCR amplification procedure is comparable to a photocopying machine, only it is at the molecular level. The amplified PCR products are then identified by either the addition of known DNA probes (e.g., DQA1 and PM test kits) or separation by gel electrophoresis (D1S80, short tandem repeat [STR], and amelogenin [gender] analyses) followed by chemical staining. Such detection procedures eliminate the need for critically sensitive DNA probes, thus reducing the analysis time from several weeks to 24–48 hours. The resulting DNA profiles are routinely interpreted by direct comparison to DNA standards. Probability calculations are determined based upon classical population genetic principles.

## Mitochondrial DNA Analysis

Mitochondrial DNA (mtDNA) typing is increasingly used in human identity testing when biological evidence may be degraded, when quantities of the samples in question are limited, or when nuclear DNA typing is not an option. Biological sources of mtDNA include hairs, bones, and teeth. In humans, mtDNA is inherited strictly from the mother. Consequently, mtDNA analysis cannot discriminate between maternally related individuals (e.g., mother and daughter, or brother and sister). However, this unique characteristic of mtDNA is beneficial for missing person cases when mtDNA samples can be compared to samples provided by the maternal relative of the missing person.

In humans, the mtDNA genome is approximately 16,000 bases (A, T, G, and C) in length containing a “control region” with two highly polymorphic regions. These two regions, termed Hypervariable Region 1 (HV1) and Hypervariable Region 2 (HV2), are 342 and 268 base pairs (bp) in length, respectively, and are highly variable within the human population. This sequence (the specific order of bases along a DNA strand) variability in either region provides an attractive target for forensic identification studies. Moreover, because human cells contain several hundred copies of mtDNA, substantially more template DNA is available for amplification than nuclear DNA.

Mitochondrial DNA typing begins with the extraction of mtDNA followed by PCR amplification of the hypervariable regions. The amplified mtDNA is purified and subjected to sequencing (Sanger et al., 1977), with the final products containing a fluorescently labeled base at the end position. The products from the sequencing reaction are separated, based on their length, by gel or capillary electrophoresis. The resulting sequences or profiles are then compared to sequences of a known reference sample to determine differences and similarities between samples (Anderson et al., 1981; Andrews et al., 1999). Samples are not excluded as originating from the same source if each base (A, T, G, or C) at every position along the hypervariable regions is similar. However, due to the size of the mtDNA database and to the unknown number of mtDNA sequences in the human population, a reliable frequency estimate is not provided. Consequently, mtDNA sequencing is becoming known as an exclusionary tool as well as a technique to complement other human identification procedures.

Chapter 2

# Types of Biological Samples

With the exception of white blood cells, DNA is found in every human cell. Consequently, DNA is present in a variety of body fluids and tissues that have been demonstrated to be suitable for DNA typing. However, if the sample or evidence collection is performed improperly the sample's integrity may be compromised, leading to contamination and/or degradation. Improper handling procedures during storage and transport from the crime scene to the laboratory can result in samples unfit for analysis. The importance of sample integrity cannot be overemphasized because ambiguous data or information can compromise the investigation and/or the outcome of the case.

In the past, DNA typing tests such as restriction fragment length polymorphism (RFLP) were successful in generating complete DNA profiles provided that adequate and nondegraded samples were utilized. The introduction of the polymerase chain reaction (PCR) in the mid- to late 1980s extended the range of possible samples available for DNA analysis regardless of their condition. Some of the biological samples that have been tested successfully with PCR-based typing methods are listed in Table 1. The minimum or corresponding amount of DNA available from each biological sample is also shown.

Prior to DNA isolation and typing, biological samples must first be collected either from a known contributor (victim and/or suspect) or from the crime scene (evidentiary sample). Once the sample is collected, the DNA is extracted and subjected to PCR analysis. In general, the PCR procedure typically requires as little as 1 nanogram (1 billionth of a gram) of high molecular weight genomic DNA. DNA thought to be degraded can also be subjected to PCR analysis because intact, high molecular weight DNA is not necessary to generate a complete DNA profile. Table 2 illustrates the types of physical evidence collected at crime scenes, the various locations of the DNA, and the biological source.

Biological evidence will attain its full forensic value only when the DNA types can be compared to known profiles obtained from the victims and suspects. The following is a brief description of the various biological samples suitable for DNA typing with accompanying guidance for collection and storage.

## Whole Blood

Whole blood from a known source should be collected in a sterile tube containing the preservative EDTA (ethylenediamine tetraacetic acid). In addition to acting as a preservative, EDTA also inhibits the activity of enzymes that are responsible for degrading DNA. Tubes containing blood samples should be stored at

**TABLE 1**
**DNA Contents of Biological Samples**

| Biological Sample or Source | Approximate DNA Content |
|---|---|
| Liquid blood | 20–40 µg/ml (1 µl from 4–11 × $10^3$ white blood cells) |
| Blood stain (1 $cm^2$) | 250–500 ng |
| Semen | 150,000–300,000 ng/ml |
| Postcoital vaginal swab | 0–3000 ng/ml |
| Saliva | 1000–10,000 ng/ml |
| Oral swab | 1000–1500 ng |
| Hair roots | 1–750 ng/plucked hair root |
| Hair (shed) | 1–12 ng/hair |
| Urine | 1–20 ng/ml |
| Bone | 3–10 ng/mg bone |
| Tissue (15 mg) | 3–15 µg/mg |
| Fibroblast cell line | 6.5 µg/1 × $10^6$ cells |

refrigerated (for short periods of time) or frozen temperatures (for long-term storage). Whole blood can also be "spotted" onto an FTA© collection card (an absorbent cellulose-based paper that contains chemical substances to inhibit bacterial growth and to protect the DNA from enzymatic degradation), allowed to dry, and stored at room temperature for several years.

## Bloodstains or Mixed Stains

Garments or clothing containing stains are packaged in a suitable manner (e.g., in a bag and/or box) and transported to the laboratory and stored until analysis. It should be noted that all stained material should be

**TABLE 2**
**Physical Evidence Collected at Crime Scenes**

| Physical Evidence | Location of DNA | Biological Source of DNA |
|---|---|---|
| Used cigarette | Cigarette butt | Saliva |
| Toothpick | Tips | Saliva |
| Stamps and envelopes | Licked area | Saliva |
| Bottle or can | Mouthpiece | Saliva |
| Used condom | Inside or outside surface | Semen, or vaginal or rectal cells |
| Blanket, sheet, or pillow | Surface | Semen, sweat, hair, saliva, or urine |
| Bite mark | Clothing or person's skin | Saliva |
| Fingernail | Scrapings | Blood, skin, or sweat |
| Tape or ligature | Inside or outside surface | Skin or a surface |
| Bullet | Outside surface | Blood, tissue, or skin |
| Clothing | Surface area | Blood, sweat, or semen |
| Hat, mask, or bandanna | Inside surface | Sweat, hair, or dandruff |
| Knife, bat, or similar object | Outside surface or handle | Blood, skin, tissue, or sweat |

dried thoroughly prior to packaging and submission to the laboratory for analysis. Short-term storage should be at room temperature in a humidity-controlled room out of direct sunlight. For long-term storage, samples should be stored in a low-temperature, frost-free freezer. Once the packaged material arrives at the laboratory, the removal of the stain from the item is performed by the forensic DNA analyst. Stains comprising a size of approximately 5 $mm^2$ (half the size of a dime) or greater, and with a volume of less than 5 µl, have been successfully analyzed by current DNA typing techniques.

## Hairs

In general, forensic hair analysis involves either head or pubic hair. The collection of 12–24 full-length hairs from the scalp will provide more than enough material for analysis. Hair samples that contain an intact root will provide enough nuclear DNA for short tandem repeat (STR) typing (see Exercise 8). A hair shaft contains sufficient mitochondrial DNA (mtDNA) for successful mtDNA typing (see Exercise 11). As with stains, short-term storage may be at room temperature in a humidity-controlled room and out of direct sunlight. For long-term storage of hair samples, a low-temperature, frost-free freezer is recommended.

## Swabs from Biological Material or Inanimate Objects

DNA has been successfully analyzed from swabs containing biological material (e.g., buccal cells from the inside of the cheek or epithelial cells from a vaginal swab) and from swabs of various inanimate objects such as cigarettes, envelopes, soda cans, and stamps. Using a sterile and moistened cotton swab, the area in question is "swabbed," then the swab is dried and placed in a container for storage or placed in a vial containing a small volume of sterile solution such as 1X TE buffer for short-term storage. Swabs containing the biological material for analysis are stored as recommended as with stains.

## Bone, Teeth, and Tissue

In some instances, the probability of obtaining a sufficient quantity or quality of biological material such as blood or semen for DNA typing will be low. This insufficient quality or quantity of material may be due to sample degradation, availability, or even accessibility. In these cases, samples such as bones, teeth, skin, and/or muscle tissue will usually provide sufficient DNA for analysis. Generally, a 1 $cm^2$ section (slightly smaller than a dime) of such biological material is suitable for testing. Following collection, the samples should be frozen and transported to the laboratory on ice. Upon arrival at the laboratory, the samples should be kept frozen until the DNA typing analysis begins. To ensure sample integrity, avoid multiple freeze-thaw conditions.

## Paraffin-Embedded Tissues, Smears, or Slides

When known biological samples are unavailable, but needed, investigation into local medical facilities may yield a source: specimens collected from biopsies or surgical procedures that were processed, analyzed, and stored. Such specimens may include vaginal or pap smears or histological sections. Histological sections contain samples fixed in formalin, processed, and embedded in paraffin. The paraffin "blocks" are sectioned using a microtome and placed on polylysine-coated slides. Usually, a minimum of one block, smear,

or slide is typically necessary for a successful DNA typing analysis. Histological sections, smears, or slides may be stored at room temperature indefinitely prior to analysis.

## Semen and Sperm

Sperm specimens, collected from a vaginal swab, will contain epithelial (skin) cells from the female and, in some instances, from the male. Sperm can be preferentially separated from the rest of the material in such a mixture using specific extraction methods discussed in Exercise 1 (see the section entitled "Differential Extraction"). Samples or stains that are thought to, or have been shown to, contain only spermatozoa can be collected and processed as described above (see "Bloodstains or Mixed Stains"). Sperm specimens should be stored frozen prior to analysis. To ensure sample integrity, avoid multiple freeze-thaw conditions.

## Urine

Similar to perspiration and sebaceous oils, urine when concentrated will contain a sufficient amount of epithelial cells to generate a DNA profile. Ideally, a minimum sample volume of 10 ml is required for analysis, with an optimum approaching 30 ml. However, a sufficient number of cells might be obtained from a stain or swab of a known source. For long-term storage, the specimen should be stored frozen. Specimens can be stored in refrigerated temperatures for short periods of time prior to analysis. To maintain specimen integrity, it is critical to avoid multiple freeze-thaw conditions.

Chapter 3

# Exercise 1
## *DNA Extraction*

## Introduction

There are a number of different approaches for the isolation of genomic DNA. Each procedure begins with some form of cellular lysis, followed by deproteinization and recovery of DNA. The main differences between the various approaches lie in the extent of deproteinization and the size of the DNA isolated. In addition, the isolation or extraction of DNA will vary according to the type of biological sample, the amount of evidence or biological sample, and the type of cell(s) present in the sample.

DNA must first be separated from the rest of the cellular components, as well as from any nonbiological material present. The removal of extraneous substances following cell lysis minimizes sample (DNA) degradation due to cellular enzymes while ensuring maximum enzymatic efficiency during the typing procedure.

## Objectives

In this exercise, you will learn different isolation techniques used in forensic DNA analysis to extract DNA from various biological sources. The techniques used in this exercise are representative examples of modern techniques used in forensic laboratories to isolate whole genomic DNA from known samples as well as from evidentiary samples. The extraction procedures described below are relatively brief and easy to perform. The optimum isolation procedure is highlighted for each sample or cell type. When completed, the DNA isolated in this exercise can be utilized in the subsequent exercises.

## Extraction Methods

The use of disposable gloves and aerosol-resistant pipet tips is highly recommended to prevent cross-contamination. A helpful organizational sheet is provided at the end of the exercise to record data and other necessary information.

### *1. Chelex Extraction*

When a minimal amount of sample is available (e.g., a spot of blood), the Chelex extraction method has been used. The sample is boiled in a solution containing minute beads of a chemical called Chelex. The boiling causes the cells to lyse, releasing the DNA. The Chelex binds to the extraneous cellular material, and the entire "complex" is removed by centrifugation, leaving the DNA in the supernatant. Because the high temperatures disrupt the two strands of the DNA, generating single-stranded molecules, this extraction process is generally reserved for PCR-based typing techniques.

#### Equipment and Material

1. 15 ml sterile polypropylene test tube
2. Sterile 1.5 ml Eppendorf or microcentrifuge tubes
3. 5 ml pipettor with sterile tips
4. Adjustable-volume digital micropipets (20–1000 µl range)
5. 10% suspension of Chelex resin beads
6. Aerosol-resistant pipet tips
7. Cultured human cell lines (see Exercise 7 for details)
8. 1.5 ml test tube holder
9. 0.9% saline
10. Sterile cotton swabs
11. Disposable gloves
12. Boiling water bath in a 1000 ml beaker
13. Ice in buckets
14. Tabletop clinical centrifuge
15. Microcentrifuge

#### Procedure

**Collection of Cells (e.g., Buccal Cells, Liquid Blood, and Cultured Human Cells)**

1. Label a 15 ml polypropylene test tube and the top of a 1.5 ml Eppendorf tube (also referred to as a microcentrifuge tube) with your name and any other appropriate information.
2. Pipet 10 ml of suspended cells (maximum $5 \times 10^6$ cells/ml) or liquid sample into the polypropylene test tube (for harvesting cultured human cells, see the "Salting Out" procedure below, steps 1–6). For buccal cells, rinse your mouth with 10 ml of saline solution and vigorously swish against your cheeks for 10 sec. Expel saline solution back into the labeled 15 ml polypropylene test tube over the sink.

Or,

if sterile swabs are available, place the swab inside your mouth and press it firmly against the inside of your cheek. Roll the swab back and forth over the inside surface of your cheek at least 10 times. Repeat on the other cheek. Place the swab into a labeled 15 ml test tube containing 10 ml saline solution.

**Concentrate Cells by Centrifugation**

3. Centrifuge the samples at 300 × g for 5 min. The cells form a firm pellet below the saline supernatant. **SAVE THE PELLET AND DISCARD THE SUPERNATANT** by decanting into the sink with running water, taking care not to disturb the cell pellet at the bottom of the tube.
4. Add 500 µl of Chelex beads into the 15 ml test tube containing the cell pellets. Resuspend the cell pellet either by slowly pipetting "in and out" several times or by tapping with your finger.
5. Transfer a 500 µl aliquot of the cell–Chelex slurry into a sterile 1.5 ml Eppendorf tube. Make sure the Eppendorf tube is labeled for identification purposes.

**Lysing the Cells and Collecting the DNA**

6. Place the capped (closed) Eppendorf tubes in a "float," and place in a boiling water bath for 10 min.
7. After the heat treatment, place the samples on ice for 5 min.
8. Place the Eppendorf tubes containing the lysed cells in a microcentrifuge, and spin at the maximum speed for 1 min. The pellet contains the Chelex beads bound to the denatured proteins. The supernatant contains the DNA.
9. Using a 1000 µl micropipettor with a sterile tip, transfer all of the clear supernatant to a fresh 1.5 ml Eppendorf tube.
10. Label the tube, and place on ice until you are ready to proceed to the next step.

## *2. Organic Extraction*

Organic extraction is a general method used for many situations when stained fabric or clothing is suspected of containing biological material. The stain on the material is cut away from the fabric, soaked in a warm solution (stain extraction buffer) to release the cells from the fabric, and incubated with proteinase K, and the DNA is isolated using organic solvents. The organic extraction method maintains the integrity of the DNA (i.e., large segments are maintained) while "cleaning" the DNA.

### Equipment and Material

1. 2.0 ml screw-cap tube
2. 0.5 ml microcentrifuge tube
3. Adjustable-volume digital micropipets (100–1000 µl range)
4. Aerosol-resistant pipet tips
5. Stain extraction buffer
6. Proteinase K (10 mg/ml)

7. Phenol:$CHCl_3$:isoamyl alcohol (25:24:1)
8. Chloroform ($CHCl_3$)
9. Disposable gloves
10. Incubator or water bath at 56°C
11. Vortex mixer
12. Microcentrifuge

### Procedure

1. Place the "cutting" from the stained material in a 2.0 ml screw-cap tube.
2. Add 400 µl of stain extraction buffer and 10 µl of proteinase K to each tube. Mix and centrifuge for 2 sec. Incubate the tubes containing the "cutting" at 56°C overnight.
3. Briefly centrifuge the samples for 5 sec.
4. Punch a hole in the bottom of a 0.5 ml microcentrifuge tube. Remove the cutting from the 2.0 ml tube using sterile forceps, and place in the 0.5 ml tube. Place the 0.5 ml tube into the 2.0 ml tube from which the cutting was removed.
5. Centrifuge the 2.0 ml tube containing the 0.5 ml "inserted" tube at maximum speed for 5 min in the microcentrifuge.
6. Remove the 0.5 ml tube, and save the cutting.
7. Replace the screw cap on the 2.0 ml tube.

**Note:** The following steps should be carried out in an exhaust hood.

8. Add 500 µl to each tube, vortex the tube for 20 sec, and centrifuge in the microcentrifuge for 2 min.
9. Transfer the top aqueous layer containing the DNA to a new 2.0 ml tube. Do not disturb the interface. Dispose of the phenol:$CHCl_3$:isoamyl alcohol solution in the collection tube in a biohazard waste container.

**Note:** Steps 10 and 11 are optional unless using Centricon concentration.

10. Add 500 µl of $CHCl_3$ to each tube, vortex, and centrifuge for 2 min.
11. Transfer the top aqueous layer to a new 2.0 ml tube. Dispose of the $CHCl_3$ solution in the collection tube in a biohazard waste container. The sample is now ready for precipitation or concentration.

## *3. "Salting Out"*

The salting-out procedure is relatively easy to use with liquid samples (known or evidentiary samples) and with cell cultures that might be used as "mock" evidence samples or as controls. The salting-out DNA isolation procedure involves the preferential hydrolysis and precipitation of cellular proteins. The protein-free genomic DNA is subsequently recovered by either method described in Exercise 2 or 3.

### Equipment and Material

1. TE-9 buffer
2. Proteinase K

3. Adjustable-volume digital micropipets (20–200 µl range)
4. Aerosol-resistant pipet tips
5. Cell culture (T-75 flask)
6. 15 ml conical tubes
7. 50 ml conical tubes
8. Disposable gloves
9. 10% SDS
10. Saturated NaCl
11. TE buffer
12. 1X trypsin-EDTA
13. 1X PBS
14. Ice in buckets
15. Inverted microscope
16. Incubator or water bath at 48°C
17. Tabletop clinical centrifuge

## Procedure

1. Decant the growth medium from the cell culture flask. Place the growth medium in a 15 ml conical tube, and save for Step 5.
2. Wash the cell monolayer twice with 1X PBS (free of calcium and magnesium), decant, and discard.
3. Add 2 ml of 1X trypsin-EDTA to each cell culture flask. Incubate the flask in the palm of your hands for 30–60 sec. Decant, and discard the trypsin. Under the inverted microscope, observe the "rounding up" of the cells.

**Note:** In the absence of a microscope, the "rounding up" of the cells can be assessed by holding the flask up to a light source. As the refractive index changes due to the cells rounding up, the bottom of the flask (which the cells are attached to) will appear cloudy or foggy. The extent of this foggy appearance will depend on the degree of cell rounding and the density of the cell population. It is extremely important not to lyse the cells in the presence of trypsin.

4. To completely dislodge the cells, strike the flask against the palm of your hand. It might be necessary to strike the flask several times against your hand to completely dislodge the cells.
5. To inactivate the trypsin, add 5 ml of the saved medium from Step 1.
6. Transfer the cell suspension to a 15 ml conical tube (or suitable centrifuge tube), and centrifuge at 150–200 × g for 3–5 min.
7. Decant the supernatant, and resuspend the cell pellet 4.5 ml in of TE-9 buffer. Add 500 µl of 10% SDS, and invert the tube to mix.
8. Add 125 µl of proteinase K to each tube, and invert to mix. Incubate the samples at least 30 min at 48°C.
9. Add 1.5 ml of saturated NaCl solution to each tube, and shake for 15 sec. The lysate should become and remain cloudy.
10. Centrifuge at 500 × g for 10 min to pellet proteins.

11. Decant the supernatant containing the DNA into a fresh 15 ml tube. Centrifuge for an additional 10 min.
12. Decant the supernatant containing the DNA into a fresh 50 ml conical tube, and place on ice (see Exercise 2 for instructions on how to concentrate the DNA).

## *4. Differential Extraction*

Differential extraction is the method of choice when biological samples are suspected of containing cells from more than one contributor. Differential extraction is commonly used to isolate the male and female components from a sample containing DNA from a male and female contributor. Consequently, differential extraction is used to separate sperm cells from "nonsperm" cells in sexual assault cases. This nonsperm category includes the epithelial cells (or skin cells) found in saliva, buccal swabs, vaginal swabs, urine, and feces. The different properties of sperm cells are exploited to separate them from these "nonsperm" or epithelial cells. The separation of the sperm (sometimes referred to as the male fraction) from the epithelial cells (referred to as the female fraction) provides a DNA profile that is easier for the forensic DNA analyst to interpret in a rape case.

### Equipment and Material

1. TNE buffer
2. 20% sarkosyl
3. Sterile deionized water
4. Proteinase K (20 mg/ml)
5. Adjustable-volume digital micropipets (20–1000 µl range)
6. Aerosol-resistant pipet tips
7. 1 M DTT
8. 2.0 ml screw-cap microcentrifuge tube
9. 0.5 ml microcentrifuge tube
10. 2.0 ml screw-cap tubes (conical tubes)
11. Disposable gloves
12. Phenol:$CHCl_3$:isoamyl alcohol (25:24:1)
13. Stain extraction buffer
14. Vortex mixer
15. Microcentrifuge
16. Incubator or water bath at 37°C and 56°C

### Procedure

**Extraction of DNA from Mixtures (or Mixed Stains)**

1. The questioned sample containing a stain thought to contain a mixture of sperm and epithelial cells is placed in a 2.0 ml screw-capped Eppendorf or microcentrifuge tube.
2. Mild detergents are then added to remove the stain containing the cells from the material.

Add:
400 µl TNE
25 µl 20% sarkosyl
75 µl sterile deionized water
5 µl 20 mg/ml proteinase K

3. Mix the sample, and centrifuge for 2 sec. Incubate the sample at 37°C for 2 hrs.
4. Punch a hole in the bottom of a 0.5 ml microcentrifuge tube. Place stained swab into the 0.5 ml tube, and place the 0.5 ml tube into the 2.0 ml tube from which the stained swab was removed. Align the tab on the 0.5 ml tube with the case number label on the 2.0 ml tube.
5. Spin the tube for 5 min.
6. Remove the 0.5 ml tube, and place the swab in a clean 2.0 ml screw-capped tube. This is Fraction 2 (F2).
7. Transfer the supernatant to a clean 2 ml screw-capped tube. This is Fraction 1 (F1). Set aside. The remaining pellet in the tube is the male fraction (M).
8. Add the following components to the pellet labeled (M):
   150 µl TNE
   150 µl $H_2O$
   50 µl 20% sarkosyl
   40 µl 1M DTT
   10 µl 20 mg/ml proteinase K
9. Mix, and incubate at 37°C for 2 hrs.
10. Add 500 µl of stain extraction buffer to the swab (F2).
11. Incubate overnight at 56°C.

**Note:** The following steps should be carried out in an exhaust hood.

12. Add 400 µl phenol:$CHCl_3$:isoamyl alcohol to each male fraction and 500 µl of the same to each female fraction.
13. Vortex, and centrifuge for 2 min.
14. Transfer the top aqueous layer to a new tube. Dispose of the phenol:$CHCl_3$:isoamyl alcohol solution in the collection tube, and dispose of the test tube in the appropriate biohazard waste container.

**Note:** Steps 15, 16, and 17 are optional unless using Centricon concentration.

15. Add 500 µl $CHCl_3$ to the tube.
16. Vortex, and spin for 2 min.
17. Transfer the supernatant to a new tube *or* remove the bottom layer and discard. Do not disturb the interface. Dispose of the $CHCl_3$ solution in the collection tube. Dispose of the test tube in an appropriate biohazard waste container. The sample is now ready for precipitation or concentration.

## 5. DNeasy Blood and Tissue Kit

The DNeasy Blood and Tissue Kit (QIAGEN, Inc., Valencia, California) is designed for the rapid isolation, purification, and concentration of total DNA from animal tissue and/or cells. The buffer system, which is supplied by the manufacturer, allows for direct cell lysis followed by selective binding of the DNA to a


**FIGURE 1**
DNeasy Blood and Tissue Procedure for the Isolation, Purification, and Concentration of DNA. *Source*: Courtesy of QIAGEN, Inc.

silica gel-membrane. The lysate is loaded onto the DNeasy minicolumn and briefly centrifuged. During centrifugation, the DNA binds to the membrane in the minicolumn while contaminants and enzyme inhibitors (e.g., proteins and divalent cations) "pass through" the membrane into a collection tube. Following two wash steps, the DNA is eluted in water or a buffer, and is ready for use (Figure 1). The entire procedure and protocol are presented in Exercise 2.

## Results

Before any analysis proceeds, it is important to determine the success of your extraction. It is important to determine the quality and quantity of DNA present and determine if any degradation of the DNA has occurred. The answers to these questions as well as guidelines for interpreting your results are described in Exercise 4.

## Samples Extracted

Analyst: _____ Lab Number: _____

Date: _____

| Item Number and/or Description | Extraction Method | Purification Method | Item Number and/or Description | Extraction Method | Purification Method |
|---|---|---|---|---|---|
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

*C = Chelex extraction. S = Salting-out extraction. O = Organic extraction. D = Differential extraction.*

**Comments**:

_____

_____

_____

_____

## Questions

1. What are the factors that a DNA analyst considers when determining the isolation and extraction procedure to use when analyzing a sample?

2. In your attempt to extract DNA from various samples (both known and evidentiary), several isolation techniques were explored. What technique(s) or method of choice would be used if an evidentiary sample was suspected to contain sperm? Why?

3. In all of the extraction procedures discussed proteinase K and/or a detergent (SDS or sarkosyl) was used in the process. What is the purpose of proteinase K? What is the purpose of the detergents?

Chapter 4

# Exercise 2

## *Concentration of Extracted DNA*

## Introduction

Following the removal and purification of the DNA from the sample, the next step is to concentrate the DNA. Various methods exist to concentrate DNA. Two widely used methods includes concentrating extracted DNA by precipitation with ethanol or using a column filtration system (DNeasy Blood and Tissue Kit, QIAGEN, Inc.) to concentrate the DNA. Both techniques are rapid and are quantitative even with nanogram amounts of DNA.

## Objective

To concentrate isolated DNA using two different techniques: ethanol precipitation and column filtration (DNeasy Blood and Tissue Kit).

## Equipment and Material

1. Phosphate-buffered saline (PBS)
2. 1X TE buffer
3. DNeasy Blood and Tissue Kit
4. 1.5 ml Eppendorf or microcentrifuge tubes
5. Adjustable-volume digital micropipets (2–200 µl range)
6. Aerosol-resistant pipet tips

7. Absolute ethanol (EtOH; 70% and 96–100%)
8. Disposable gloves
9. Ice in buckets, –20°C, or –70°C freezer
10. Incubator or water bath at 37°C, 56°C, and 70°C
11. Microcentrifuge
12. Tabletop clinical centrifuge

## Procedure

The use of disposable gloves and aerosol-resistant pipet tips is highly recommended to prevent cross-contamination. Helpful organizational sheets are provided at the end of the exercise.

### *A. Precipitation of DNA Using Ethanol*

1. Estimate the volume of the DNA solution, and add exactly two volumes of ice-cold absolute ethanol (EtOH). The extracted samples from Exercise 1 contain approximately 500 µl of DNA solution. Add 1 ml of cold absolute EtOH to these samples or tubes (containing the aqueous layer). Mix by hand.
2. Place the tube containing the EtOH and sample on ice for 30 min, *or* place the tube in the freezer at –70°C for 30 min. Usually 30–60 min at –20°C is sufficient to allow the DNA precipitate to form.
3. Centrifuge the DNA solution containing the EtOH for 15 min. For most purposes, 10 min using a microcentrifuge at 12,000 × g is sufficient. After centrifugation, decant EtOH.
4. Rinse the DNA pellet with 1 ml of 70% EtOH (room temperature), and centrifuge for 10 min. After centrifugation, decant EtOH.
5. Stand the tube in an inverted position on a layer of absorbent paper until dry (approximately 30 min), or air-dry samples in a secure place.
6. Dissolve the DNA pellet in 36 µl or the desired volume of 1X TE.
7. Resuspend the DNA at 56°C for no more than 2 hrs. To assist in dissolving the pellet, the sample can be heated to 37°C.
8. Store the sample at 4°C in an Eppendorf or microcentrifuge tube.

### *B. Concentration of DNA Using the DNeasy Blood and Tissue Kit*

1. Centrifuge the sample for 5 min at 300 × g at room temperature. If human cell lines (e.g., HepG, HeLa, or K562) are used, centrifuge approximately 1–5 × $10^6$ cells/ml under the same conditions. Cell lines that are archorage dependent will need to be trypsinized prior to harvesting (see Exercise 1, Section 3, entitled "Salting Out").
2. After centrifugation, decant the supernatant, and add 200 µl of PBS to the pellet.
3. Add 200 µl of "Buffer AL" (provided by the manufacturer of the DNeasy Blood and Tissue Kit) to the resuspended cell pellet (see Figure 1). Mix thoroughly, and incubate at 70°C for 10 min.

4. Add 200 µl of 96–100% EtOH to the sample, and mix thoroughly. The sample containing the Buffer AL should be mixed thoroughly with the EtOH to ensure a homogeneous solution. A white precipitate may form with the addition of EtOH.
5. Place the DNeasy spin column in a 2 ml collection tube (spin columns and collection tubes are provided in the kits by the manufacturer).
6. Place the extracted DNA (or mixture from Step 4) into the spin column, and centrifuge at greater than 6000 × g at room temperature for 1 min. The "flow through" that contains the unwanted cellular material is discarded along with the collection tube.
7. The DNeasy spin column, containing the DNA, is placed in a *new* 2.0 ml collection tube (provided in the kit).
8. Add 500 µl of "Buffer AW1" to the spin column, and centrifuge the column and tube at 6000 × g at room temperature for 1 min.
9. Following centrifugation, the "flow through" again is discarded along with the collection tube, and the spin column placed in a new 2.0 ml collection tube.
10. Pipet 500 µl of "Buffer AW2" into the spin column, and centrifuge the column or tube at full speed for 3 min. This centrifugation step ensures that no residual EtOH is carried over during the following elution.
11. Following centrifugation, the "flow through" is discarded, and the spin column placed in a 1.5 or 2 ml microcentrifuge tube. Pipet 200 µl of "Buffer AE" onto the column, and incubate at room temperature for 1 min.
12. The spin column and microcentrifuge tube are then centrifuged at 6000 × g for 1 min to elute the DNA.

## Results

Before any analysis proceeds, it is important to determine the success of this concentration procedure. It is important to determine the quality and quantity of DNA present. It is also important to determine if any degradation of the DNA has occurred. The answers to these questions as well as guidelines for interpreting your results are described in Exercise 4.

## Precipitation of DNA Using Ethanol

Analyst: _____ Lab Number: _____

Date: _____

| Item Number and/or Description | Extraction Method | Purification Method | Item Number and/or Description | Extraction Method | Purification Method |
|---|---|---|---|---|---|
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

**Comments**:

_____

_____

_____

_____

## Qiagen Extraction/Purification Method
## Manual Spin Columns

Analyst: _____ Date: _____

System: _____ Gel Number: _____

| Reagent | Lot/Source |
|---|---|
| 1 X PBS | |
| Protese | |
| AL Buffer | /Qiagen |
| 95% EtOH | |
| AW1 Buffer | /Qiagen |
| AW2 Buffer | /Qiagen |
| AE Buffer | /Qiagen |

Lysate Transfer Witness: _____

Elution Tube Transfer Witness:_____

## Questions

1. What is the purpose of concentrating the extracted DNA?

2. Two different concentration techniques were discussed: ethanol precipitation and column filtration. What are the advantages and disadvantages when using these techniques to concentrate DNA?

Chapter 5

# Exercise 3

## *Microcon Concentration and Purification of Extracted DNA*

## Introduction

After DNA extraction, centrifugal filter devices, such as the Microcon purification procedure, can serve as powerful tools in DNA concentration and desalting procedures. Ultrafiltration (UF) is a pressure-driven, convective process that uses semipermeable membranes to separate DNA by molecular size and shape. Ultrafiltration is highly efficient, allowing for concentration and purification at the same time. Unlike the use of chemical precipitation methodologies (as in Exercises 1 and 2 using ethanol or phenol/chloroform), there is no phase change or possible degradation of the DNA with UF. Ultrafiltration routinely concentrates DNA, without the use of co-precipitants, in a short time period with 99% recovery of the starting material. Centrifugal concentrator devices are ideal for separating high and low molecular weight DNA molecules.

The Microcon purification procedure is often used when the biological sample that was extracted was deposited on a substrate (e.g., denim or velvet) known to inhibit DNA amplification or the polymerase chain reaction (see Exercise 6) due to the substrate releasing excessive amounts of dye during the extraction process. In this exercise, the DNA sample is concentrated, then diluted to the original volume with the desired buffer and concentrated again, thus "washing out" inhibitors or the original solvent.

## Objective

The purpose of this exercise is to concentrate and remove unwanted components from isolated DNA using ultrafiltration and the Microcon purification procedure.

## Equipment and Material

1. Microcon 100 Concentrator Assembly (Millipore Corp.)
2. Adjustable-volume digital micropipets (2–200 µl range)
3. Aerosol-resistant pipet tips
4. 1X TE buffer
5. 1.0 or 1.5 ml microcentrifuge tubes
6. Sterile $H_2O$
7. Transfer pipets
8. Disposable gloves
9. Phenol:$CHCl_3$:isoamyl alcohol (25:24:1)
10. Bromophenol blue tracking dye (loading buffer)
11. Microcentrifuge

## Procedure

The use of disposable gloves and aerosol-resistant pipet tips is highly recommended to prevent cross-contamination. A helpful organizational sheet is provided at the end of the exercise.

### *A. Concentrating the DNA Using a Microcon Concentrator*

1. Use a Microcon concentrator that is adequate for your DNA size (e.g., use the Microcon 50 concentrator for minute biological stains or materials). Add 500 µl of prewarmed (room temperature) phenol:$CHCl_3$:isoamyl alcohol to each tube containing the DNA.
2. Cap the tube, and mix thoroughly by hand for 2–3 sec or until the solution has a milky appearance.
3. Centrifuge the tube(s) for 3 min in a microcentrifuge at greater than 6000 × g to separate the two phases.
4. Insert a labeled Microcon 100 concentrator into a labeled collection vial. Add 500 µl of sterile $H_2O$ to the concentrator. Using a transfer pipet, transfer the aqueous or top phase containing the DNA (from Step 3, above) to the Microcon concentrator. Place the cap from the collection vial on the concentrator.
5. Centrifuge the Microcon assembly in a microcentrifuge for 10–30 min at approximately 5000 rpm (× g or relative centrifugal force [RCF] determined by the rotor used) until the volume is reduced.
6. After centrifugation, remove the concentrator "unit" from the Microcon assembly, and discard the fluid from the filtrate cup. Return the concentrator to the top of the filtrate cup.
7. Remove the cap from the concentrator, and add 200 µl of sterile $H_2O$. Replace the cap, and centrifuge at 5000 rpm (× g or RCF determined by the rotor used) for 10–30 min until the volume is reduced.
8. Remove the cap from the concentrator, and add 30 µl of 1X TE buffer.
9. Remove the concentrator from the filtrate cup, and carefully invert the concentrator onto the retentate cup. Discard the filtrate cup.
10. Centrifuge the Microcon assembly (the retentate cup end first) at 5000 rpm (× g or RCF determined by the rotor used) for 5 min.

*Optional*:

11. Remove 4 µl of the sample, and place in a separate 1.0 ml tube. Add 2 µl loading buffer to the sample (see Exercise 4).
12. Run the sample on a test agarose gel for quantitation (see Exercise 4).

## Results

Before any analysis proceeds, it is important to determine the success of your extraction, purification, and concentration of the DNA. It is important to determine the quality and quantity of DNA present. It is also important to determine if any degradation of the DNA has occurred. The answers to these questions as well as guidelines for interpreting your results are described in Exercise 4.

## Microcon Concentration and Purification of Extracted DNA

Analyst: _____ Lab Number: _____

Date: _____

| Item Number and/or Description | Extraction Method | Purification Method | Item Number and/or Description | Extraction Method | Purification Method |
|---|---|---|---|---|---|
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

**Comments**:

_____

_____

_____

_____

## Questions

1. What are the advantages of the Microcon Concentrator Assembly over the previous extraction processes described?

2. What are the advantages over the two techniques (ethanol precipitation and column filtration) described previously to concentrate extracted DNA?

Chapter 6

# Exercise 4

## *Assessing the Quality and Quantity of Isolated DNA*

## Introduction

To determine the quality and quantity of extracted or isolated DNA recovered from a sample (known or evidentiary), preliminary tests are conducted. Two tests are often used to assess the quantity (how much DNA is present) or quality (how much, if any, degradation has occurred) of the DNA.

In the first test, a miniature-agarose gel or yield gel is used to estimate both the quality and quantity of DNA recovered from each sample. A yield gel is prepared, and a small portion of each DNA sample is "loaded" into separate wells of the gel. The DNA is analyzed by agarose gel electrophoresis, stained to visualize the DNA by UV illumination, and photographed (or the images are generated using computer software). Documentation for each gel is maintained and indicates the DNA samples that have been included on a particular yield gel, along with the appropriate controls (i.e., visual marker, HindIII-digested lambda DNA; human K562 DNA control or intact lambda DNA for calibration or quantity determination; and undigested K562 DNA). Large, intact, and undegraded DNA will appear as a compact band near the origin of the gel, similar to the standards in the adjacent lanes. Degraded DNA will form a smear and will migrate further through the gel depending on the various sizes of the DNA fragments. Degraded DNA will also be observed following hybridization and autoradiography and/or chemiluminography (Figures 2 and 3). Extremely degraded DNA may not be visible because these smaller fragments will migrate toward the end of the gel. The quantity of the DNA in question can be compared to DNA standards of known quantity that have been run in adjacent lanes.

The second method consists of the slot or dot blot technique, which is used to determine only the quantity of the DNA recovered from a sample. A small portion of the sample DNA in question is applied to a membrane along with a set of standard samples of known quantity. After the samples have been "fixed" to the membrane, a human DNA probe is added and allowed to hybridize to the fixed DNA. The DNA probe used in this instance is tagged or labeled (e.g., with an enzyme or fluorescent dye) for easy detection of the DNA (Figure 4). The slot or dot blot technique does not provide any information on the quality or the level of degradation of the DNA.

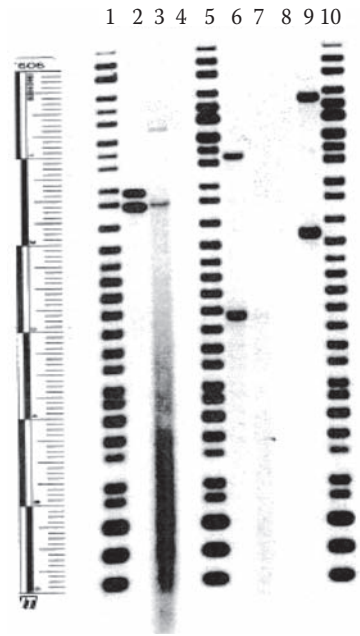



**FIGURE 2**
Restriction Fragment Length Polymorphism (RFLP) Lumigraph (see Exercise 9) Demonstrating DNA Degradation. **Note:** Lanes 1, 5, and 10 contain molecular ladders. Lanes 2 and 9 serve as positive controls and contain a known reference sample. Lane 3 contains degraded DNA from the nonsperm fraction from the victim. Lane 6 contains the suspect's reference sample. Lanes 4 and 8 are blank. Lane 7 contains the sperm fraction from the sexual assault sample. The suspect is a heterozygote (2 DNA fragments) at this locus because two (2) DNA fragments were observed. However, only one (1) faint DNA fragment (the lower molecular weight fragment) was observed at this locus from the sexual assault sample, which would lead the DNA analyst to possibly render an inconclusive result.


**FIGURE 3**
Restriction Fragment Length Polymorphism (RFLP) Lumigraph (see Exercise 9) Demonstrating DNA Degradation. **Note:** Lanes 1, 5, and 10 contain molecular ladders. Lanes 2 and 9 serve as positive controls and contain a known reference sample. Lane 3 contains degraded DNA from the nonsperm fraction from the victim. However, two (2) DNA fragments are observed. Lane 6 contains the suspect's reference sample. Lanes 4 and 8 are blank. Lane 7 contains the sperm fraction from the sexual assault sample. The suspect's DNA profile is observed as well as the victim's profile in this sexual assault sample. It should be noted that the "carryover" is evident from the nonsperm fraction into the sperm fraction. Also evident in the sperm fraction are DNA fragments that align with the suspect's two-band profile.

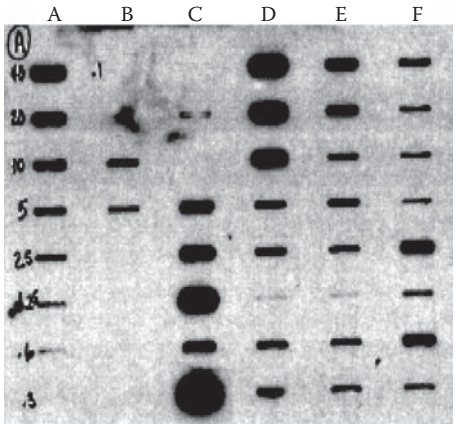



**FIGURE 4**
Slot Blot Used to Determine the Quantity of Human DNA recovered from a Sample. **Note:** The first column or lane (A) contains the quantitation standards in decreasing order from top to bottom (40, 20, 10, 5, 2.5, 1.25, 0.6, and 0.3 ng DNA). The remaining lanes contain "responses" from the evidence (Lanes B and C) and reference samples (Lanes D and E) as well as from the positive (Lane F) and negative (bottom of Lane B) controls. The amount of DNA contained in a sample (evidentiary or reference) is obtained by comparing the response to the standards.

## Objective

To determine the quality and quantity of DNA isolated from a known sample, from an evidentiary sample, or from a human cell line (e.g., HepG, HeLa, and K562) using agarose gel electrophoresis.

## Equipment and Material

1. Agarose
2. Ethidium bromide or coomassie blue
3. Ice in buckets
4. TBE buffer
5. K562 DNA
6. 125 ml Erlenmeyer flask
7. Adjustable-volume digital micropipets (2–200 µl range)
8. 1.0 or 1.5 ml microcentrifuge tubes
9. Aerosol-resistant pipet tips
10. Bromophenol blue tracking dye
11. TAE buffer
12. Disposable gloves
13. Power pack or power supply
14. Microwave or hot plate
15. Incubator or water bath at 56°C
16. Electrophoresis systems (gel tray or combs)
17. UV transilluminator
18. Polaroid camera with film

## Procedure

The use of disposable gloves and aerosol-resistant pipet tips is highly recommended to prevent cross-contamination. Helpful organizational sheets are provided at the end of the exercise to record your data and observations.

1. Remove 4 µl of extracted and/or resolubilized DNA, and combine with 2 µl tracking dye. This may be done in a centrifuge tube or in a microtiter plate.
2. Preparation of 5% agarose test gel:
   A. Add 1.25 g of DNA Typing Grade Agarose in 25 ml of TAE buffer containing ethidium bromide (EB) at a ratio of 10 µl EB/100 ml TAE buffer.

**Note:** Some laboratories will stain the gel with ethidium bromide after electrophoresis instead of adding ethidium bromide to the TAE containing the agarose or the reservoir buffer.

   B. Heat the solution in a microwave on high for about 40 sec, swirling the flask by hand every 10–15 sec, or briefly bring to a boil to dissolve the agarose using a hot plate.
   C. Cool the liquid agarose to about 56°C.
   D. While the liquid agarose is cooling, prepare the gel casting tray or gel form according to the manufacturer's guidelines. When cool, pour the agarose into the gel form. Use either one or two 14-well combs.

**Note:** The number of wells (or teeth) in a comb will vary according to the manufacturer.

   E. Let the liquid agarose "stand" or cool in the gel tray for 10 min to solidify.
   F. Remove gel dams, and pour approximately 175 ml of the TAE–EB buffer into an electrophoresis gel tank.
   G. Remove the comb(s).

**Note:** If the gel is not ready to be loaded 10 min after pouring, add the buffer anyway so that the gel will not dry out.

3. The DNA sample, mixed with loading buffer (tracking dye), is pipetted into the well with the gel submerged. The final sample volume should contain 10% tracking dye, and the total volume should not exceed 20 µl. Be careful not to push the pipet tip through the bottom of the well in the gel.
4. Include on your gel the following K562 DNA Standards:
   500 ng/4 µl
   250 ng/4 µl
   125 ng/4 µl
   63 ng/4 µl
   31 ng/4 µl
   15 ng/4 µl

**Note:** Intact lambda DNA can be used in quantities ranging from 300 ng to 10 ng.

5. Set the voltage (100 volts), and "RUN" the samples until the bromophenol blue tracking dye has moved 1–2 cm from the origin (i.e., well) or until the dye front is approximately 2 cm from the end of the gel. This should take less than 20 min.
6. Remove the gel from the electrophoresis tank. Stain the gel in SYBR Green or ethidium bromide (if not added previously to the agarose gel or buffer reservoir).

7. Examine the gel on an ultraviolet (UV) transilluminator. Take a photograph of your gel. Intact DNA will move as a band not far from the origin. A smear from the origin to or past the dye front indicates that the DNA has been fragmented and may not be suitable for further use.

**Warning:** Avoid excessive exposure to the UV light. Always wear a full face shield when working with the transilluminator.

## Yield Gel

Analyst: _____ Lab Number: _____

Date: _____

| Well No. | Sample | DNA (ng) |
|---|---|---|
| 1 | Visual marker | |
| 2 | Standard | 500 |
| 3 | Standard | 250 |
| 4 | Standard | 125 |
| 5 | Standard | 63 |
| 6 | Standard | 31 |
| 7 | Standard | 15 |
| 8 | Sample | |
| 9 | “ | |
| 10 | “ | |
| 11 | “ | |
| 12 | “ | |
| 13 | “ | |
| 14 | “ | |
| 15 | “ | |
| 16 | “ | |
| 17 | “ | |
| 18 | “ | |
| 19 | “ | |
| 20 | “ | |

| Reagents | Lot Number | Source |
|---|---|---|
| Agarose | | |
| 1X TAE (Gel Buffer) | | |
| 1X TAE (Tank Buffer) | | |
| Loading Buffer | | |
| Ethidium Bromide | | |
| Visual Marker | | |
| 500 ng standard | | |
| 250 ng standard | | |
| 125 ng standard | | |
| 63 ng standard | | |
| 31 ng standard | | |
| 15 ng standard | | |

## Gel Electrophoresis

| | | |
|---|---|---|
| Time on: | Voltage: | mAMPs: |
| Time off: | Voltage | mAMPs: |

Gel Prepared By: _____ Date: _____

Tape Your Gel Photo Here

Lane #1 _____

Lane #2 _____

Lane #3 _____

Lane #4 _____

Lane #5 _____

Lane #6 _____

Lane #7 _____

Lane #8 _____

Lane #9 _____

Lane #10 _____

Lane #11 _____

Lane #12 _____

Lane #13 _____

Lane #14 _____

Lane #15 _____

Lane #16 _____

Lane #17 _____

Lane #18 _____

Lane #19 _____

Lane #20 _____

## Yield Gel

Analyst: _____ Lab Number: _____

Date: _____

| Well No. | Sample | DNA (ng) |
|---|---|---|
| 1 | | |
| 2 | | |
| 3 | | |
| 4 | | |
| 5 | | |
| 6 | | |
| 7 | | |
| 8 | | |
| 9 | | |
| 10 | | |
| 11 | | |
| 12 | | |
| 13 | | |
| 14 | | |
| 15 | | |
| 16 | | |
| 17 | | |
| 18 | | |
| 19 | | |
| 20 | | |

# Results

## Interpreting Your Results

1. While under UV illumination, take a photograph of the gel and attach it to the Reporting Form.
2. From the photograph, estimate the quality of the DNA in the test specimens by comparison to the uncut K562 DNA standards.
   - Intact DNA: Large, intact, and undegraded DNA will appear as a compact band near the origin of the gel, similar to the standards in the adjacent lanes.
   - Degraded DNA: Degraded DNA will form a smear along the lane and will migrate further through the gel depending on the various sizes of the DNA fragments. Degraded DNA will also be observed following hybridization and autoradiography and/or chemiluminography (see Figures 2 and 3). Extremely degraded DNA may not be visible because these smaller fragments will migrate toward the end of the gel.
3. Record the quality of DNA (i.e., intact versus degraded) in the "Results" section, using the "Sample" column in the "Yield Gel" form.
4. From the photograph, estimate the quantity of DNA in test specimens. The quantity of the DNA in question can be compared to DNA standards of known quantity that have been run in adjacent lanes.
   - A single estimate should be made (not a range), and the quantity of total DNA remaining per 4 µl and 32 µl of the sample should be recorded on the worksheet. The estimation is multiplied by 8 to obtain the total quantity of DNA in the remaining 32 µl of the sample. Record the estimated quantity of DNA in the "Results" section using the "Yield Gel" form.

## Questions

1. Once the DNA has been isolated and before any analysis can proceed, it is imperative to determine the quality and quantity of DNA present. Why is it important to determine the quantity and quality of DNA in a sample?

2. How would a DNA analyst determine, in one (1) experiment, the quantity and quality of DNA from a given sample?

3. If the evidentiary sample to be analyzed was degraded, what are the many "forms" of DNA that would be expected following gel electrophoresis?

Chapter 7

# Exercise 5

## *DNA Analysis Using Restriction Fragment Length Polymorphisms (RFLP)*

## Introduction

The first widespread use of DNA tests involved restriction fragment length polymorphism (RFLP) analysis, a test designed to detect variations in the DNA from different individuals. In the RFLP method, DNA is isolated from a biological specimen (e.g., blood, semen, and vaginal swabs) and digested or cut by enzymes called restriction endonucleases (or RENs). The resulting DNA or restriction fragments are separated by size into discrete bands by gel electrophoresis. Following electrophoresis, the separated restriction fragments are transferred onto a membrane by Southern "blotting," and identified using probes (known DNA sequences that are "tagged" with a chemical tracer). The resulting DNA profile is visualized by exposing the membrane to a piece of X-ray film, which allows the scientist to determine which specific fragments the probe identified among the thousands in a sample of human DNA. A "match" is made when similar DNA profiles are observed between an evidentiary sample and a suspect's DNA or known reference sample. A determination is then made as to the probability that a person selected at random from a given population would match the evidence sample as well as the suspect. The entire analysis may require several weeks to complete.

## Objective

In this exercise, you will gain experience with RFLP, the first test adapted for forensic DNA analysis. During this exercise, you will learn how to use restriction enzymes to digest or cut DNA and how to separate the resulting DNA fragments by agarose gel electrophoresis. You will also learn how to transfer DNA fragments onto a membrane (for support) by Southern blotting to allow for the detection of allelic patterns following hybridization.

## Equipment and Material

1. 1.0, 1.5, and 2.0 ml Eppendorf or microcentrifuge tubes
2. HaeIII restriction enzyme
3. Adjustable-volume digital micropipets (2–200 µl range)
4. Aerosol-resistant pipet tips
5. HindIII-digested lambda DNA
6. HaeIII-digested K562 DNA
7. KpnI-digested Adenovirus DNA
8. Molecular weight markers (526 to 22,621 bp)
9. Herring sperm DNA (10 mg/ml)
10. Ethidium bromide or coomassie blue
11. Bromophenol blue tracking dye
12. Kimwipes™
13. Neutralization solution
14. 125 ml Erlenmeyer flask
15. TBE buffer
16. Agarose
17. 1X TAE buffer
18. Sterile distilled water
19. Ice in buckets
20. 0.5X Wash I solution
21. 1X Wash I solution
22. 1X Wash I solution concentrate
23. Labeled DNA probes
24. Plastic box or tray
25. Glass baking dish
26. 30 or 50 ml conical tube
27. Nylon membrane
28. Denaturation solution
29. Disposable gloves
30. 20% SDS
31. 20X SSPE
32. 10X SSC
33. 50% PEG
34. TE buffer
35. Thin sponges

36. Whatman 3MM chromatography paper
37. Kodak X-Omat RP film and cassette
38. Blotting pads or paper towels
39. Glass or plastic plate
40. Glad or Saran wrap
41. Orbital shaker or rotator
42. Vortex mixer
43. Microwave or hot plate
44. Electrophoresis systems (gel tray or combs)
45. Incubator or water bath at 37°C, 55°C, and 65°C
46. UV transilluminator
47. Microcentrifuge
48. Vacuum oven
49. Power pack or supply
50. Polaroid camera with film

## Procedure

The use of disposable gloves and aerosol-resistant pipet tips is highly recommended to prevent cross-contamination. Helpful organizational sheets and semilog graph paper are provided at the end of the exercise.

### *A. Digesting DNA with Restriction Endonucleases (RENs)*

Restriction endonucleases are enzymes that recognize specific sequences within double-stranded DNA. Hundreds of different RENs have been isolated, and most recognize different sequences. To ensure uniformity and consistency in the forensic community, the most often used REN for forensic casework is HaeIII. As with all RENs, HaeIII has a set of optimal reaction conditions specified on the product insert or information sheet provided by the manufacturer. The major variables in the restriction digests are the temperature of incubation and the composition of the reaction buffer. Restriction digests or reactions typically contain approximately 1 µg of DNA in a volume of 20 µl or less. Once the concentration of DNA in the sample(s) has been determined (see Exercise 4), the volume of DNA sample to be added to the reaction is adjusted accordingly.

1. If the quantity of DNA in the sample exceeds 1000 ng, dilute the sample in a microcentrifuge tube and bring the final volume up with sterile water to: a) a minimum of 18 µl to, b) a maximum of 32 µl.
2. To each sample containing DNA, add: a) 2.0 µl of 10X digestion buffer (supplied with enzyme) or, b) 4.0 µl of 10X digestion buffer.
3. Add 1 unit of the restriction enzyme (HaeIII), mix by tapping the tube, and centrifuge the microcentrifuge tube containing the sample(s) for 2 sec.

**Note:** One unit of enzyme is defined as the amount required to digest 1 µg of DNA to completion in one (1) hr in the recommended buffer and at the recommended temperature, usually 37°C. The volume of restriction enzyme added should never be more than 10% of the final digestion volume.

**Note:** Always keep HaeIII or any restriction enzyme on ice.

4. Incubate the reaction tube at 37°C for 1 hr.
5. Centrifuge the reaction tube for 5 sec.

## *B. Digest Gel to Measure Completeness of Restriction Digestion*

The purpose of the digest gel is to assess the completeness of the HaeIII digestion of the specimen DNA before proceeding to an RFLP analytical gel (see below). Controls include HindIII-digested lambda DNA and approximately 200 ng (3 µl) of HaeIII-digested K562 DNA.

1. To determine that the reaction is complete and that the sample DNA has been completely digested, remove 4 µl of the HaeIII-digested DNA, and combine with 2 µl loading buffer (tracking dye). This may be done in a microcentrifuge tube.
2. Run the digested sample(s) on an agarose test gel to determine if restriction digestion is complete (see Exercise 4 for details on preparing an agarose test gel).
3. Completely digested DNA will be present on this gel as a uniform smear composed of different-sized fragments "running" from the origin down the length of the lane to the dye front (Figure 5, Lane 4). Proceed to the next step ("C. Resolution of DNA Fragments on an Analytical Gel") if the samples are completely digested. Uncut DNA will form a compact, fluorescent large band near the origin of the gel (Figure 5, Lane 3). Partially digested DNA will appear as multiple high and low molecular weight DNA bands (Figure 5, Lane 5).

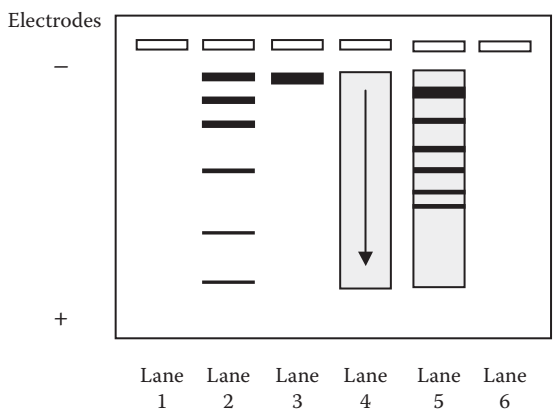



**FIGURE 5**
Digest Gel Demonstrating Completely and Incompletely Digested DNA Samples. *Note*: Lanes 1 and 6 are blank (no DNA); Lane 2 contains the molecular weight markers; Lane 3 contains a single, high molecular weight band demonstrating undigested DNA; Lane 4 contains completely digested DNA illustrating a uniform "smear" of high to low molecular weight DNA fragments (also refer to Figures 2 and 3, Lane 3); and Lane 5 contains partially digested DNA showing evidence of a substantial amount of high molecular weight DNA.

## C. Resolution of DNA Fragments on an Analytical Gel

DNA fragments generated by REN digestion are separated by conventional gel electrophoresis using a larger agarose gel format that is often referred to as an analytical gel. The analytical gel is designed to separate the REN-digested DNA (i.e., the HaeIII-digested DNA isolated from the different specimens) based on size. The analytical gels contain several controls: KpnI-digested Adenovirus DNA (nine fragments ranging in size from 1086 to 771 bp), molecular weight markers (30 viral DNA fragments ranging in size from 526 to 22,621 bp), and HaeIII-digested K562 DNA (human DNA control). The analytical gels are generally composed of 1% agarose in 1X TAE buffer. The gel dimensions are 11 × 14 × 0.65 cm (usually less than 100 ml total volume is needed).

1. Prepare the analytical gel:
   A. For each analytical gel, prepare 1 liter of 1X TAE buffer (50 ml of 20X TAE combined with 950 ml of distilled $H_2O$).
   B. To a 125 ml Erlenmeyer flask, add 1.0 g of agarose into a flask. Add 100 ml of 1X TAE buffer containing ethidium bromide (EB; 10 µl EB/100 ml of TAE buffer).

**Optional:** Ethidium bromide (20 mg/ml) can be added directly to the gel at this stage, or the gel can be stained in EB following electrophoresis.

   C. Microwave on high for about 40 sec. Swirl the flask and continue to microwave the agarose solution on high for another 40 sec, *or* bring to boil to dissolve agarose using a hot plate.
   D. Prepare the gel-casting tray (according to the manufacturer's guidelines) so that the open ends are sealed or secured and the tray is level.
   E. Place a comb into the gel-casting tray with the flat edge of the comb nearest you. The number of samples to be analyzed will help determine the number of "teeth," or wells, on the comb needed.
   F. Allow the agarose to cool to 50–60°C prior to casting. After pouring the agarose into the gel tray, let the solution "cool" for at least 15 min to solidify.
2. Pour the remaining 900 ml of the 1X TAE buffer into the gel tank.
3. Remove the "ends" from the gel-casting tray, and place the tray into the tank with the well comb nearest you. The TAE buffer should cover the gel to a depth of at least 0.5 cm. Remove the comb from the gel.
4. Load the samples into the analytical gel:
   A. Aliquot 12 µl of the KpnI-digested Adenovirus DNA (analytical visual marker) into a 1.5 ml microcentrifuge tube.
   B. To prepare the molecular weight (MW) marker, determine how many marker lanes you need. Follow the manufacturer's directions to prepare the MW markers.
   C. To prepare sample DNAs, add 14 µl of HaeIII-digested DNA (see Step A, above) and 4 µl of loading buffer to a microcentrifuge tube. Mix, and centrifuge for 2 sec. Repeat for all samples to be analyzed. If less than 14 µl of the digested DNA is used, add TE to bring the volume to 14 µl.
   D. Carefully pipet each sample into the appropriate well as follows:

| Well | Sample |
|---|---|
| 1 | Blank |
| 2 | Analytical visual marker (10 µl) |
| 3 | DNA analysis marker (10 µl) |
| 4 | HaeIII-digested K562 DNA (18 µl) |
| 5 | Test sample |
| 6 | Test sample |
| 7 | DNA analysis marker (10 µl) |
| 8 | Test sample |
| 9 | Test sample |
| 10 | DNA analysis marker (10 µl) |
| 11 | Test sample |
| 12 | Known male standard* |
| 13 | DNA analysis marker (10 µl) |
| 14 | Blank |

*Note:* The first and last lanes or wells should be left empty unless they are needed.

* A known male bloodstain is extracted and run with other test samples with casework.

5. Connect the electrodes from the power pack or supply to the gel electrophoresis apparatus. An electric field is then applied to the agarose gel by setting the voltage to 100 volts. Because the DNA molecules are negatively charged, the fragments will migrate toward the positive (+) electrode (red). Consequently, the wells containing the samples are located at the opposite end of the gel, or close to the negative (–) electrode (black).
6. After electrophoresis (determined when the "dye front" is approximately 2 cm from the end of the gel), examine the gel on the UV transilluminator to evaluate migration. Under UV illumination, the ethidium bromide (which has bound to the DNA) fluorescence should appear as a smear without the appearance of any bands.

**Warning:** Avoid excessive exposure to the UV light. Always wear a full face shield when working with the transilluminator.

## *D. Southern Blotting DNA from Agarose Gels*

To detect specific polymorphic DNA fragments, the DNA must be transferred from the agarose or analytical gel to a solid support. This transfer is accomplished by a method described by Southern (1975) and is referred to as the "Southern blot." The polymorphic fragments, separated by agarose gel electrophoresis based on size, are denatured (i.e., separated into single strands) using an alkaline solution, transferred to a solid support (e.g., a nylon membrane is preferred over nitrocellulose), immobilized, and detected with DNA probes through a process called hybridization. The DNA profile or pattern is captured on film (e.g., autoradiography or chemiluminescence) for analysis and long-term storage (for an overview of the RFLP process, including Southern blotting or the DNA transfer setup, see Figure 6).

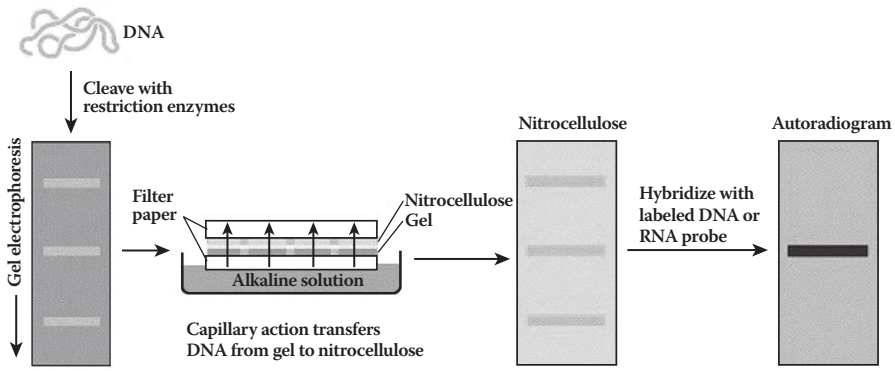



**FIGURE 6**
Overview of the RFLP Procedure. *Source*: From the University of Strathclyde in Glasgow, http://www.strath.ac.uk/~dfs99109/BB211/RecombDNAtechlect2.html.

1. Slide the gel from the tray into a plastic box that contains enough denaturation solution to cover the gel. Place the box containing the gel on an orbital shaker, and gently stir for 15 min.
2. While the gel is soaking in denaturation solution, place thin sponges (approximately 5 cm larger than the gel on all sides) in a glass dish or transfer tray, and add 10X SSC until sponges are saturated and solution level is just above the bottom sponge.
3. In a separate tray containing 10X SSC, immerse an 11.5 × 14.5 cm prelabeled (using pencil) nylon membrane. The transfer nylon membrane should be handled at the edges.

**Note:** Always wear disposable gloves when handling nylon membranes. Oils from your skin will prevent proper wetting of the membrane and subsequent transfer of DNA.

4. Decant the denaturation solution, and rinse the gel in deionized or distilled water for 20 sec. *Gently* shake by hand, and then decant water. Add enough neutralization solution to cover the gel, and place the tray containing the gel on an orbital shaker and shake gently for 15 min. Ensure that the solution covers the gel and that the gel is not adhering to the bottom of the tray.
5. Cut three pieces of Whatman 3MM chromatography paper to the same size of the sponge, saturate with 10X SSC, and place on top of the sponge.
6. Carefully remove the gel from the neutralization solution, and place on the chromatography paper with the underside of the gel facing up. Remove any trapped air bubbles between the paper and the gel by rolling a pipet over the surface of the gel.
7. Place the transfer nylon membrane (from Step 3, above) on the surface of the gel, and remove any air bubbles as in the preceding step.
8. Cover the membrane with a piece of Whatman 3MM chromatography paper that has been cut to the size of the gel (11 × 14 cm) and presoaked with 10X SSC. Remove any air bubbles as before with a pipet.
9. Place 9 dry blotting pads or paper towels cut to the size of the gel, and place on top of the Whatman 3MM chromatography paper.
10. Place a glass or plastic plate on top of the blot pads or paper towels.
11. Place a weight on top of the glass or plastic plate to hold the assembly in place.
12. Allow the transfer to proceed at room temperature for at least 2 hr or until all blot pads are saturated. Transfer should not exceed 6 hr. Due to the limited volume of 10X SSC, check periodically and add 10X SSC as needed. **Do not allow the Southern blot to dry out.**

13. Remove the blotting pads or paper towels and the Whatman 3MM chromatography paper.
14. Grasp the membrane at the right corner (origin end) with your gloved hand, turn it over, place in a plastic tray, and wash once with 0.2 M Tris, pH 7.5, and 2X SSC for 15 min with gentle shaking on a rotator. Place the membrane on a sheet of Whatman 3MM chromatography paper, and allow to dry at room temperature.
15. To immobilize the DNA to the membrane, either place the membrane in an envelope made from Whatman 3MM chromatography paper and place in an 80°C vacuum oven for 30 min, or irradiate the membrane (DNA side face down) for 1 to 5 min using a UV transilluminator (254 nm wavelength).
16. The membrane can be hybridized at this point or stored in a Ziploc plastic bag at room temperature.

## *E. Hybridization and Detection*

### Chemiluminescent Probe

A number of methods have been used in forensic laboratories for detecting DNA following electrophoretic separation and Southern blotting. These methods were effective but were expensive and time consuming. During the past decade, fluorescence-based detection systems have gained in popularity and are widely used in forensic laboratories due to their ease of use and rapid formats.

With RFLP analysis, the single-stranded DNA probe is tagged with an enzyme and hybridized to the DNA fragments immobilized on the Southern blot. When the Southern blot is exposed to a certain chemical, a discharge of light is emitted from the enzymatically labeled probe that has bound to its complementary strand during hybridization. This effect or process is referred to as chemiluminescence. The light that is emitted from the chemical is captured on X-ray film as dark black bands in the region detected by the probe as well as with computer-aided imaging systems.

1. Prepare 500 ml of 1X Wash I solution per hybridization container and 250 ml of 0.5X Wash I solution. Preheat solutions to 55°C prior to hybridization. Both solutions can be prepared a day in advance and stored at 55°C until they are ready for use, *or* incubate at 55°C approximately 2 hr prior to use.

**Note:** Preheat Wash I Concentrate at 55°C prior to making dilution to ensure that all solids are dissolved. If concentrate becomes cloudy, leave at room temperature until clear.

2. Add membranes, DNA side up, to the appropriate volume (e.g., 1–4 membranes use 30 ml of prehybridization or hybridization solution, and 5–8 membranes use 60 ml of prehybridization or hybridization solution) of hybridization solution (Wash I Concentrate). Ensure that each membrane is covered with solution prior to adding the next membrane. This "blocking" or prehybridization step may be performed in a sealable bag or a tray.
3. Prehybridize membranes for 20 min in a rotating water bath (60–70 rpm)* at 55°C.
4. During the prehybridization period, transfer the appropriate amount of hybridization solution (see Step 2, above) to a conical tube and heat to 55°C. To the conical tube, add the appropriate amount of chemiluminescent-labeled probe (as described in the manufacturer's product information; between 15 and 30 µl per 30 ml hybridization solution) and the molecular weight marker probe.
5. Remove the hybridization box from the water bath, and decant the prehybridization solution.

* In an orbital shaker; speed control and/or settings may vary between instruments.

6. Add the appropriate volume of labeled probe and molecular weight marker probe to a 50 ml conical bottom centrifuge tube containing the appropriate volume of hybridization solution (see Step 4, above).
7. Vortex the hybridization solution and labeled probes solution briefly, and add to the hybridization container. Rotate the container gently by hand to distribute the hybridization solution, ensuring that each membrane is covered with solution.
8. Hybridize membranes for 30 min in a rotating water bath (60–70 rpm) at 55°C in hybridization solution. To ensure equal distribution of the probe, the membranes should not adhere to each other.

### Radioactive Probe

1. Mix the following solutions (hybridization solution) into a hybridization container:
   40.8 ml sterile $H_2O$
   24.0 ml 50% PEG
   9.0 ml 20X SSPE
   42.0 ml 20% SDS
   (As many as 6 nylon membranes can be added to the hybridization container.)
2. Place 60 ml of the hybridization solution into the hybridization container.
3. Using gloves, place the membrane(s) into the hybridization container one at a time. Make sure that each membrane is saturated thoroughly with the hybridization solution before adding the next membrane to the tube.
4. Place the hybridization container in the 65°C incubator shaker to equilibrate.
5. Fill a beaker with water, and place on a hot plate to boil.
6. For each hybridization, label a 15 ml conical screw-capped centrifuge tube with the name of the probe (e.g., D2S44 and D10S28).
7. Pipet 1.5 ml of herring sperm DNA into each conical screw-capped tube.
8. Pipet the entire contents of the vial of labeled probe* into the tube(s) containing the herring sperm DNA. Dispose of the pipet tip and probe vial in an appropriate radioactive waste container. Cap the tube securely.

**Note:** DNA labeling kits are commercially available through various vendors.

9. Place the conical tube(s) in the boiling water for 5 min.
10. Remove the hybridization container from the incubator shaker, and remove the lid.
11. Lift the hybridization container to one side so that all of the "prehybridization solution" collects in one corner.
12. Carefully pour the contents (or probes) of the conical tube(s) into the corner of the container, and carefully rotate the container to mix the solutions. Dispose of the empty conical tube in a radioactive waste container.
13. Replace the lid on the hybridization container, and incubate at 65°C overnight with constant shaking.

* A short segment of DNA that are "tagged" with a chemical tracer and used to detect alleles at certain loci.

## F. Posthybridization Washes

### Chemiluminescent Procedure

1. Decant the hybridization solution from the hybridization container.
2. Rinse the membranes in the following washes in a rotating water bath (i.e., orbital shaker):

**Note:** The membranes will stick to the bottom of the container. To ensure effective washing, make sure that the membranes are not adhering to each other during the posthybridization washes. Use enough wash solution to cover the membranes and half-fill the container. The speed control and/or settings may vary between shakers.

A. 15 min in 1X Wash I solution at 55°C at 60–70 rpm
B. 15 min in 1X Wash I solution at 55°C at 60–70 rpm
C. 15 min in 0.5X Wash I solution at 55°C at 60–70 rpm
D. 5 min in 1X Final Wash solution at room temperature with gentle agitation

3. Place membrane(s) DNA side up on a clean sheet of Whatman 3MM chromatography paper to remove excess solution. **Do not blot the membrane(s)**. After 5–10 min, place the air-dried membranes in a plastic container with the appropriate volume of LumiPhos Plus (see below). Make sure each membrane is covered with LumiPhos Plus before adding the next membrane. Place the container on a rocking or shaking platform, and gently shake for 5 min.

**Membrane Number and Appropriate LumiPhos Plus Volumes**

| Number of Membranes | LumiPhos Plus Volume |
|---|---|
| 1–2 | 15 ml |
| 3–4 | 20 ml |
| 5–6 | 25 ml |
| 7–8 | 30 ml |

4. Carefully remove each membrane from the LumiPhos Plus solution using blunt-end forceps. To remove excess LumiPhos Plus, "drag" the membrane along the side of the container.
5. Place each membrane in a plastic folder, wipe folder with a Kimwipe™ to press out air bubbles, and heat-seal folder.
6. Trim the excess plastic close to the outer edge of the heat seal. Wipe the edges with a Kimwipe™ to remove any excess LumiPhos Plus. The plastic folders can accommodate two membranes per folder.

### Radioactive Procedure

1. Decant the hybridization solution from the hybridization container slowly into a radioactive waste container. Wipe the corner of the container with a Kimwipe™, and discard the waste into a radioactive waste container.
2. Rinse the membranes in the following washes in a rotating water bath (i.e., orbital shaker):

**Note:** The membranes will stick to the bottom of the container. To ensure effective washing, make sure that the membranes are not adhering to each other during the post-hybridization washes. Use enough wash solution to cover the membranes and half-fill the container.

A. 15 min in 2X SSC + 0.1% SDS at room temperature with slow "rocking"

B. 15 min in 2X SSC + 0.1% SDS at room temperature with slow "rocking"

C. The final *high-stringency* wash conditions (0.1X SSC + 0.1% SDS and 65°C) will vary according to the probe used (see below):

| Probe | Number of Washes | Length of Each Wash |
|---|---|---|
| D2S44 | 1 | 10 min |
| D17S79 | 1 | 10 min |
| D1S7 | 2 | 30 min |
| D4S139 | 2 | 30 min |
| D1OS28 | 2 | 30 min |
| D5S11O | 2 | 30 min |

*Note:* For the final stringency washes, the 0.1X SSC + 0.1% SDS solution must be preheated to 65°C before being added to the membrane(s).

3. Carefully remove each membrane from the final wash solution using blunt-end forceps, and place on Whatman 3MM chromatography paper.

## G. Lumigraphy

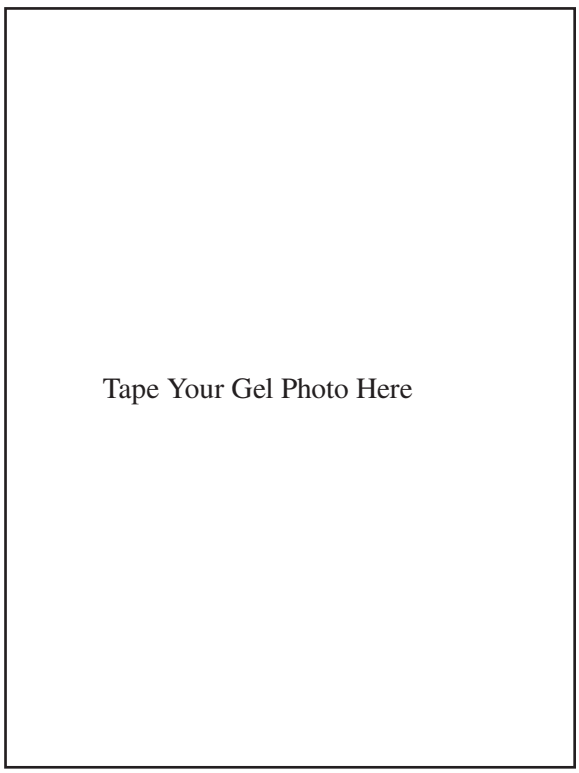
1. The plastic folders containing the membrane(s) are stored overnight *in the dark* at room temperature to allow for maximum light output.
2. In the darkroom under red light illumination, place the membrane-containing folders, DNA side down, onto Kodak X-Omat RP film. Tape the membrane packets to the film. Record the locations of the membrane(s) by writing directly on the film with a ballpoint pen. Place a second sheet of film onto the back of the membrane, and place in a cassette. Close the cassette, and keep at room temperature.
3. The two sheets of Kodak X-Omat RP film can be developed after an exposure period has been determined for the film in use. This exposure period can range from 15 min to 60 min for the back film, and 30 min to 2 hr for the front film. Film exposure times in excess of 2 hr will, in general, not increase the band or signal intensity but will increase background noise or signal.

## H. Autoradiography

1. Wrap the damp membrane(s) in Glad wrap. The wrap prevents contamination of holders and prevents the membrane from sticking to the film.
2. In the darkroom under red light illumination, place the membranes onto the X-ray film. Tape the membrane(s) securely on the film. Record the locations of the membrane(s) by writing directly on the film with a ballpoint pen. Place another sheet of film onto the back of the membranes. Close the cassette, and place at –80°C.
3. The X-ray film on the back side of the membrane(s) can be removed after a short exposure period, usually within a few hrs or as long as a few days. The back sheet of film, when developed, is used as a guide for determining the length of exposure time for the front film.

## Results

### Digest Gel




Lane #1 _____

Lane #2 _____

Lane #3 _____

Lane #4 _____

Lane #5 _____

Lane #6 _____

Lane #7 _____

Lane #8 _____

Lane #9 _____

Lane #10 _____

Lane #11 _____

Lane #12 _____

Lane #13 _____

Lane #14 _____

Lane #15 _____

Lane #16 _____

Lane #17 _____

Lane #18 _____

Lane #19 _____

Lane #20 _____

## Analytical Gel for RFLP Analysis

Analyst: _____ Lab number: _____

Date: _____

| Well No. | Sample | DNA (ng) |
|---|---|---|
| 1 | Blank | |
| 2 | HindIII-digested lambda DNA (18 µl) | |
| 3 | HaeIII-digested K562 DNA (18 µl) | |
| 4 | Test sample | |
| 5 | Test sample | |
| 6 | Test sample | |
| 7 | Test sample | |
| 8 | DNA analysis marker (10 µl) | |
| 9 | Test sample | |
| 10 | Test sample | |
| 11 | Test sample | |
| 12 | Test sample | |
| 13 | DNA analysis marker (10 µl) | |
| 14 | Test sample | |
| 15 | Test sample | |
| 16 | Test sample | |
| 17 | Test sample | |
| 18 | Known male standard | |
| 19 | DNA analysis marker (10 µl) | |
| 20 | Blank | |

| Reagents | Lot No. | Source |
|---|---|---|
| Agarose | | |
| 1X TAE (gel buffer) | | |
| 1X TAE (tank buffer) | | |
| Loading buffer | | |
| Ethidium bromide | | |
| Visual marker | | |
| | | |
| | | |
| | | |
| | | |

## Analytical Gel for RFLP Analysis

Analyst: _____ Lab number: _____

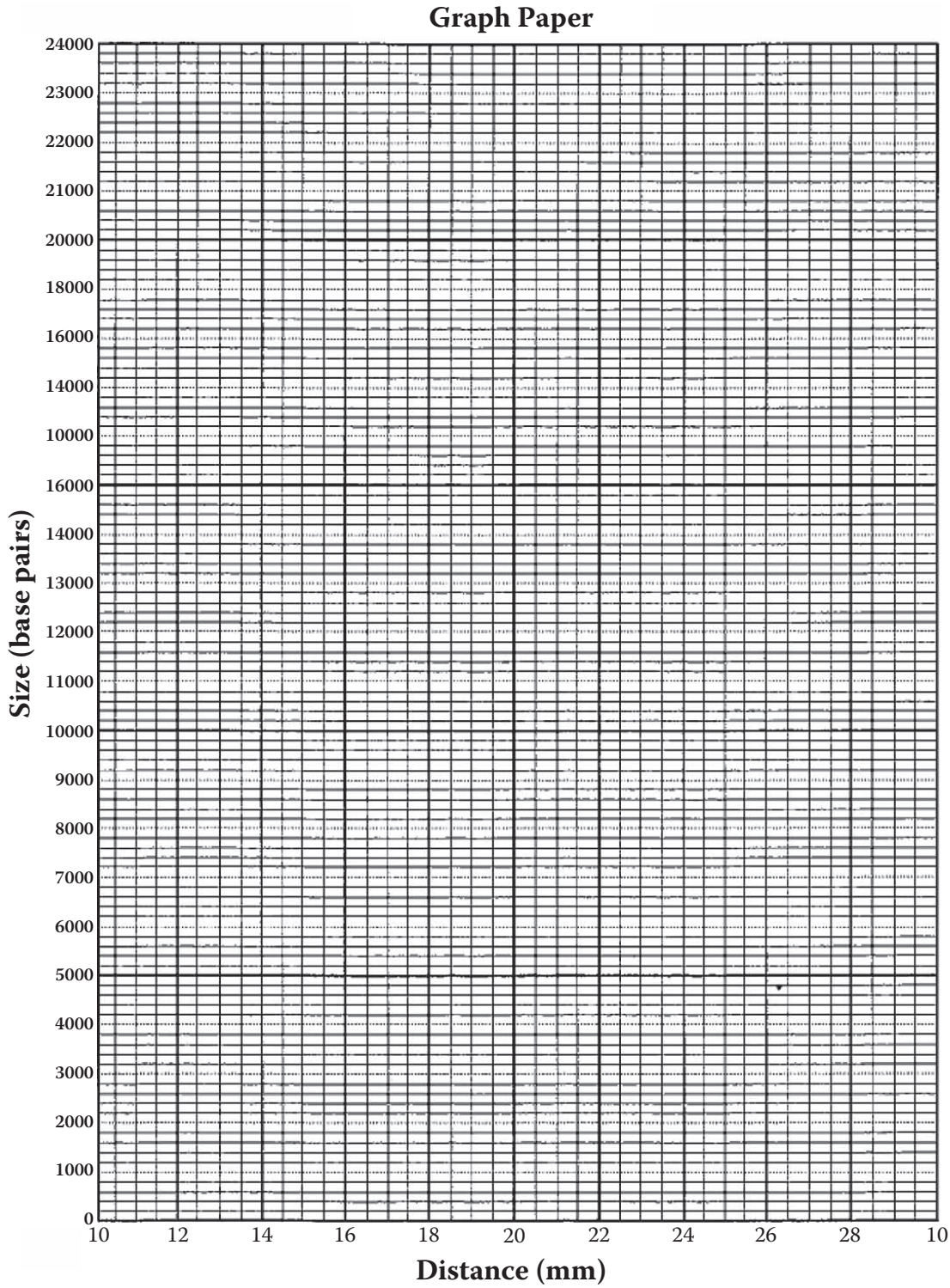
Date: _____

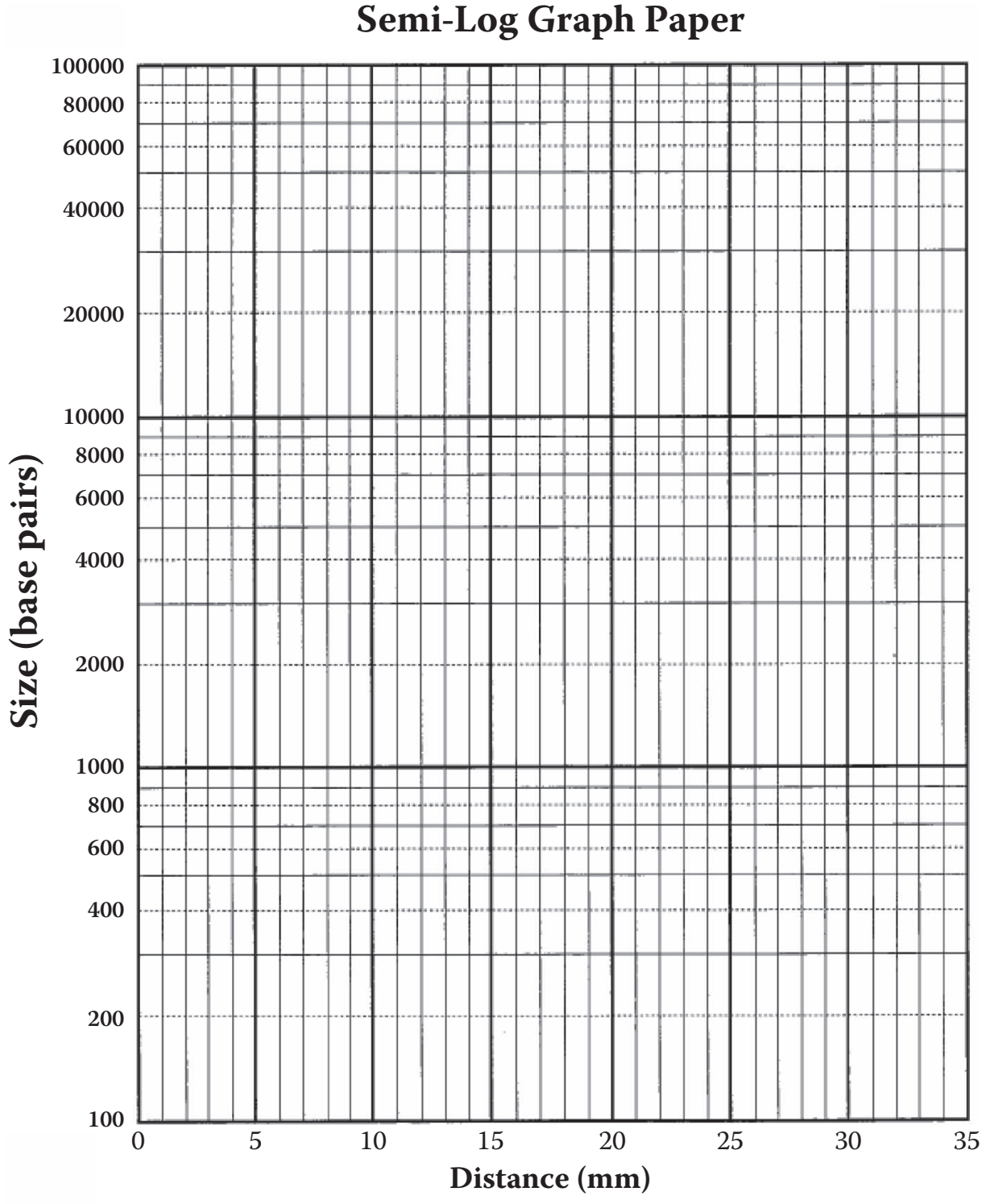
| Well No. | Sample | DNA (ng) |
|---|---|---|
| 1 | | |
| 2 | | |
| 3 | | |
| 4 | | |
| 5 | | |
| 6 | | |
| 7 | | |
| 8 | | |
| 9 | | |
| 10 | | |
| 11 | | |
| 12 | | |
| 13 | | |
| 14 | | |
| 15 | | |
| 16 | | |
| 17 | | |
| 18 | | |
| 19 | | |
| 20 | | |

## Quantitative Analysis of DNA Fragment Sizes

To make a comparison between the test samples (or crime scene samples) and the known reference samples, other than a visual comparison, a quantitative measurement of the DNA fragments observed following hybridization and visualization is needed. Determining the molecular weight of each DNA fragment is performed as follows:

1. Using a ruler, measure the distance that each DNA fragment (i.e., the molecular weight standards, the known reference samples, and the test samples) or band has migrated from the bottom of the well (the origin) to the center of each DNA fragment.
2. To determine the molecular weight or size of each DNA fragment, a standard curve is created using the distance (x-axis) and fragment size (y-axis) data from the molecular weight markers (HindIII-digested lambda DNA and/or the DNA analysis markers). Using both the linear and semilog paper (provided at the end of this exercise), plot the distance that each DNA band has migrated (in mm) versus the size (in base pairs) for each band of the molecular weight markers. Record the molecular weight of each DNA fragment in either table below. After each data point has been "plotted" on the graph paper, use a ruler and draw the "best-fit" line through the data points. Extend the line through the entire graph paper.
3. Compare the results of your graphs. Decide which graph should be used to estimate the size of the DNA fragments observed from the test samples.
4. To estimate the size of a DNA fragment from the test sample, determine the distance the fragment migrated. Locate the distance migrated on the x-axis of your graph. Read "up" to the standard line, and then follow the graph line over to the y-axis. This point is the approximate size (in base pairs) of the unknown DNA fragment. Record the molecular weight of each DNA fragment in either table below. Repeat this process for each DNA fragment.
5. Compare the sizes of the DNA fragments of the test samples to the reference samples. Is there a match?


Graph Paper
Size (base pairs)
24000
23000
22000
21000
20000
18000
16000
14000
10000
16000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
10
12
14
16
18
20
22
24
26
28
10
Distance (mm)


Semi-Log Graph Paper
Size (base pairs)
100000
80000
60000
40000
20000
10000
8000
6000
4000
2000
1000
800
600
400
200
100
0
5
10
15
20
25
30
35
Distance (mm)

## RFLP Typing Results

| Sample | Probe/Locus | | | | |
|---|---|---|---|---|---|
| | | | | | |
| 1 | | | | | |
| 2 | | | | | |
| 3 | | | | | |
| 4 | | | | | |
| 5 | | | | | |
| 6 | | | | | |
| 7 | | | | | |
| 8 | | | | | |
| 9 | | | | | |
| 10 | | | | | |
| 11 | | | | | |
| 12 | | | | | |
| 13 | | | | | |
| 14 | | | | | |
| 15 | | | | | |
| 16 | | | | | |
| 17 | | | | | |
| 18 | | | | | |
| 19 | | | | | |
| 20 | | | | | |

## RFLP Typing Results

| | Lambda/Hind III Size Marker | | Test Samples (Crime Scene) | | Reference Sample | | Reference Sample | |
|---|---|---|---|---|---|---|---|---|
| **Band** | Distance (mm) | Actual Size* | Distance (mm) | Actual Size* | Distance (mm) | Actual Size* | Distance (mm) | Actual Size* |
| 1 | | 23,130 | | | | | | |
| 2 | | 9,416 | | | | | | |
| 3 | | 6,557 | | | | | | |
| 4 | | 4,361 | | | | | | |
| 5 | | 2,322 | | | | | | |
| 6 | | 2,027 | | | | | | |

* The actual size of the DNA fragments is an approximation of the molecular weight in base pairs (bp).

## RFLP Typing Results

| | Lambda/Hind III Size Marker | | Test Samples (Crime Scene) | | Suspect 1 | | Suspect 2 | |
|---|---|---|---|---|---|---|---|---|
| **Band** | Distance (mm) | Actual Size* | Distance (mm) | Actual Size* | Distance (mm) | Actual Size* | Distance (mm) | Actual Size* |
| 1 | | 23,130 | | | | | | |
| 2 | | 9,416 | | | | | | |
| 3 | | 6,557 | | | | | | |
| 4 | | 4,361 | | | | | | |
| 5 | | 2,322 | | | | | | |
| 6 | | 2,027 | | | | | | |

* The actual size of the DNA fragments is an approximation of the molecular weight in base pairs (bp).

## Questions

1. Restriction endonucleases are used to cleave the DNA into many fragments. If the sample was not completely digested, what type of restriction pattern would you expect to observe following gel electrophoresis? Assuming that the sample will need to be redigested, what steps would you take to ensure that the sample was completely digested?

2. During gel electrophoresis, an electric field is created with positive and negative poles at the ends of the gel. After the DNA sample is loaded into the wells, to which electric pole would you expect DNA to migrate? Explain your answer.

3. After the DNA samples have been loaded into the sample wells, what size fragments (large versus small) would you expect to move toward the opposite end of the gel the quickest?

4. When looking at the data plotted on the linear and semilog paper, which graph provides the best-fit line that would allow you to estimate the test samples' or known reference samples' size?

Chapter 8

# Polymerase Chain Reaction (PCR)

## Introduction

Early forensic detection systems relied on the quality and quantity of the DNA sample to be analyzed. The large amount (a "dime-size" stain) of isolated DNA, whether from an evidentiary or a reference sample, had to be relatively fresh or undegraded—essentially, unadulterated—for these detection systems to yield a sufficient profile. For samples considered too minuscule (i.e., low concentration) or determined to be degraded, the polymerase chain reaction (PCR) or amplification process is now performed. The process initially involves the isolation of DNA from a biological specimen followed by a quantitative and qualitative assessment of recovery. The PCR amplification technique is used to produce millions of copies of a specific portion of a targeted chromosome(s) that contains polymorphic DNA selected for forensic and parentage evaluations. The abundance of the PCR product allows for the direct visualization of "blue dots" or bands that represent the allele(s) at specific loci. This evaluation is accomplished by reverse dot blot analysis or gel electrophoresis followed by chemical staining. DNA typing using PCR and gel electrophoresis eliminated the need for critically sensitive DNA probes previously used with the restriction fragment length polymorphism (RFLP) procedure. Analysis time ranges between 24 to 48 hours. Computer-assisted image analysis of test results is helpful but not always necessary because the resulting genetic profiles are routinely interpreted by visual or direct comparison to allele standards at specific loci. Population frequencies are conservative estimates based upon classical population genetic principles.

The PCR methodology also has demonstrated consistency of results from tissue to tissue, and from body fluid to body fluid, within an individual. Therefore, the detection of an allele(s) in an unknown biological stain allows for comparison to reference samples regarded as a possible source or contributor to such biological materials. If the collection of genetic information (DNA profile) associated with an unknown stain is consistent with the results generated from a reference sample, then the possibility that a common genetic source exists for both sets of samples cannot be eliminated. The demonstration of independent Mendelian inheritance, as mentioned earlier, allows for a conservative estimate of the frequency of such a DNA profile occurring among unrelated individuals randomly selected in various racial groups.

## Objective

The following exercises (Exercises 6–10) will introduce the student to different PCR-based tests: the AmpliType PM/DQA1 system, the D1S80 system, and short tandem repeat (STR) analysis.

## Laboratory Setup

Due to the sensitivity of PCR-based tests, certain precautions are necessary to avoid contamination of samples with other sources of DNA. To minimize the potential for laboratory-induced DNA contamination, several aspects of the PCR process should be considered: 1) DNA extraction, 2) PCR setup, and 3) amplified DNA analysis. Each aspect of the PCR process should be separated by time and space. The following section addresses special precautions that must be taken to minimize contamination in the laboratory.

1. The work area for DNA extractions and non-amplified DNA should include dedicated equipment and supplies.
2. DNA extractions and PCR setup should be conducted within self-contained hoods. If hoods are unavailable, use an area of a benchtop that is dedicated for this use only.
3. Use disposable gloves at all times, and change frequently. Prior to leaving the laboratory area, always remove the gloves and wash your hands.
4. DNA extraction of questioned samples (i.e., evidentiary samples) should be performed separately from the extraction of known samples. This will minimize the potential for cross-contamination between samples.
5. Every sample to be analyzed should be properly labeled and recorded. Evidentiary and known reference samples to be analyzed in a forensic laboratory are given a unique identification number that is used throughout the entire analysis.
6. To minimize sample-to-sample contamination, extract samples containing high levels of DNA (e.g., whole blood) separately from samples containing low levels of DNA (e.g., small bloodstains, stamps, and envelopes).
7. Always use sterile solutions or reagents and, whenever possible, sterile disposable supplies (i.e., pipet tips and microcentrifuge tubes).
8. Always change pipet tips between handling each sample even when dispensing reagents.
9. Sterilize reagents, and store as small aliquots to minimize the number of times a given tube of reagent is opened. It is recommended that the small aliquots be retained until typing of the set of samples for which the aliquots were used is complete. Then dispose of the tube containing the reagent.
10. Include reagent blank controls with each set of DNA extractions.
11. Before and after setting up the DNA extractions, clean all work surfaces thoroughly with a 10% solution of bleach. In addition, the use of disposable bench paper will prevent the accumulation of human DNA on permanent work surfaces.

Chapter 9

# Exercise 6

## *Polymerase Chain Reaction (PCR)–Based Tests: The AmpliType PM/DQA1 System*

## Introduction

If the evidentiary sample contains an insufficient quantity of DNA or if the DNA is degraded, a polymerase chain reaction (PCR)–based test may be used to obtain a DNA profile. The PCR-based tests generally provide rapid results that can serve as an alternative or as a complement to other DNA testing. The process involves the isolation of DNA from a biological specimen (e.g., blood, semen, saliva, or fingernail clippings) followed by an assessment of DNA quality and quantity. Next, the PCR amplification technique is used to produce millions of copies of a specific portion of a targeted DNA segment (Figure 7). The PCR amplification procedure is similar to a molecular photocopying machine. The amplified PCR products are then separated and identified by the reverse dot blot technology or by gel electrophoresis followed by enzymatic conversion of a colorless substrate or by chemical staining using ethidium bromide or coomassie blue, respectively. The resulting DNA profiles are routinely interpreted by direct comparison to DNA and/or allele standards. Probability calculations are determined based upon classical population genetic principles.

The first PCR-based test that became available to the forensic community was the HLA DQ alpha system (developed by Cetus), now referred to as DQA1. The human leukocyte antigen (HLA) locus was found to contain a significant number of variations that, when applied to human identification, were able to distinguish 28 DQA1 genotypes. However, because only one locus with limited variability was analyzed in this system, the power of discrimination was extremely low. A second commercially available PCR typing kit, called the AmpliType PM kit or Polymarker (the manufacturer-distributor's name has changed from Roche Molecular to Applied Biosystems, Foster City, California), was introduced in the mid-1990s with the ability to type 5 additional loci along with the HLA DQA1 locus. The combination of 6 genetic loci increased the power of discrimination while retaining the advantages of a PCR-based system.

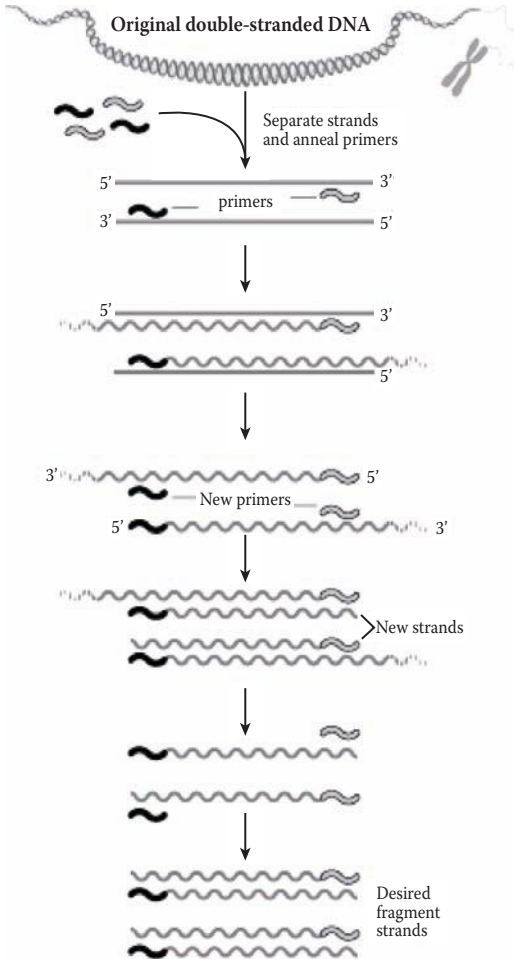



**FIGURE 7**
Overview of the Polymerase Chain Reaction (PCR). **Note:** A defined region of DNA is copied using the PCR amplification technique to produce millions of copies of the specified region on the DNA strand.

The format for the PM/DQA1 typing process is quite different than the format for RFLP. The PM/DQA1 test took advantage of the reverse dot blot technology where the DNA probe is bound to a solid substrate (i.e., a nylon membrane) and the amplified PCR products—from known and evidentiary samples—are hybridized to two separate nylon strips containing the immobilized probes for either the DQA1 amplified products or the 5 PM loci. The final product(s) or the positive responses are visualized upon the enzymatic conversion of a colorless substrate to a blue-colored precipitate (Figure 8). The pattern of "blue dots" corresponds to the alleles detected in the samples tested.

## Objective

In the next series of exercises, you will gain experience with one of the most important techniques in modern molecular biology, the polymerase chain reaction (PCR), using the first commercially available PCR-based kit used in the identification of individuals in criminal and paternity cases. While learning these techniques, you will examine data, specific to a case study, generated using the AmpliType PM/DQA1 kit (Applied Biosystems). Following DNA isolation and amplification, the PM/DQA1 products from the known

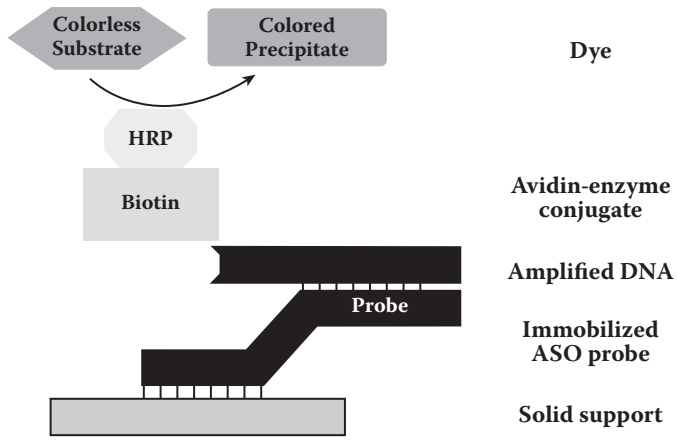



**FIGURE 8**
Detection of PCR Products Using the AmpliType PM/DQA1 Test and the Reverse Dot Blot Technology. *Note*: The nylon membrane strip contains the immobilized allele-specific oligonucleotide (ASO) probes. The amplified PCR products from the known and/or evidentiary samples are labeled with a streptavidin (avidin)–biotin–enzyme (horse radish peroxidase, or HRP) complex and allowed to hybridize to the immobilized probes. Following hybridization, a colorless substrate is added, and if the amplified DNA bound to the probe, the enzyme will convert the colorless substrate to a blue precipitate.

and evidentiary samples will be hybridized to the nylon strip containing the immobilized DNA probes, and the responses recorded to determine the various genotypes and profiles used for identification.

## Equipment and Material

1. Ice in buckets
2. Chelex beads
3. 0.5, 1.0, and 1.5 ml Eppendorf or microcentrifuge tubes
4. Mineral oil (optional)
5. 15 ml polypropylene test tube
6. Double-distilled water
7. 30 or 50 ml conical tubes
8. Adjustable-volume digital micropipets (2–200 µl range)
9. Aerosol-resistant pipet tips
10. Perkin-Elmer Cetus GeneAmp Kit
11. Taq DNA polymerase (if not supplied in kit)
12. Disposable gloves
13. TAE buffer
14. TBE buffer
15. Agarose
16. Molecular weight markers (526 to 22,621 bp)
17. Ethidium bromide or coomassie blue

18. Bromophenol blue tracking dye
19. 125 ml Erlenmeyer flask
20. Microwave or hot plate
21. Incubator or water bath at 56°C
22. Electrophoresis systems (gel tray or combs)
23. Power pack or supply
24. Microcentrifuge
25. DNA thermal cycler
26. Polaroid camera with film

## Procedure

The use of disposable gloves and aerosol-resistant pipet tips is highly recommended to prevent cross-contamination. There are several steps in the AmpliType PM/DQA1 test:

1. DNA is extracted from the known and evidentiary samples (see Exercise 1 for extraction procedures). Due to the increased level of sensitivity when using PCR, the number of cells required is significantly fewer than that required for RFLP analysis.
2. The DNA from the sample is amplified using PCR, tagged or labeled using the streptavidin (avidin)–biotin–enzyme complex described above, and allowed to hybridize to the PM/DQA1 probes bound to the nylon strips.
   **Note:** In RFLP, the target DNA is bound to the blot, and the probe DNA is added. For the PM/DQA1 test, the probe DNAs are immobilized on a nylon strip and the target DNA is added.
3. The pattern of blue dots determines the DNA type or profile at the HLA DQA1 locus. The pattern of blue dots in Figure 10 is an example of how the DQA1 profile is determined.
4. The AmpliType PM nylon strips contain 5 genetic loci: LDLR, GYPA, HBGG, D7S8, and GC. Each locus represents a distinct location or site on the DNA. When compared to the allelic variations for the DQA1 locus, the 5 PM loci have rather simple allelic variations. For example, there are only two alleles for the LDLR, GYPA, and D7S8 loci designated allele A and/or B. The HBGG and GC loci each have 3 possible alleles: A, B, and C. The "S" dot is the control dot. The response or signal at this site is designed to be less than that observed for the other dots. A faint response at the "S" dot indicates that a threshold has been met during the hybridization process rendering support for the other hybridization responses (Figure 11).

### *A Case Study*

A man named Harry lived alone. Instead of cooking for himself, Harry usually went out for his meals. One evening, Harry decided to go to a restaurant for dinner. While he was out, his home was robbed. When Harry returned home from dinner, he saw a man driving away from his house in a van. Although Harry did not get a good look at the face of the burglar, he could identify him as a Caucasian male in his twenties. Harry called the police, and sometime before midnight they had determined that the stolen items included a flat-screen TV, a stereo CD player, a digital video camera, a desktop computer, and a laptop.

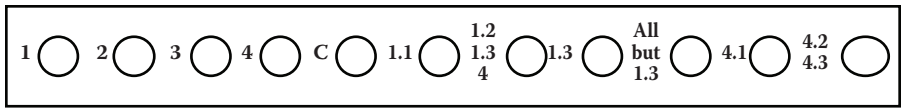



FIGURE 9
DQA1 Typing Strip. **Note:** Before hybridization, the nylon typing strips are colorless. The invisible dot identified as a "1" has a DNA probe for the allele variation designated number 1 for the DQA1 test. The invisible dot identified above the "2" contains the DNA probe for the "2" allele. The invisible dot above the 3 and 4 alleles contains the DNA probe for these alleles. The "C" dot is the control dot. The response or signal at this site is designed to be less than that observed for the other dots. A faint response at the "C" dot indicates that a threshold has been met during the hybridization process rendering support for the other responses. The invisible dot containing the 1 allele has several variations: the 1.1, 1.2, and 1.3 subtypes, also called alleles. The remaining invisible dots contain various combinations of allelic probes. This format has no specific or single dot or probe for the 1.2, 4.2, or 4.3 subtypes; thus, the design of the typing strip is unable to distinguish between these subtypes. The "circles" surrounding an area on the nylon strip are only present in these illustrations to indicate the location of the DNA probes.

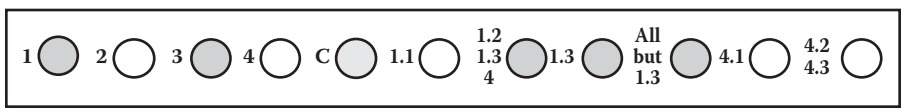



FIGURE 10
HLA DQA1 Typing Results. **Note:** Following hybridization, a colorless substrate is added, and if the amplified DNA bound to the probe the enzyme will convert the colorless substrate to a blue precipitate. The pattern of the "blue dots" is an example of a DQA1 typing strip that was hybridized with amplified DNA from a person with a DQA1 type of 1.3, 3. The typing results are interpreted from left to right. In this example, a faint response is observed at the "C," or control, dot. There is a response at the "1" allele; a response at the 3 allele (confirming a 3 allele); a response at the 1.2, 1.3, and 4 alleles; a response at the 1.3 allele (confirming a 1.3 allele); and a response at the "all but 1.3" alleles. Based on these responses, the DQA1 type or profile is 1.3, 3. The circles surrounding an area on the nylon strip are only present in these illustrations to indicate the location of the DNA probes.

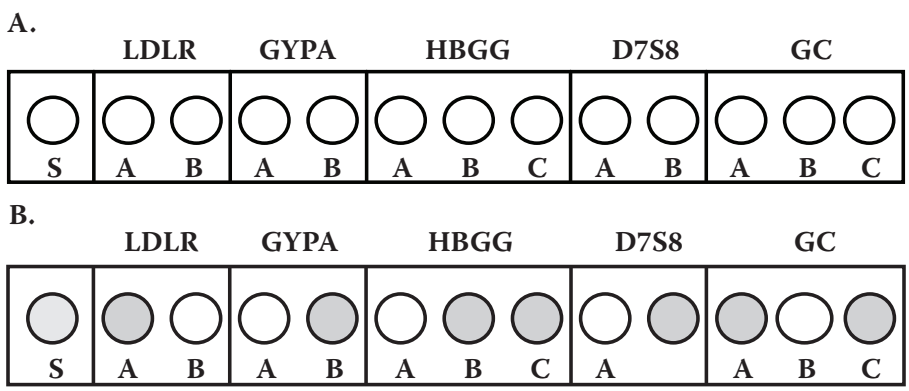
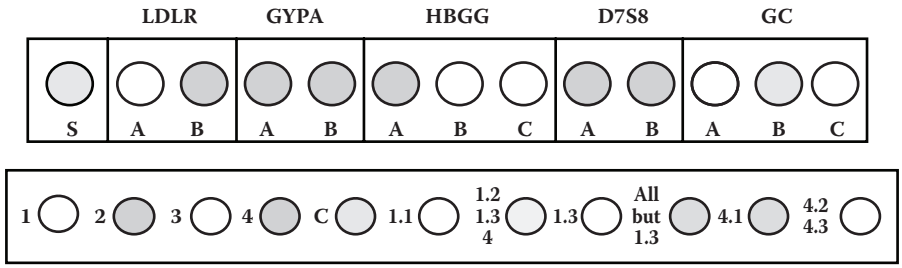



FIGURE 11
PM Typing Strip. **Note:** 1) Prior to hybridization, the nylon typing strips are colorless. The DNA probes are immobilized onto the nylon strip and identified with the corresponding locus (e.g., LDLR and GYPA). The circles surrounding an area on the nylon strip are only present in these illustrations to indicate the location of the DNA probes. Each locus will have 2 or 3 invisible dots that represent the allelic variations at that locus. 2) In this example, a faint response is observed at the "S," or control, dot. There is a response at the "A" allele of the LDLR locus, a response at the B allele of the GYPA locus, two responses at the B and C alleles of the HBGG locus, a response at the B allele of the D7S8 locus, and a response at the A and C alleles of the GC locus. Based on these responses, the AmpliType PM type or profile is AA/BB/BC/BB/AC or A/B/BC/B/AC.

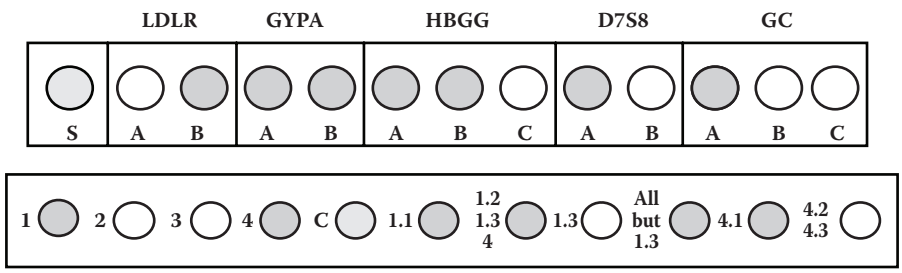
During the investigation, the police located the digital video camera and the laptop at a local pawnshop. The pawnshop owner reviewed his sales records and provided the investigators on the case with several names whom he thought sold him the stolen merchandise. Sometime later and after careful questioning, the investigators had identified three male suspects who often sold "stolen" merchandise to local area pawnshops. Two Caucasian males in their late twenties were "picked up" for questioning. Both male suspects had their blood drawn and sent to the local forensic laboratory for DNA analysis. The digital video camera and the laptop, the only two stolen items recovered, were also sent to the laboratory for analysis. The forensic DNA analyst subjected the swab of the digital video camera and a small drop of blood, found on the laptop, to AmpliType PM/DQA1 analysis. Reference samples from each of the two suspects were also subjected to the PM/DQA1 analysis. The results are shown below.

## Results

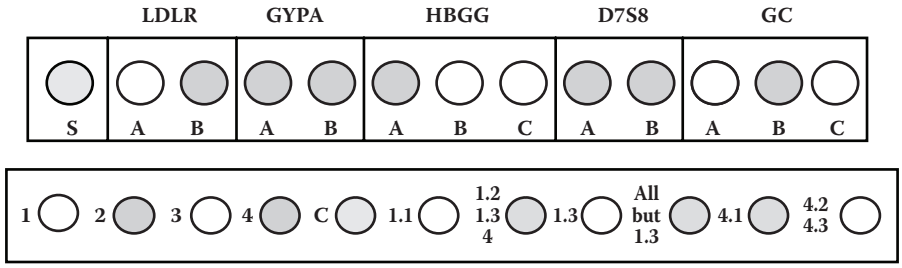
### *Suspect #1*



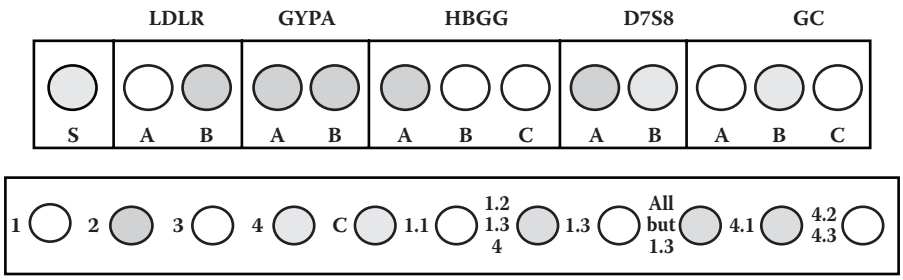

### *Suspect #2*



### *Swab from Digital Video Camera*




### *Blood from Laptop*




## Interpreting Your AmpliType PM/DQA1 Typing Results

### *Basis of the AmpliType PM/DQA1 Typing System*

- Six loci were amplified simultaneously with a pair of primers for each locus.
- At each locus, 2 or more alleles have been observed in a given population.
- An individual is either homozygous or heterozygous for any of the two possible alleles:
  - LDLR (low-density lipoprotein receptor): alleles A and/or B
  - GYPA (glycophorin A): alleles A and/or B
  - HBGG (hemoglobin G gamma globulin): alleles A, B, and/or C
  - D7S8 (chromosome 7): alleles A and/or B
  - GC (group-specific component): alleles A, B, and/or C
  - DQA1 (human leukocyte antigen): alleles 1.1, 1.2, 1.3, 2, 3, 4.1, 4.2, and/or 4.3
- A control spot (an "S" spot on the PM strip and a "C" spot on the DQA1 strip) is included on each of the nylon test strips. The control spots are included to determine the minimum amount of response needed (i.e., the amount signal from the enzymatic reaction) for a positive reaction. Any signal or spot lighter than the control spot is considered a negative response, and the test invalid. Any signal or spot darker than the control spot is considered a positive response and valid.

Record your observations or the PM/DQA1 DNA typing profiles of the known reference samples (i.e., Suspects #1 and #2) and the evidentiary samples (i.e., swab from camera and blood from the laptop) using the table below.

AmpliType PM/DQA1 Typing Results

| Sample | Polymarker (PM) Loci | | | | | DQA1 |
|---|---|---|---|---|---|---|
| | LDLR | GYPA | HBGG | D7S8 | GC | |
| Suspect #1 | | | | | | |
| Suspect #2 | | | | | | |
| Swab from Camera | | | | | | |
| Blood from Laptop | | | | | | |

Your group will work together to interpret the data generated from the AmpliType PM/DQA1 typing analysis. First, determine the allelic profile at each locus for all samples. Then compare the DNA typing results from each suspect's known reference sample to the PM/DQA1 profiles generated from the swab of the digital video camera and the blood from the laptop. For each sample, determine if the DNA pattern appears to be heterozygous or homozygous at the different PM/DQA1 loci. If an allelic response at any locus from the evidentiary samples does not match the allelic responses observed from either of the known reference samples, then that suspect(s) is excluded. If all of the alleles for each locus "match" (i.e., between the known reference sample and the evidentiary sample), then that individual (Suspect #1 or #2) cannot be excluded as being the contributor to the questioned samples. When considering the results, assume that all controls (e.g., positive and negative) responded appropriately. After completing your analysis, answer the questions below.

## Questions

1. What is the genotype (at all 6 loci) of Suspect #1? Explain your reasoning.

2. What is the genotype (at all 6 loci) of Suspect #2? Explain your reasoning.

3. Is Suspect #1 included or excluded as the contributor of the swab from the digital video camera? From the blood from the laptop? Explain your reasoning.

4. Is Suspect #2 included or excluded as the contributor of the swab from the digital video camera? From the blood from the laptop? Explain your reasoning.

5. Do the DNA profiles from the swab of the camera and the blood from the laptop match? Explain your answer.

6. If, in analyzing evidence from a crime scene using the AmpliType PM/DQA1 system, two or more suspects showed the same allelic pattern as that seen in this case study, what could you do to resolve the question?

Chapter 10

# Exercise 7

## *Polymerase Chain Reaction (PCR)–Based Tests: The D1S80 System*

## Introduction

The nucleus of a human somatic cell contains 6.4 billion base pairs of DNA. Specific portions of this DNA encode for over 100,000 genes, whereas the remainder of the DNA is comprised of noncoding regions often referred to as "junk DNA." Approximately 99.5% of the DNA code is identical for all people. The remaining 0.5% is of interest to forensic scientists because of the variations in the DNA that exist between individuals. Consequently, forensic DNA analysis focuses on these differences or hypervariable regions of DNA between individuals. One such form of variability is called a variable number of tandem repeats (or VNTR). The most forensically characterized VNTR is the polymorphism at the locus denoted D1S80 (Nakamura et al., 1988). This locus, located on human chromosome 1, is composed of repeating units of DNA segments that are 16 nucleotides in length (Kasai et al., 1990). The number of tandem repeats varies from one individual to the next, and is known to range from 15 to over 41. This variability in the number of tandem repeats is the basis for identification in the D1S80 typing system.

The D1S80 system was used by some forensic laboratories to complement the existing PCR-based assays, the AmpliType PM/DQA1 system, and, in many instances, RFLP analysis. Thus, the advent of PCR offered a viable alternative to RFLP analysis, especially if the questioned or evidentiary sample contained an insufficient quantity of DNA or the DNA was suspected to be degraded. The D1S80 system was amenable to PCR amplification due to the relatively small size of the locus with amplified products less than 700 bp in length. Moreover, the D1S80 system combined the advantages inherent with any PCR-based system with the greater variations seen in RFLP analysis. The use of PCR reduced assay time and cost, and served as an alternative or as a complement to other DNA testing. Similar to the DQA1 test, only one locus is analyzed in this system, thus limiting the D1S80's power of discrimination. However, when used in conjunction with other DNA tests, the power of discrimination increased dramatically.

## Objective

In this exercise, you will gain experience with one of the most important techniques in modern molecular biology, the polymerase chain reaction (PCR), while using the D1S80 typing system. While learning these techniques, you will extract DNA from cells in your mouth (buccal swabs) and/or from a human cell line, incubate the isolated DNA with appropriate PCR reagents, and amplify the alleles at the D1S80 locus. Following amplification, the amplified products will be separated and identified using agarose gel electrophoresis.

## Equipment and Material

1. 0.5, 1.0, and 1.5 ml Eppendorf or microcentrifuge tubes
2. TAE buffer
3. Chelex beads
4. Disposable gloves
5. 15 ml polypropylene test tube
6. Double-distilled water
7. 30 or 50 ml conical tube
8. Mineral oil (optional)
9. Aerosol-resistant pipet tips
10. Perkin-Elmer Cetus GeneAmp kit
11. Taq DNA polymerase (if not supplied in kit)
12. Adjustable-volume digital micropipets (2–200 µl range)
13. Ice in buckets
14. Primers (D1S80) 0.1 µg/µl
    A. 5´ GAA ACT GGC CTC CAA ACA CTG CCC 3´
    B. 5´ GTC TTG GAG ATG CAC GTG CCC CTT GC 3´
15. Genomic DNA from human cell lines* (10 ng/µl)
    A. HEP G2: hepatocellular carcinoma (liver)
    B. HTB 180 NCI-H345: small cell carcinoma, lung
    C. CCL 86: Raji Burkitt lymphoma
    D. HTB 184 NCI-H510A: small cell carcinoma, extrapulmonary origin
    E. CRL 1905 H: normal skin cell line
    F. HeLa: epithelial carcinoma cell line
    G. K562: erythromyeloblastoid leukemia cell line, chronic myeloid leukemia
16. Agarose
17. TBE buffer
18. Bromophenol blue tracking dye

* Examples of human cell lines that can be used to demonstrate a D1S80 profile.

19. 125 ml Erlenmeyer flask
20. Ethidium bromide or coomassie blue
21. Molecular weight markers (526 to 22,621 bp)
22. Incubator or water bath at 56°C and 100°C
23. Electrophoresis systems (gel tray or combs)
24. Power pack or supply
25. DNA thermal cycler
26. Microwave or hot plate
27. Microcentrifuge
28. Tabletop clinical centrifuge
29. Polaroid camera with film

## Procedure

The use of disposable gloves and aerosol-resistant pipet tips is highly recommended to prevent cross-contamination. Helpful organizational sheets are provided at the end of the exercise.

### *Collection of Cells (e.g., buccal cells, liquid blood, and cultured human cells)*

1. Label a 15 ml polypropylene test tube and the top of a 1.5 ml Eppendorf or microcentrifuge tube with your name and any other appropriate information.
2. Pipet 10 ml of cultured human cells (maximum $5 \times 10^6$ cells/ml) or liquid sample into the polypropylene test tube. The cultured human cells can serve as the positive control or as the "mock" evidentiary sample. For buccal cells, pour 10 ml of saline solution into your mouth, and vigorously swish against your cheeks for 10 sec. Expel saline solution back into the labeled 15 ml polypropylene test tube over the sink.

   Or,

   if sterile swabs are available, place the swab inside your mouth, and press it firmly against the inside of your cheek. Roll the swab back and forth over the inside surface of your cheek at least 10 times. Repeat on the other cheek. Place the swab into a labeled 15 ml test tube containing 10 ml saline solution.

### *Concentrate Cells by Centrifugation*

3. Centrifuge the samples at 300 × g for 5 min. The cells will form a firm pellet at the bottom of the tube.
4. Decant the saline supernatant into a liquid waste container. Be careful not to disturb the cell pellet at the bottom of the tube.
5. Add 500 µl of Chelex beads into the 15 ml test tube containing the cell pellets. Resuspend cells with Chelex either by pipetting in and out several times or by tapping with your finger.
6. Transfer a 500 µl aliquot of the cell–Chelex slurry into a sterile 1.5 ml Eppendorf tube. Make sure the Eppendorf tube is labeled.

## Lysing the Cells and Collecting the DNA

7. Place the capped (closed) Eppendorf tubes in a "float," and place in a boiling water bath for 10 min.
8. After the heat treatment, place the samples on ice for 5 min.
9. Place the Eppendorf tubes containing the lysed cells in a microcentrifuge, and spin at the maximum speed for 1 min. The pellet contains the Chelex beads bound to the denatured proteins. The supernatant contains the DNA.
10. Using a sterile pipet tip, transfer all of the clear supernatant to a fresh 1.5 ml Eppendorf tube. Label the tube, and place on ice until you are ready to proceed to the next step.

## Setting Up the PCR Amplification

11. Label 3 0.5 ml microcentrifuge tubes as follows: 1) "DNA/human cell line—positive control"; 2) "buccal swab"; and 3) "no DNA—negative control."
12. Dispense 10 µl of appropriate genomic DNA (10 ng/µl) into the labeled tubes and 10 µl of distilled $H_2O$ into the tube labeled "no DNA."
13. Prepare the following "master mix" for 6.5 reactions (see Table 3). To determine the final volume of the master mix needed for all of the reactions, calculate the required amount of each component of the PCR master mix.
14. Add 15 µl of the master mix to the 3 tubes containing genomic DNA, mix, and overlay with 50 µl of mineral oil if needed. Depending on the thermal cycler model used, this step may be omitted.
15. Place the reaction tubes into a thermal cycler programmed to run at least 35 cycles with the following parameters:

| | | | |
|---|---|---|---|
| Step 1 | 2.0 min | 94°C | Denaturation |
| Step 2 | 1.0 min | 94°C | Denaturation |
| Step 3 | 1.0 min | 64°C | Annealing |
| Step 4 | 1.5 min | 70°C | Extension |
| Step 5 | Repeat Steps 2–4 for a total of 35 times. | | |
| Step 6 | Soak | 4°C | |

**TABLE 3**
**PCR Master Mix for the D1S80 Locus**

| PCR Master Mix Component | Volume | Final Concentration |
|---|---|---|
| 10X PCR buffer | 16.25 µl | 1X |
| 10 mM dNTP | 13.00 µl | 800 µM |
| D1S80 primer (a) | 3.25 µl | 50 ng/reaction |
| D1S80 primer (b) | 3.25 µl | 50 ng/reaction |
| *Taq* DNA polymerase | 3.25 µl | |
| Distilled $H_2O$ | 58.50 µl | |
| **Total volume** | 97.50 µl | |

**Note:** The parameters outlined above may vary according to the thermal cycler used for the PCR amplification.

16. Start or "run" the PCR incubation reaction. When the thermal cycler's program is completed (approximately 3 hr), the tubes containing the PCR products will be removed by your instructor and stored at 0°C.

## *Visualization of the D1S80 PCR Products*

17. Because small PCR products (or DNA fragments) in the range of 200 to 600 base pairs are expected, a high concentration of agarose is required for adequate separation during gel electrophoresis. Prepare the agarose gel as described in Step 2 of the "Procedure" section of Exercise 4; however, TBE buffer is preferred (instead of TAE buffer) for separation of DNA molecules less than 1 kb in size.
18. Remove 16 µl of the PCR product(s), and place into separate microcentrifuge tubes. Add 4 µl of loading or tracking dye to each tube.
19. The DNA sample, mixed with loading buffer (6 µl total volume), is pipetted into the well with the gel submerged in TBE buffer. Be careful not to push the pipet tip through the bottom of the well in the gel.
20. Include on your gel the K562 DNA standards (concentration determined previously). Load the gel as follows:

| | | |
|---|---|---|
| Lane 1 | K562 DNA (positive control) | 16 µl |
| Lane 2 | Molecular weight markers | 16 µl |
| Lane 3–18 | DNA from buccal swabs | 16 µl |
| Lane 19 | Molecular weight markers | 16 µl |
| Lane 20 | No DNA control | 16 µl |

21. Set the voltage (100 volts), and turn on the power supply. "RUN" the samples until the bromophenol blue tracking dye has moved 1–2 cm from the origin (i.e., well) or until the dye front is approximately 2 cm from the bottom of the gel. This should take less than 1 hr.
22. Stain the gel in SYBR green or ethidium bromide (if not added previously to the agarose gel or reservoir buffer). Photograph the gel, and determine the allele response and size at the D1S80 locus.

## Sample Setup for Thermal Cycler

Analyst: _____ Date: _____ Gel No: _____

Thermal Cycler: _____ Start Time: _____

Date of Last Calibration: _____ System: _____

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | A11 | A12 |
| B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | B10 | B11 | B12 |
| C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 |
| D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 | D9 | D10 | D11 | D12 |
| E1 | E2 | E3 | E4 | E5 | E6 | E7 | E8 | E9 | E10 | E11 | E12 |
| F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 | F11 | F12 |
| G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | G10 | G11 | G12 |
| H1 | H2 | H3 | H4 | H5 | H6 | H7 | H8 | H9 | H10 | H11 | H12 |

## Results

### D1S80 GEL

Analyst: _____ Lab Number: _____

Date: _____

| Well No. | Sample | DNA (ng) |
|---|---|---|
| 1 | K562 DNA | |
| 2 | Molecular Weight Marker | |
| 3 | DNA from Buccal Swab | |
| 4 | " | |
| 5 | " | |
| 6 | " | |
| 7 | " | |
| 8 | " | |
| 9 | " | |
| 10 | " | |
| 11 | " | |
| 12 | " | |
| 13 | " | |
| 14 | " | |
| 15 | " | |
| 16 | " | |
| 17 | " | |
| 18 | " | |
| 19 | Molecular Weight Marker | |
| 20 | No DNA Control | |

| Reagents | Lot Number | Source |
|---|---|---|
| Agarose | | |
| 1X TBE (Gel Buffer) | | |
| 1X TBE (Tank Buffer) | | |
| Loading Buffer | | |
| Ethidium Bromide | | |
| Visual Marker | | |
| | | |
| | | |

## Gel Electrophoresis

| | | |
|---|---|---|
| Time on: | Voltage: | mAMPs: |
| Time off: | Voltage | mAMPs: |

Gel Prepared By: _____ Date: _____

## Reporting Form

Tape Your Gel Photo Here

Lane #1 _____

Lane #2 _____

Lane #3 _____

Lane #4 _____

Lane #5 _____

Lane #6 _____

Lane #7 _____

Lane #8 _____

Lane #9 _____

Lane #10 _____

Lane #11 _____

Lane #12 _____

Lane #13 _____

Lane #14 _____

Lane #15 _____

Lane #16 _____

Lane #17 _____

Lane #18 _____

Lane #19 _____

Lane #20 _____

## D1S80 GEL

Analyst: _____ Lab Number: _____

Date: _____

| Well No. | Sample | DNA (ng) |
|---|---|---|
| 1 | | |
| 2 | | |
| 3 | | |
| 4 | | |
| 5 | | |
| 6 | | |
| 7 | | |
| 8 | | |
| 9 | | |
| 10 | | |
| 11 | | |
| 12 | | |
| 13 | | |
| 14 | | |
| 15 | | |
| 16 | | |
| 17 | | |
| 18 | | |
| 19 | | |
| 20 | | |

## Interpreting Your Results

Your group will work together to interpret the photograph of your gel with the test results. Attach the gel photograph to the Reporting Form (see previous). For each lane, state the approximate size of each fragment and if the person appears to be heterozygous or homozygous at the D1S80 locus. Use the table below to record your observations. Also, examine the other groups' test results, and attempt to determine the number of responses or the different alleles present in the class. Are any of the profiles similar? Are any two genotypes the same?

### *Basis of the D1S80 Typing System*

- The locus consists of repeating units of segments of DNA that are 16 nucleotides in length.
- The number of tandem repeats varies from one individual to the next.
- The alleles range from 15 to over 41. This variability in the number of tandem repeats is the basis for identification in the D1S80 system.

**D1S80 Typing Results**

| Sample | D1S80 Locus |
|---|---|
| 1 | |
| 2 | |
| 3 | |
| 4 | |
| 5 | |
| 6 | |
| 7 | |
| 8 | |
| 9 | |
| 10 | |
| 11 | |
| 12 | |
| 13 | |
| 14 | |
| 15 | |
| 16 | |
| 17 | |
| 18 | |
| 19 | |
| 20 | |

## Questions

1. What are the 3 steps in the PCR process?

2. What are the necessary components of the PCR process?

3. How are the PCR products analyzed to ensure that the PCR reaction was successful?

4. What is the "power of discrimination" with the D1S80 typing system?

Chapter 11

# Experiment 8

## *Polymerase Chain Reaction (PCR)–Based Tests: Short Tandem Repeat (STR) Analysis*

## Introduction

A DNA segment that appears more than once on the same chromosome is known as a repeat. Human genomes contain 5–10% of such repetitive sequences that occur in tandem or adjacent to each other. These repetitive sequences vary in size and length and show sufficient variability among individuals in a population. Regions of DNA that contain these short repeated segments are referred to as short tandem repeats (STRs) and are important markers for human identity testing in the forensic community.

There are literally thousands of STR markers scattered throughout the human genome, and they occur, on average, in one in every 10,000 nucleotides. The DNA sequence repeated in an STR motif is usually from 2 to 7 base pairs (bp), with 4 bases being the preferred size for forensic systems (Edwards et al., 1991, 1992; Warne et al., 1991). An example of a 4 bp or a tetranucleotide repeat is shown below, where the TCTA motif is repeated 4 times.

…ATGTGA TCTA TCTATCTATCTATTGG…

PCR-based STR systems offer many advantages over earlier DNA typing techniques (e.g., restriction fragment length polymorphisms, or RFLP). STR systems provide a rapid and sensitive method to evaluate small amounts (1 ng) of human DNA. This small amount of DNA needed for STR systems is 50 times less than what is normally required for RFLP analysis. Also, the repeating sequences in an STR are relatively short, with the entire STR strand or allele generally less than 400 bp in length. This short length renders STR systems amendable to the analysis of samples suspected of being degraded. STR analysis often allows the DNA analyst to recover a complete DNA profile even from an evidentiary sample that was exposed to unfavorable conditions (e.g., body or stains subject to extreme decomposition). This is in sharp contrast to

RFLP systems, which require a large sample size for analysis and full-length fragments, which often consist of thousands of bases, to generate a complete DNA profile.

STRs and corresponding loci are easily amplified by PCR. Further, PCR amplification of many different STR loci is commonly performed simultaneously in the same tube. The simultaneous amplification of 2 or more loci is commonly known as multiplexing, or multiplex PCR. For a multiplexing reaction to be successful, the system must be designed to ensure that the sizes of the amplified products do not overlap, thereby allowing each STR allele for a specific locus to be clearly visualized on a gel or by capillary electrophoresis. This "requirement design" of overlapping fragments became less important with the development of multiple color detection systems.

Different detection methods are available to visualize the STR products. The STR loci and corresponding alleles may be separated by gel electrophoresis and detected using ethidium bromide and silver staining or exotic dyes (e.g., SYBR green). Several STR systems have been developed where fluorescent dyes or labels are used to detect the STR alleles either during separation (i.e., capillary electrophoresis) or after separation (i.e., gel electrophoresis). The resulting STR profiles are routinely interpreted by direct comparison to DNA standards, allelic ladders (an artificial mixture of common alleles present in the human population for a particular STR marker or locus), and reference standards (known DNA profiles from the victim and suspect). Probability calculations are determined based upon classical population genetic principles.

For STR markers to be effective across various jurisdictions, a common set of standardized markers is used. Currently, the forensic scientific community in the United States has established a set of 13 core STR loci that, in turn, can be entered into a national database known as the Combined DNA Index System (CODIS), a collection of DNA profiles from known offenders. A summary of the 13 CODIS loci is contained in Table 4.

**TABLE 4**
**Information on the 13 Core Short Tandem Repeat Loci Listed in CODIS**

| STR Locus | Chromosome Number | Sequence |
|---|---|---|
| FGA | 4 | CTTT |
| vWA | 12 | [TCTG][TCTA] |
| D3S1358 | 3 | [TCTG][TCTA] |
| D21S11 | 21 | [TCTA][TCTG] |
| D8S1179 | 8 | TATC |
| D7S820 | 7 | GATA |
| D13S317 | 13 | TATC |
| D5S818 | 5 | AGAT |
| D16S539 | 16 | GATA |
| CSF1PO | 5 | AGAT |
| TPOX | 2 | AATG |
| THO1 | 11 | TCAT |
| D18S51 | 18 | AGAA |

## Objective

In this exercise, you will extract DNA from cells in your mouth (buccal swabs) and/or from a human cell line, incubate the isolated DNA with appropriate PCR reagents, and amplify the alleles at multiple STR loci using the GenePrint STR Systems (Promega Corporation, Madison, Wisconsin). Following amplification, the amplified STR products will be separated and identified using agarose gel electrophoresis.

## Equipment and Material

1. 0.5, 1.0, and 1.5 ml Eppendorf or microcentrifuge tubes
2. 30 or 50 ml conical tubes
3. Mineral oil (optional)
4. 15 ml polypropylene test tube
5. Double-distilled water
6. Sterile cotton swabs
7. Adjustable-volume digital micropipets (2–200 µl range)
8. Aerosol-resistant pipet tips
9. GenePrint STR Systems CSF1PO, TPOX, and TH01 (Promega Corporation)
10. Taq DNA polymerase (not supplied in kit)
11. Disposable gloves
12. Ice in buckets
13. Genomic DNA from human cell lines* (10 ng/µl)
    A. HEP G2: Hepatocellular carcinoma (liver), male
    B. HTB 180 NCI-H345: Small cell carcinoma, lung
    C. CCL 86: Raji Burkitt lymphoma
    D. HTB 184 NCI-H510A: Small cell carcinoma, extrapulmonary origin
    E. CRL 1905 H: Normal skin cell line
    F. HeLa: Epithelial carcinoma cell line
    G. K562: Erythromyeloblastoid leukemia cell line, chronic myeloid leukemia
14. Molecular weight markers (526 to 22,621 bp)
15. Ethidium bromide or coomassie blue
16. Bromophenol blue tracking dye
17. 125 ml Erlenmeyer flask
18. TBE buffer
19. TAE buffer
20. Agarose
21. DNA thermal cycler

* Examples of human cell lines that can be used to demonstrate an STR profile and serve as a positive control.

22. Electrophoresis systems (gel tray or combs)
23. Power pack or supply
24. Microwave or hot plate
25. Incubator or water bath at 56°C
26. Microcentrifuge
27. Tabletop clinical centrifuge
28. Polaroid camera with film

## Procedure

To prevent cross-contamination, the use of disposable gloves and aerosol-resistant pipet tips is highly recommended. Helpful organizational sheets are provided at the end of the exercise.

1. Refer to Exercise 1 for the methods and/or steps used in the collection and concentration of cells and for methods outlining cell lysis and the collection of DNA.

### *Setting Up the PCR Amplification*

1. Thaw the PCR reagents (STR 10X buffer and STR 10X primer pairs), and keep on ice. These reagents will be combined to form the PCR "master mix" for the multiplex reactions.
2. Determine the number of reactions to be set up. Positive and negative controls should also be included when determining the number of reactions.
3. For each reaction, label one sterile 0.5 ml microcentrifuge tube, and place into a rack.
4. To determine the final volume of the master mix needed for all of the reactions, calculate the required amount of each component of the PCR master mix (see Table 5). Multiply the volume (µl) per sample by the total number of reactions (from Step 2) to obtain the final volume (µl). To compensate for pipetting error, add enough components to the master mix for two additional reactions.
5. In the order listed in Table 5, add the final volume of each component to a sterile microcentrifuge tube. Once completed, mix the components gently, and place on ice.
6. Add 22.50 µl of the PCR master mix to each tube (from Step 4, above), and place on ice.
7. For amplification, add the appropriate volume (use 5 ng) of template DNA (extracted DNA from the buccal swabs and the human cell lines) to each reaction tube.
8. For the positive control, pipet 2.5 µl of genomic DNA (5 ng of human cell line DNA) into a 0.5 ml microcentrifuge tube containing 22.5 µl of the PCR master mix.
9. For the negative control, pipet 2.5 µl of sterile water (not template DNA) into a 0.5 ml microcentrifuge tube containing 22.5 µl of the PCR master mix.
10. Add 1 drop of mineral oil to each microcentrifuge tube to prevent evaporation. Close the tubes, and centrifuge briefly (5 sec). Depending on the thermal cycler model used, this step may be omitted.
11. Place the reaction tubes into a thermal cycler programmed to run at least 35 cycles with the following parameters:

**TABLE 5**
**Multiplex Reactions Containing Three Loci**

| PCR Master Mix Component | Volume per Sample (μl) | Number of Reactions | Final Volume (μl) |
|---|---|---|---|
| Sterile water | 17.35 | | |
| STR 10X buffer | 2.50 | | |
| Multiplex 10X primer pair mix | 2.50 | | |
| *Taq* DNA polymerase (at 5u/μl)* | 0.15 (0.75u) | | |
| **Total volume** | **22.50** | | |

*Note:* If the DNA is stored in TE buffer, the volume of the DNA sample should not exceed 20% of the final volume because components of the buffer compromise PCR amplification efficiency and quality. This rule does not apply to DNA stored in sterile water.

* The volumes or values given for Taq DNA polymerase assume a concentration of 5u/Fl. If the final volume is less than 0.5 μl, the enzyme can be diluted with STR 1X buffer, and then a larger volume added. Because the enzyme cannot be stored diluted, only prepare the amount that you will need. The amount of sterile water can be adjusted accordingly so that the final volume of each reaction is 25 Fl.

| | | | |
|---|---|---|---|
| Step 1 | 2.0 min | 94°C | Denaturation |
| Step 2 | 1.0 min | 94°C | Denaturation |
| Step 3 | 1.0 min | 64°C | Annealing |
| Step 4 | 1.5 min | 70°C | Extension |
| Step 5 | Repeat Steps 2–4 for a total of 35 times. | | |
| Step 6 | Soak | 4°C | |

*Note:* The parameters outlined above may vary according to the thermal cycler used for the PCR amplification.

12. Start or "run" the PCR incubation reaction. When the thermal cycler's program is completed (approximately 3 hr), the tubes containing the PCR products will be removed by your instructor and stored at 0°C.

## *Visualization of the STR PCR Products*

1. Because small PCR products (or DNA fragments) in the range of 150 to 400 base pairs are expected, a high concentration of agarose is required for adequate separation during gel electrophoresis. Prepare the agarose gel as described in Step 2 of the "Procedure" section of Exercise 4.
2. Remove 2.5 μl of the PCR product(s), and place into separate microcentrifuge tubes. Add 2.5 μl of STR 2X Loading Solution (supplied with kit) to each tube.
3. Add 2.5 μl (50 ng) of pGEM DNA markers (supplied with kit) to 2.5 μl of STR 2X loading solution.

**Note:** The pGEM DNA markers are visual standards used to confirm allelic size ranges for each locus. The markers consist of 15 DNA fragments with weights (bp) of 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51, and 36.

4. Add 2.5 µl of the STR allelic ladder (supplied in kit) to 2.5 µl of STR 2X loading solution for each ladder lane (at least 2 per gel).
5. Using a different pipet tip for each sample, load the DNA samples, mixed with loading solution (5 µl total volume), into the wells with the gel submerged. Be careful not to push the pipet tip through the bottom of the well in the gel.
6. Include on your gel the positive and negative controls. Load the gel as follows:

| | | |
|---|---|---|
| Lane 1 | pGEM markers | 5 µl |
| Lane 2 | STR allelic ladder* | 5 µl |
| Lane 3 | Human cell line DNA (positive control) | 16 µl |
| Lane 4 | Negative control (no DNA) | 16 µl |
| Lanes 5–11 | DNA from buccal swabs | 16 µl |
| Lane 12 | STR allelic ladder | 5 µl |
| Lanes 13–19 | DNA from buccal swabs | 16 µl |
| Lane 20 | STR allelic ladder | 5 µl |

* For ease of interpretation, the allelic ladders can be run in lanes adjacent to each sample.

7. Set the voltage (100 volts), and "RUN" the samples until the bromophenol blue tracking dye has moved 1–2 cm from the origin (i.e., well) or until the dye front is approximately 2 cm from the bottom of the gel. This should take less than 1 hr.
8. Photograph the gel, and determine the allele response and size at each locus. Direct comparison between the allelic ladders and amplified samples of the same locus should allow for the numerical assignment of each allele (see Table 6 and Figure 12).
   Artifacts or unidentifiable DNA bands may be detected with this system. For questions related to these STR "by-products," refer to the "Troubleshooting Guide" in the technical manual for the Geneprint System.

**TABLE 6**
**Locus-Specific Information for the Geneprint STR Systems (CSF1PO, TPOX, and TH01)**

| Component Loci | Allelic Ladder Size Range (Bases) | STR Ladder Alleles (Number of Repeats) | Other Known Alleles | K562 DNA Allele Sizes |
|---|---|---|---|---|
| **CSF1PO** | 295–327 | 7, 8, 9, 10, 11, 12, 13, 14, 15 | 6 | 9, 10 |
| **TPOX** | 224–252 | 6, 7, 8, 9, 10, 11, 12, 13 | None | 8, 9 |
| **THO1** | 179–203 | 5, 6, 7, 8, 9, 10, 11 | 9.3 | 9.3, 9.3 |

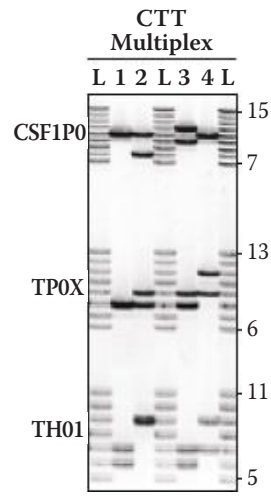



**FIGURE 12**
Profiles from the GenePrint STR Systems (CSF1PO, TPOX, and TH01). *Note*: Genomic DNA (Lanes 1–4) was amplified using the CTT Multiplex STR System, separated in a 4% polyacrylamide denaturing gel, and detected using silver stain. The lanes labeled "L" contain the allelic ladders for each locus (i.e., CSF1PO, TPOX, and TH01). The numbers to the right of the image indicate the smallest to the largest number of repeats or numerical designation for each allele. *Source*: Courtesy of the Promega Corporation.

## Sample Setup for Thermal Cycler

Analyst: _____ Date: _____ Gel No: _____

Thermal Cycler: _____ Start Time: _____

Date of Last Calibration: _____ System: _____

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | A11 | A12 |
| B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | B10 | B11 | B12 |
| C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 |
| D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 | D9 | D10 | D11 | D12 |
| E1 | E2 | E3 | E4 | E5 | E6 | E7 | E8 | E9 | E10 | E11 | E12 |
| F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 | F11 | F12 |
| G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | G10 | G11 | G12 |
| H1 | H2 | H3 | H4 | H5 | H6 | H7 | H8 | H9 | H10 | H11 | H12 |

## Results

### STR Test Gel

Analyst: _____ Lab Number: _____

Date: _____

| Well No. | Sample | DNA (ng) |
|---|---|---|
| **1** | pGEM Marker | |
| **2** | STR Allelic Ladder | |
| **3** | Human Cell Line (Positive Control) | |
| **4** | No DNA (Negative Control) | |
| **5** | DNA from Buccal Swabs | |
| **6** | " | |
| **7** | " | |
| **8** | " | |
| **9** | " | |
| **10** | " | |
| **11** | " | |
| **12** | STR Allelic Ladder | |
| **13** | " | |
| **14** | " | |
| **15** | " | |
| **16** | " | |
| **17** | " | |
| **18** | " | |
| **19** | " | |
| **20** | STR Allelic Ladder | |

| Reagents | Lot Number | Source |
|---|---|---|
| Agarose | | |
| 1X TBE (Gel Buffer) | | |
| 1X TBE (Tank Buffer) | | |
| Loading Buffer | | |
| Ethidium Bromide | | |
| Visual Marker | | |
| | | |
| | | |

## Gel Electrophoresis

| | | |
|---|---|---|
| Time on: | Voltage: | mAMPs: |
| Time off: | Voltage | mAMPs: |

Gel Prepared By: _____ Date: _____

## Reporting Form

Tape Your Gel Photo Here

## STR Test Gel

Analyst: _____ Lab Number: _____

Date: _____

| Well No. | Sample | DNA (ng) |
|---|---|---|
| 1 | | |
| 2 | | |
| 3 | | |
| 4 | | |
| 5 | | |
| 6 | | |
| 7 | | |
| 8 | | |
| 9 | | |
| 10 | | |
| 11 | | |
| 12 | | |
| 13 | | |
| 14 | | |
| 15 | | |
| 16 | | |
| 17 | | |
| 18 | | |
| 19 | | |
| 20 | | |

## Interpreting Your STR Results

Your group will work together to interpret the photograph of your gel with the test results. Attach the gel photograph to the reporting form (see previous page). Determine the allele response and size at each locus. Direct comparison between the allelic ladders and amplified samples of the same locus should allow for the numerical assignment of each allele (see Figure 12 and Table 6).

For each lane, state the approximate size of each fragment and if the person appears to be heterozygous or homozygous at the different STR loci. Also, examine the other groups' test results, and attempt to determine the number of responses or the different alleles present in the class. Are any of the profiles similar? Are any two genotypes the same?

**STR Typing Results**

| Sample | LOCUS | | |
|---|---|---|---|
| | CSF1PO | TPOX | THO1 |
| 1 | | | |
| 2 | | | |
| 3 | | | |
| 4 | | | |
| 5 | | | |
| 6 | | | |
| 7 | | | |
| 8 | | | |
| 9 | | | |
| 10 | | | |
| 11 | | | |
| 12 | | | |
| 13 | | | |
| 14 | | | |
| 15 | | | |
| 16 | | | |
| 17 | | | |
| 18 | | | |
| 19 | | | |
| 20 | | | |

## Questions

1. What are the advantages of using STR systems versus some of the earlier DNA typing techniques?

2. STR loci chosen for use in the forensic community have many characteristics. Describe 3 favorable characteristics of STR loci.

3. What are some of the challenges that a forensic DNA analyst confronts with STR typing?

4. Why are STRs preferred genetic markers?

Chapter 12

# Experiment 9

## *Polymerase Chain Reaction (PCR)–Based Tests: Short Tandem Repeat (STR) Analysis to Determine Paternity (a Case Study)*

## Introduction

DNA typing is the most accurate form of paternity testing possible. DNA typing can indicate with 100% certainty if the tested male is excluded as the biological father or will demonstrate with a high degree of scientific certainty (i.e., greater than 99.9% probability) if the tested male is the biological father. DNA paternity tests can be used to answer questions and/or issues such as the following:

- Paternity or maternity identification and verification
- Child support and custody disputes
- Suspected incest cases
- Inconclusive paternity results from other methods
- Single-parent cases where paternity or maternity is in question
- Newborn testing
- Prenatal paternity cases
- Identification of father in surrogate mother cases
- Estate or trust disputes

Parentage testing is performed by collecting biological samples (e.g., blood or buccal swabs) from the mother, the child, and the alleged biological father. For newborns, testing can be performed using umbilical blood from the umbilical cord. In unusual circumstances, DNA can be collected from other sources, as

previously described (see "Types of Biological Samples"). DNA testing is based on genetic information that is passed on from the parents to their children (see Table 7). In cases where the alleged father is unavailable for testing, partial pedigree analysis can be conducted using DNA samples from the parents of the alleged father. If necessary, siblings of the alleged father can also be used.

## Objective

In this exercise, DNA has been extracted from samples (i.e., buccal swabs) collected from the mother, the child, and the alleged father. The isolated DNA was amplified at multiple STR loci using PCR and the GenePrint STR Systems (Promega Corporation). Following amplification, the amplified STR products were separated and identified using polyacrylamide gel electrophoresis (PAGE). In this exercise, you will be analyzing data, specific to a case study, generated using PAGE. As the amplified STR products are separated, the fluorescently labeled DNA molecules are excited by the laser light source, and the emission is captured by a detection system and recorded as a chemilumigraph (e.g., bar code format) by the computer.

## Equipment and Material

1. 0.5, 1.0, and 1.5 ml Eppendorf or microcentrifuge tubes
2. Agarose
3. Mineral oil (optional)
4. 15 ml polypropylene test tube
5. Double-distilled water
6. Ice in buckets
7. Adjustable-volume digital micropipets (2–200 µl range)
8. Aerosol-resistant pipet tips
9. GenePrint STR Systems (PowerPlex 16 BIO System) (Promega Corporation, Madison, Wisconsin)
10. Taq DNA polymerase (not supplied in kit)
11. Disposable gloves
12. TAE buffer
13. Genomic DNA (10 ng/µl) from mother, child, and alleged father
14. Molecular weight markers (526 to 22,621 bp)
15. Ethidium bromide or coomassie blue
16. Bromophenol blue tracking dye
17. 125 ml Erlenmeyer flask
18. TBE buffer (loading buffer)
19. 30 or 50 ml conical tubes
20. Incubator or water bath at 56°C
21. Electrophoresis systems (gel tray or combs)
22. Power pack or supply

**TABLE 7**
**PowerPlex 16 BIO Typing Results**
DNA was extracted from samples collected from the mother, the child, the boyfriend, and the alleged father and amplified at the STR loci using the PowerPlex 16 BIO System.

| Locus | 15-Year-Old Mother | Child | 18-Year-Old Boyfriend | Alleged Father |
|---|---|---|---|---|
| FGA | 21, 23 | 22, 23 | 21, 22 | 20, 22 |
| TPOX | 8, 10 | 8, 11 | 11, 11 | 11, 11 |
| D8S1179 | 12, 13 | 12, 13 | 13, 13 | 13, 13 |
| vWA | 14, 18 | 14, 15 | 17, 18 | 14, 15 |
| Penta E | 10, 11 | 11, 16 | 10, 11 | 14, 16 |
| D18S51 | 13, 14 | 14, 14 | 14, 17 | 14, 17 |
| D21S11 | 31.2, 32.2 | 30, 32.2 | 30, 30 | 29, 30 |
| TH01 | 6, 9 | 6, 9.3 | 5, 6 | 6, 9.3 |
| D3S1358 | 16, 17 | 16, 17 | 15, 15 | 15, 17 |
| Penta D | 11, 14 | 11, 11 | 11, 14 | 11, 12 |
| CSF1PO | 8, 12 | 8, 12 | 8, 11 | 12, 12 |
| D16S539 | 11, 13 | 9, 11 | 9, 13 | 9, 10 |
| D7S820 | 9, 10 | 8, 9 | 8, 8 | 8, 9 |
| D13S317 | 11, 12 | 10, 11 | 7, 12 | 10, 13 |
| D5S818 | 7, 8 | 7, 11 | 11, 12 | 11, 12 |
| Amelogenin | XX | XX | XY | XY |

23. DNA thermal cycler
24. Microcentrifuge
25. Microwave or hot plate

## Procedure

To prevent cross-contamination, the use of disposable gloves and aerosol-resistant pipet tips is highly recommended.

1. Refer to Exercise 7 for the methods and/or steps used in the collection and concentration of cells and for methods outlining cell lysis and the collection of DNA.
2. Refer to Exercise 7 for the methods and/or steps used in setting up the PCR amplification and for the visualization of STR PCR products.

### *A Case Study*

In 2007, a pregnant 15-year-old female claimed that her father had sexually assaulted her, which resulted in her pregnancy. The father, in his early 50s, was arrested and charged with incestuous pedophilia and sexual abuse. The father denied all claims and stated that his daughter was sexually active with her 18-year-old boyfriend. To establish a genetic profile of the fetus and to determine the biological father, a chorionic villus sample (CVS) from the fetus was collected. Samples (i.e., buccal swabs) were also collected from the mother (i.e., the 15-year-old daughter), the 18-year-old boyfriend, and the alleged father. All samples were sent to the state laboratory for DNA analysis. DNA was extracted from all samples, purified, and subjected to STR analysis using the PowerPlex 16 BIO typing system (Promega Corporation). The fluorescently labeled STR products were then separated by gel electrophoresis, and the DNA molecules captured using a fluorescent detection system. The STR typing results of the mother, the child, the 18-year old boyfriend, and the alleged father are shown in Table 7.

## Interpreting Your STR Typing Results

Your group will work together to interpret the data generated from the STR analysis for the above-referenced case study. To determine if the alleged father or the 18-year-old boyfriend is the biological father, you should first determine which alleles (or STR fragments) of the child were donated by the mother. This analysis should be performed for each locus. Second, determine if the remaining allele at each locus of the child could have been contributed by the alleged father or the boyfriend. If one allele at any locus does not match, the alleged father or the boyfriend is excluded. If all of the remaining alleles at each locus "match" the alleged father's or the boyfriend's STR profile, then one is presumed to be the biological father. On the basis of these results obtained from all genetic systems tested, the alleged father or the boyfriend cannot be excluded as the biological father of the child. The paternity probability, determined to be 99.99%, would also support these findings that the alleged father or the boyfriend is, in fact, the biological father.

## Questions

1. Based on your analysis of the STR typing results, was the alleged father "excluded" as the biological father, or were his genetic markers consistent with those observed in the child and thus he "could not be ruled out" as the biological father?

2. Based on your analysis of the STR typing results, was the boyfriend "excluded" as the biological father, or were his genetic markers consistent with those observed in the child and thus he "could not be ruled out" as the biological father?

3. Using the same STR typing results, would you be able to determine maternity in this instance? Why or why not?

4. Assuming one of the biological parents' profile was unavailable, would you be able to determine the other parent's genetic contribution to the child? How?

5. Assuming that an alleged father (or the boyfriend) refused to provide a sample for DNA testing but you had legal access to his home, what samples would you collect for analysis? The idea is to collect enough material to generate a DNA profile.

6. If the child displayed homozygosity at one locus (e.g., for FGA: 22, 22), would you be able to assign each allele to either parent? Why or why not?

7. How accurate is DNA paternity testing? Are the results conclusive?

8. Will the DNA paternity test results stand up in court? Explain your answer.

9. The collection of cheek cells (e.g., a buccal swab) is often performed instead of collecting blood as a source of biological material for DNA paternity typing. Will the resulting DNA profile from the buccal swab be as accurate as one degenerated from blood cells? Explain your answer.

Chapter 13

# Experiment 10

## *Polymerase Chain Reaction (PCR)–Based Tests: Y-Chromosome Short Tandem Repeat (Y-STR) Analysis (a Case Study)*

## Introduction

The ability to designate whether a sample originated from a male or female contributor is extremely valuable in sexual assault cases as well as in other capital cases. The most popular method for sex typing is the amelogenin typing system because the DNA encoding gender can be amplified in conjunction with STR analysis. However, in some instances, STR and amelogenin analysis is not adequate when multiple males contribute to an evidentiary sample (e.g., a blood sample containing DNA from more than one male). Recently, Y-STR analysis has become available to the forensic community and has provided identification where STR analysis was not definitive.

Several genetic markers have been identified on the Y chromosome that are distinct from markers on the autosomes and are useful for human (male) identification (Table 8). The Y-STR markers are found on the noncoding region located on both arms (i.e., p and q) of the Y chromosome. The Y-STR markers produce a haplotype profile when amplified from male DNA. Such a profile simplifies the interpretation of a mixture containing both a male and female contributor by eliminating the female contribution from the amplification profile. This also eliminates the need to separate semen and vaginal epithelial cells prior to analysis. The Y-STR markers are extremely valuable in sexual assault cases where samples contain multiple male contributors.

Y-STR markers are also useful in the analysis of lineage and the reconstruction of family relationships. In essence, a sample from a male may be compared with those of another male and/or his brother, father, paternal grandfather, or paternal uncles for identification purposes and familiar relationships. Because these markers are only paternally inherited, they are useful in paternity-related matters. In addition, Y-STR markers' use and effectiveness in lineage studies can extend to answering questions of common ancestral geographical origin. Y-STR markers, together with mitochondrial DNA (mtDNA) markers (see Exercise 11), will complement each other in these ancestral analyses.

**TABLE 8**
**Y-STR Loci of the PowerPlex Y System**

| Y-STR Locus | Sequence |
|---|---|
| DYS391 | TCTA |
| DYS389I | [TCTG][TCTA] |
| DYS439 | GATA |
| DYS389II | [TCTG][TCTA] |
| DYS393 | AGAT |
| DYS390 | [TCTG][TCTA] |
| DYS385a/b | GAAA |
| DYS438 | TTTTC |
| DYS437 | [TCTA][TCTG] |
| DYS19 | TAGA |
| DYS392 | TAT |

## Objective

In this exercise, DNA has been extracted from male buccal swabs and/or from a human cell line of male origin, incubated with appropriate PCR reagents, and multiple Y-STR loci amplified using the PowerPlex Y System (Promega Corporation). Following PCR, the amplified Y-STR products can be separated and analyzed using polyacrylamide gel electrophoresis or capillary electrophoresis (CE; Figure 13). In this exercise, you will be analyzing data, specific to a case study, generated using CE. As the amplified Y-STR products are separated, the fluorescently labeled DNA molecules are excited by the laser light source, the emission is captured by a detection system, and it is recorded as an electropherogram by the computer.

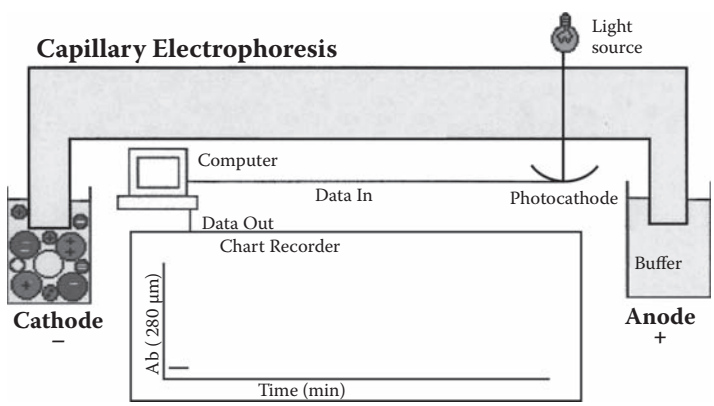



**FIGURE 13**
Schematic of the Capillary Electrophoresis System. *Note*: Samples are injected into the tube on the left (cathode) and "travel" to the right or to the anode. The fluorescently labeled DNA molecules (or amplified products) are excited by the light source, captured by a detection system, and recorded as an electropherogram by the computer. *Source*: Courtesy of http://www.CEandCEC.com.

## Equipment and Material

1. 0.5, 1.0, and 1.5 ml Eppendorf or microcentrifuge tubes
2. 30 or 50 ml conical tubes
3. Mineral oil (optional)
4. 15 ml polypropylene test tube
5. Double-distilled water
6. Ice in buckets
7. Adjustable-volume digital micropipets (2–200 µl range)
8. Aerosol-resistant pipet tips
9. GenePrint STR Systems CSF1PO, TPOX, and TH01 (Promega Corporation, Madison, Wisconsin)
10. Taq DNA polymerase (not supplied in kit)
11. Disposable gloves
12. Agarose
13. Genomic DNA from human cell lines
    A. HEP G2: hepatocellular carcinoma (liver), male (10 ng/µl)
14. Molecular weight markers (526 to 22,621 bp)
15. Ethidium bromide or coomassie blue
16. Bromophenol blue tracking dye
17. 125 ml Erlenmeyer flask
18. TBE buffer (loading buffer)
19. TAE buffer
20. Incubator or water bath at 56°C
21. Electrophoresis systems (gel tray or combs)
22. Power pack or supply
23. Microwave or hot plate
24. DNA thermal cycler
25. Microcentrifuge
26. Tabletop clinical centrifuge

## Procedure

To prevent cross-contamination, the use of disposable gloves and aerosol-resistant pipet tips is highly recommended. A helpful organizational sheet is provided at the end of the exercise to record your Y-STR typing results.

1. Refer to Exercise 1 for the methods and/or steps used in the collection and concentration of cells and for methods outlining cell lysis and the collection of DNA.

## *Setting Up the PCR Amplification*

1. Refer to Exercise 7 for setting up the PCR amplification reaction. Keep all samples and reagents on ice.
2. Determine the number of reactions to be set up. Positive and negative controls should also be included when determining the number of reactions.
3. For each reaction, label one sterile 0.5 ml microcentrifuge tube, and place into a rack.
4. To determine the final volume of the master mix needed for all of the reactions, calculate the required amount of each component of the PCR master mix (see Table 9). Multiply the volume (µl) per sample by the total number of reactions (from Step 2) to obtain the final volume (µl).
5. The reaction tubes were placed in a thermal cycler programmed to run in two phases—10 cycles at set parameters, followed by 22 cycles.

| | | |
|---|---|---|
| **For 10 cycles:** | | |
| Step 1 | 11.0 min | 95°C |
| Step 2 | 1.0 min | 96°C |
| Step 3 | 1.0 min | 94°C |
| Step 4 | 1.0 min | 60°C |
| Step 5 | 1.5 min | 70°C |
| **Then, for 22 cycles:** | | |
| Step 6 | 1.0 min | 90°C |
| Step 7 | 1.0 min | 58°C |
| Step 8 | 1.5 min | 70°C |
| **Then:** | | |
| Step 9 | 30 min | 60°C |
| Step 10 | Soak | 4°C |

**TABLE 9**
**Master Mix for the PowerPlex Y System**

| PCR Master Mix Component | Volume per Sample (µl) | Number of Reactions | Final Volume (µl) |
|---|---|---|---|
| Sterile Nuclease-free Water | | | |
| Gold ST*R 10X Buffer (Promega Corporation) | 2.50 | | |
| PowerPlex Y 10X Primer Pair Mix | 2.50 | | |
| AmpliTaq Gold DNA Polymerase (at 5u/µl)* | 0.55 (2.75 units) | | |
| **Total volume** | **25.00** | | |

*Note:* Template DNA volume (0.25–1 ng)—up to 19.45 µl.

**Note:** The parameters outlined above may vary according to the thermal cycler used for the PCR amplification.

## Detection of the Y-STR PCR Products

1. Following PCR amplification, the fluorescently labeled Y-STR alleles are separated and sized using polyacrylamide gel electrophoresis (PAGE) or capillary electrophoresis (CE).
2. Samples are denatured by heating at 95°C for 3 min (PAGE) or by diluting with a denaturant solution (CE), then are immediately chilled on ice.
3. The samples are loaded onto a gel for PAGE analysis or introduced into the capillary for CE analysis by injection. For PAGE analysis, multiple samples are separated and analyzed in 2.5 to 3 hr. For CE analysis, only one sample is injected into a capillary tube for separation and analysis; however, this process is completed in a matter of a few minutes.
4. Detection of the sample analyzed by PAGE is performed by scanning each lane and then imaged using a computer detection system. Detection of the sample is performed automatically by the CE instruments by measuring the time span from injection to sample detection with a laser near the end of the capillary. In both instances (i.e., PAGE and CE), the laser excites the fluorescently labeled DNA fragments, which causes a fluorescent light emission. This emission is captured by the detection system and plotted as a function of the relative fluorescence intensity observed from each fluorescent dye attached to the DNA molecule. These signals, recorded as bands on a gel or as an electropherogram for CE, can then be used to detect and quantify the Y-STR PCR products (Figure 14).

## Case Study

A woman was walking to her car, which was parked in an underground parking garage. As she was unlocking her car door, a man approached her from behind, forced her into the back seat of her car, and raped her. After the attacker fled the scene, she immediately called the police from her cell phone. The police took her to the local hospital, where she was examined by a sexual assault nurse examiner. Vaginal swabs and a reference sample were collected and sent to the state's forensic laboratory for analysis. The woman had described her attacker as a tall and thin African American male with a tattoo on his right hand (the only part of him that was visible to her during the attack). From that description and a file of known sexual offenders, the police arrested a male suspect. A blood sample was collected from the suspect and sent to the forensic laboratory for analysis. Because the woman was not married, did not have a boyfriend, and had not had consensual sex in several weeks, samples from consensual partners were not needed for analysis.

At the laboratory, the samples were subjected to DNA typing, specifically Y-STR analysis. The vaginal swabs taken from the victim were found to contain sperm and her own cells. The sperm cells were first separated from the victim's epithelial cells, and the DNA isolated using differential extraction. The purified DNA was amplified by PCR and analyzed at 11 Y-STR loci (DYS391, DYS389I, DYS439, DYS389II, DYS438, DYS437, DYS19, DYS392, DYS393, DYS390, and DYS385). The suspect's DNA was also analyzed at the same Y-STR loci.

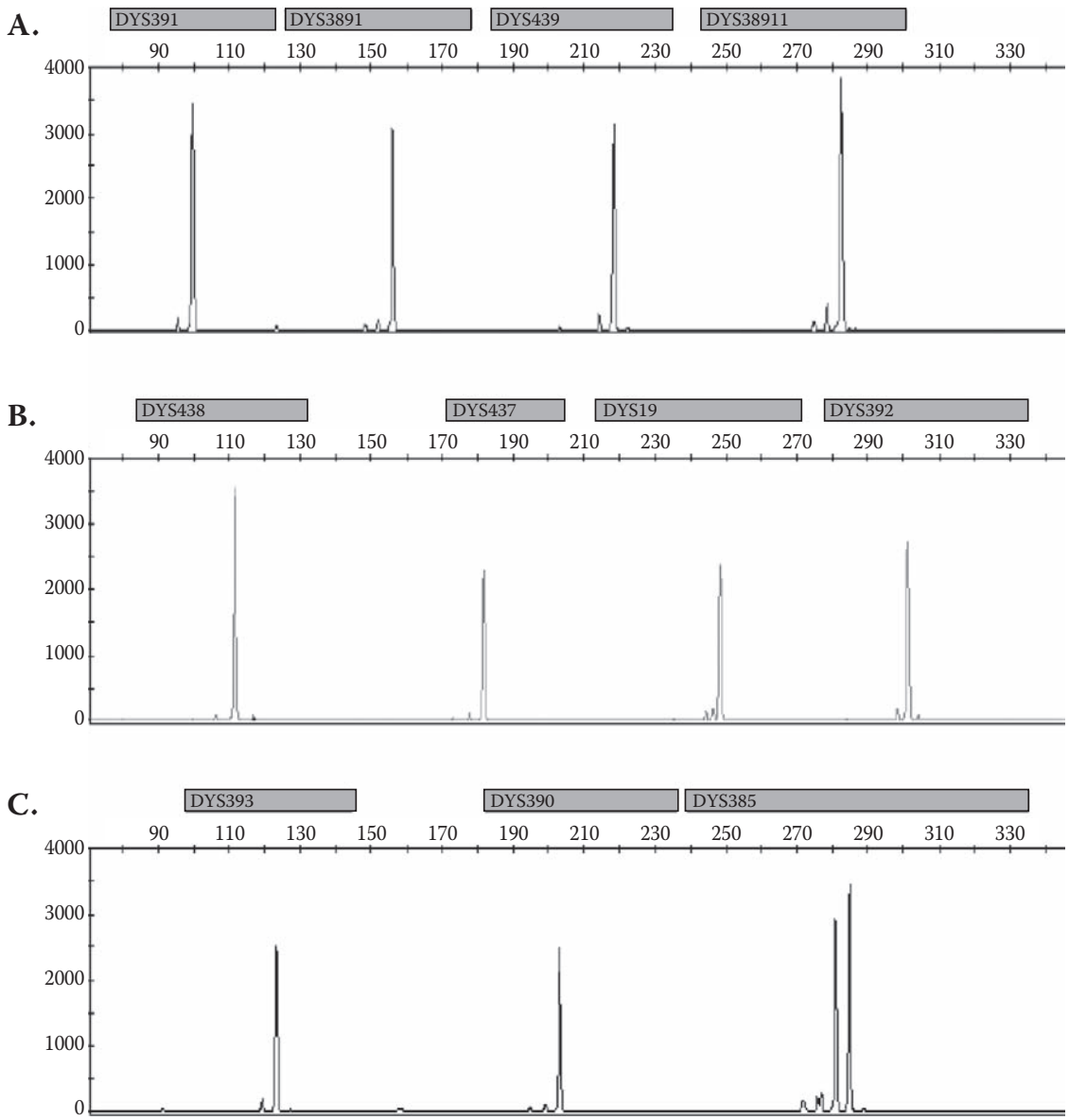



**FIGURE 14**
The PowerPlex Y System. **Note:** A single source sample from a male contributor was amplified using the PowerPlex Y System. The amplified products were captured using the Applied Biosystems 3130 Genetic Analyzer and analyzed using the GeneMapper ID software to generate the Y-STR profile. Panel A: An electropherogram of the DYS391, DYS389I, DYS439, and DYS389II loci. Panel B: An electropherogram of the DYS438, DYS437, DYS19, and DYS392 loci. Panel C: An electropherogram of DYS393, DYS390, and DYS385. *Source*: Courtesy of the Promega Corporation.

## Data Analysis

1. Following amplification, the amplified Y-STR products from the suspect's known reference sample (see Table 10 for Y-STR results) and the sperm fraction from the vaginal swab were separated by CE (Figure 14).
2. The suspect's allelic response at each Y-STR locus can be identified by direct comparison between the Y-STR profile from the sperm fraction (Figure 15) and the allelic ladders (Figure 16). The allelic

**TABLE 10**
**Y-STR Typing Results**

| Y-STR Loci | Suspect's Reference Sample |
|---|---|
| DYS391 | 10 |
| DYS389I | 14 |
| DYS439 | 11 |
| DYS389II | 32 |
| DYS438 | 10 |
| DYS437 | 14 |
| DYS19 | 15 |
| DYS392 | 12 |
| DYS393 | 14 |
| DYS390 | 23 |
| DYS385 | 15, 17 |

responses observed for each locus should allow for the numerical assignment of each allele. The electropherogram of the negative control should be devoid of any amplification products (results not shown). Conversely, the electropherogram of the positive control should consist of the male DNA standard with known allelic responses (data not shown).

## Interpreting Your Y-STR Results

Your group will work together to interpret the electropherograms in Figure 15, Figure 16, and Figure 17. Using the Y-STR typing results from the suspect's reference sample (Table 10), the Y-STR profile from the sperm fraction of the vaginal swab from the victim (Figure 15) generated by CE, and the allelic ladder (Figure 16), determine the allelic numerical designation for each locus analyzed. In addition, determine the overall Y-STR profile from the sperm fraction, and record your observation in the "Y-STR Typing Results" table (below).

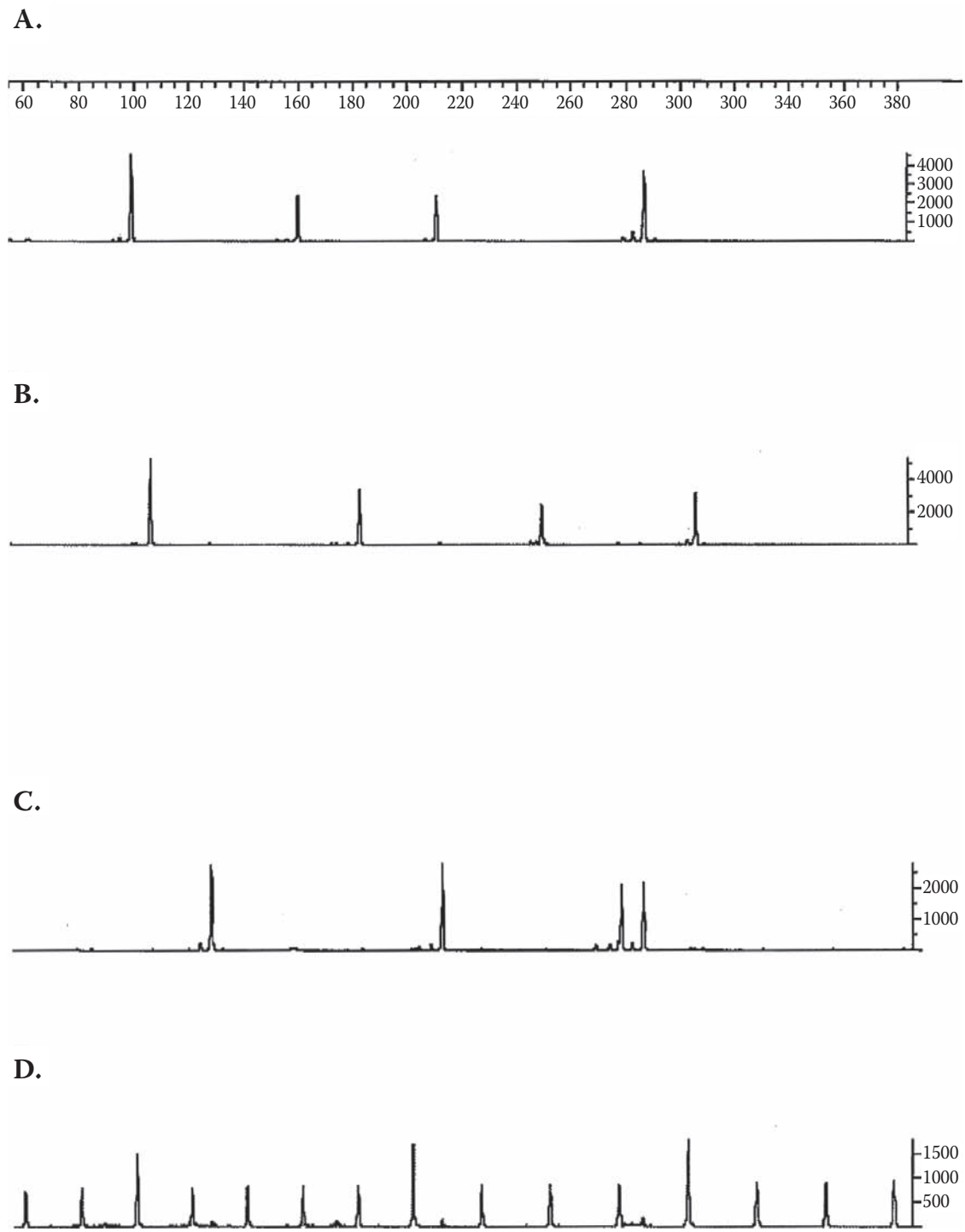



**FIGURE 15**

PowerPlex Y-STR Typing Results. **Note:** The Y-STR profile, shown in the electropherogram below, was obtained from the sperm fraction of the vaginal swab collected from the victim. Eleven Y-STR loci, specific to the male chromosome, were amplified and separated by capillary electrophoresis. Panel A: An electropherogram of the DYS391, DYS389I, DYS439, and DYS389II loci. Panel B: An electropherogram of the DYS438, DYS437, DYS19, and DYS392 loci. Panel C: An electropherogram of DYS393, DYS390, and DYS385. Panel D: An electropherogram showing the fragments of the Internal Lane Standard.

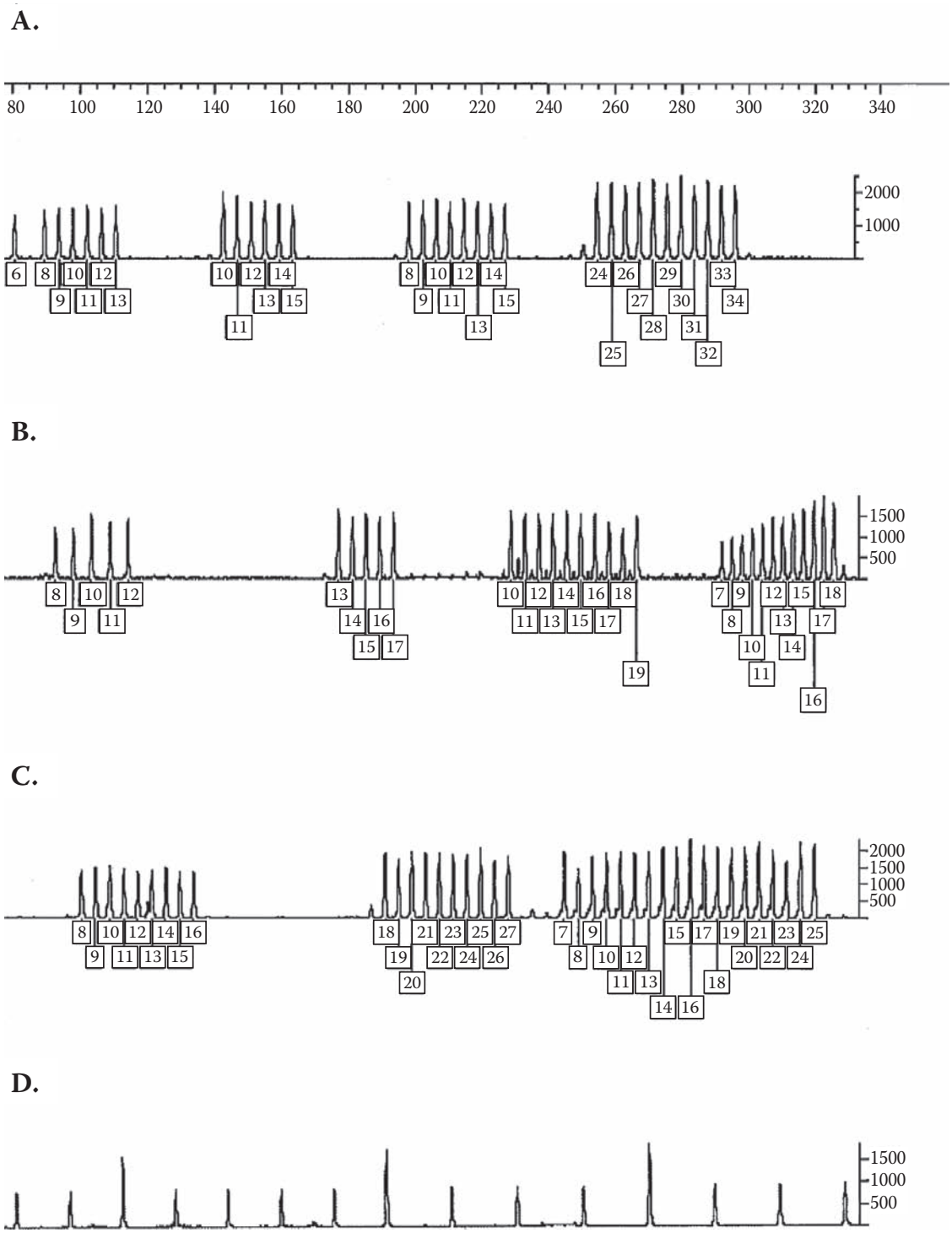



**FIGURE 16**

The PowerPlex Y Allelic Ladder Mix. **Note:** The allelic components and their allelic or numerical designations. Panel A: An electropherogram of the DYS391, DYS389I, DYS439, and DYS389II loci. Panel B: An electropherogram of the DYS438, DYS437, DYS19, and DYS392 loci. Panel C: An electropherogram of DYS393, DYS390, and DYS385. Panel D: An electropherogram showing the fragments of the Internal Lane Standard.

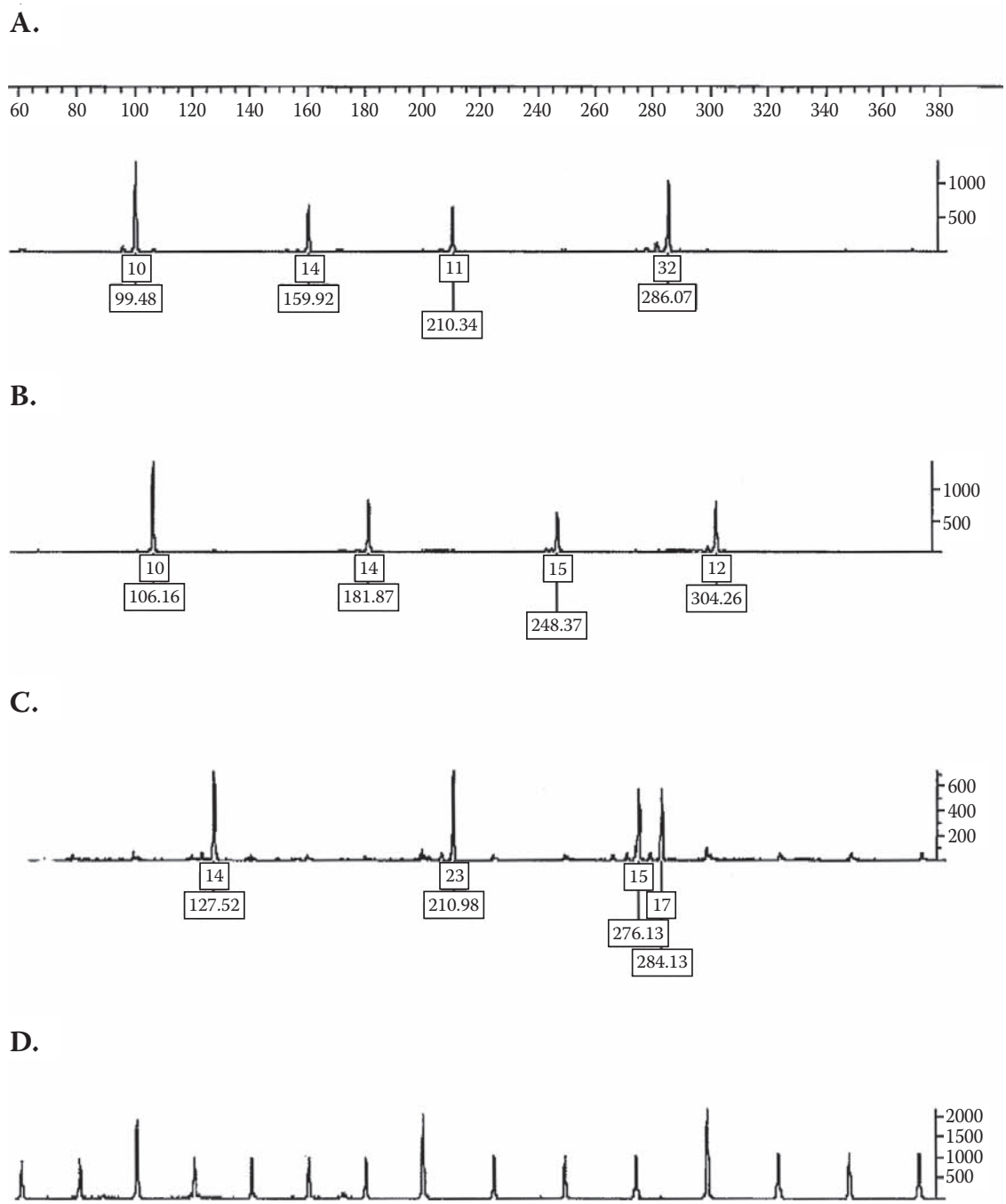



**FIGURE 17**

The PowerPlex Y-STR Typing Results: Alleles Identified. **Note:** The Y-STR profile, shown in the electropherogram below, was obtained following amplification of the STR loci specific to the male chromosome. Direct comparison between the allelic ladder (Figure 12) and the amplified sperm fraction of the same locus allowed for the numerical assignment of each allele. Panel A: An electropherogram of the DYS391, DYS389I, DYS439, and DYS389II loci. Panel B: An electropherogram of the DYS438, DYS437, DYS19, and DYS392 loci. Panel C: An electropherogram of DYS393, DYS390, and DYS385. Panel D: An electropherogram showing the fragments of the Internal Lane Standard.

**Y-STR Typing Results**

| Y-STR Loci | Sperm Fraction from Vaginal Swab | Suspect's Reference Sample |
|---|---|---|
| DYS391 | | 10 |
| DYS389I | | 14 |
| DYS439 | | 11 |
| DYS389II | | 32 |
| DYS438 | | 10 |
| DYS437 | | 14 |
| DYS19 | | 15 |
| DYS392 | | 12 |
| DYS393 | | 14 |
| DYS390 | | 23 |
| DYS385 | | 15, 17 |

## Questions

1. Are the Y-STR profiles similar between the sperm fraction and the suspect's known reference sample? Explain your answer.

2. In this case study, the sperm cells were separated from the victim's epithelial cells at the start of the Y-STR analysis. Was this step necessary? Why or why not?

3. How does the Y-STR profile, generated by your group from Figure 15, compare to the Y-STR profile observed in Figure 17?

4. If multiple males were involved in the sexual assault, how would you differentiate between each contributor using Y-STR analysis? What type of Y-STR allelic response would you expect to see at each locus?

5. Would STR analysis complement the Y-STR results? Explain your answer.

Chapter 14

# Exercise 11
## *Mitochondrial DNA (mtDNA) Analysis*

## Introduction

Mitochondrial DNA (mtDNA) typing is increasingly used in human identity testing when biological evidence may be degraded, when quantities of the samples in question are limited, or when nuclear DNA typing is not an option. Biological sources of mtDNA include hairs, bones, and teeth. In humans, mtDNA is inherited strictly from the mother. Consequently, mtDNA analysis cannot discriminate between maternally related individuals (e.g., mother and daughter, or brother and sister). However, this unique characteristic of mtDNA is beneficial for missing person cases when mtDNA samples can be compared to samples provided by the maternal relative of the missing person.

In humans, the mtDNA genome is approximately 16,569 bases (A, T, G, and C) in length, containing a "control region" with two highly polymorphic regions (Figure 18, at the top of the figure between the "F" and "P" sites). These two regions, termed Hypervariable Region 1 (HV1) and Hypervariable Region 2 (HV2), are 342 and 268 base pairs (bp) in length, respectively, and are highly variable within the human population. This sequence (the specific order of bases along a DNA strand) variability in either region provides an attractive target for forensic identification studies. Moreover, because human cells contain several hundred copies of mtDNA, substantially more template DNA is available for amplification using PCR than with nuclear DNA.

Mitochondrial DNA typing begins with the extraction of mtDNA from the mitochondria of human cells followed by PCR amplification of the hypervariable regions. The amplified mtDNA is purified, then subjected to the dideoxy terminator method of sequencing (Sanger et al., 1977), with the final products containing a fluorescently labeled base at the end position. The products from the sequencing reaction are separated, based on their length, by gel electrophoresis. The resulting sequences or profiles are then compared to sequences of a known reference sample to determine differences and similarities between samples. Samples are not excluded as originating from the same source if each base (A, T, G, or C) at every position along the hypervariable regions is similar. This sequence, if determined to be

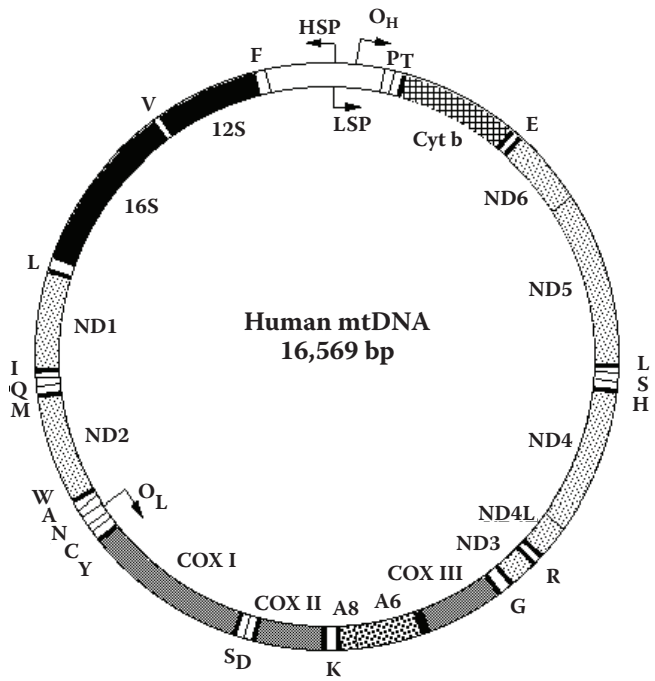



**FIGURE 18**
Mitochondrial Genome in Humans. **Note:** The two noncoding hypervariable control regions (HV1 and HV2), located within the "D-loop" (between the "F" and "P" sites) of the mtDNA genome, are positioned at the top of the figure. Each of the hypervariable regions is approximately 300 bp in length. The HV1 extends from nucleotide 16024 to 16383, and the HV2 from nucleotide 57 to 372 (Butler and Levin, 1998). *Source*: Courtesy of Columbia University.

similar between a known reference sample and an evidentiary sample, can be entered and searched in a database containing mtDNA sequences from four main racial groups (Caucasians, African Americans, Hispanics, and Asians). The search will generate a number that represents the number of observations of that sequence in each racial subgroup within the database. For example, a sequence might be seen 3 times in the database samples of Hispanic descent and not appear in the remaining database subgroups. Or, a sequence may not be observed at all in the database and is reported as not being observed. This number is usually reported as *1 out of 4800 sequences* or *0 out of 4800 sequences*. However, due to the size of the mtDNA database and to the unknown number of mtDNA sequences in the human population, a reliable frequency estimate is not provided. Consequently, mtDNA sequencing is becoming known as an exclusionary tool as well as a technique to complement other human identification techniques.

## Objective

In this exercise, you will isolate mitochondria from cells, extract mtDNA from the mitochondrial fraction, incubate the isolated mtDNA with appropriate PCR reagents, and amplify the hypervariable regions. The amplified mtDNA is then purified, subjected to sequencing, and separated by gel electrophoresis. You will compare the sequencing data from your known reference and unknown sample to a known standard.

## Equipment and Material

1. 0.5, 1.0, and 1.5 ml Eppendorf or microcentrifuge tubes
2. 30 or 50 ml conical tube
3. Mineral oil (optional)
4. 15 ml polypropylene test tube
5. Double-distilled water
6. 10% ammonium persulfate solution
7. Adjustable-volume digital micropipets (2–200 µl range)
8. Aerosol-resistant pipet tips
9. Taq DNA polymerase
10. Disposable gloves
11. Ice in buckets
12. mtDNA from human cell lines
    A. HEP G2: hepatocellular carcinoma (liver), male
13. Molecular weight markers (526 to 22,621 bp)
14. mtDNA primers
15. Ethidium bromide and coomassie blue
16. Bromophenol blue tracking dye
17. Tetramethylethylenediamine (TEMED)
18. USB Thermo Sequenase Cycle Sequencing Kit (USB Corporation, Cleveland, Ohio)
19. 20 and 60 cc Hamilton syringe and a 14 gauge needle
20. LI-COR Infrared DNA Analyzer (Model 4300) or equivalent
21. Microcentrifuge
22. DNA thermal cycler
23. Microwave or hot plate
24. Incubator or water bath at 56°C and 92°C

## Procedure

To prevent cross-contamination, the use of disposable gloves and aerosol-resistant pipet tips is highly recommended. A helpful organizational sheet is provided at the end of the exercise to record your mtDNA typing data.

The protocol provided below is a basic guide to DNA sequencing using the LI-COR Infrared DNA Analyzer (Model 4300). Various aspects of sequencing are discussed (i.e., mtDNA analysis), including template preparation, primers used, reagents, labeled primer sequencing, gel preparation, and data analysis and interpretation.

The LI-COR system (LI-COR Biosciences, Lincoln, NE) detects DNA using infrared (IR) fluorescence. In the dideoxy sequencing reaction, the DNA polymerase incorporates either a nucleotide or a primer

labeled with an IRDye™ into a newly synthesized set of chain-terminated complementary strands. The IRDye™-labeled fragments are separated by gel electrophoresis and are detected using a laser that excites the dye on the DNA fragments. The emission or signal is a series of bands displayed on a computer in a "bar code" format similar to an autoradiograph. The bar code image is captured by the DNA sequencer and analyzed using specific software (e.g., e-Seq). The sequence data are determined for each lane, and the specific order of bases (A, T, G, and C) is determined. The sequence data are presented as a standard chromatogram or as an ASCII text.

## *A. Template Preparation*

The quality and/or purity of the DNA template will dictate the sequence data quality. Several DNA extraction and purification methods were described in previous exercises that will ensure and maximize the quality and purity of the DNA template (see Exercises 1, 2, and 3). In addition to the quality of the DNA, it is important to determine the quantity of DNA in the known and/or evidentiary sample to be analyzed. Similar amounts of DNA template in each reaction will provide consistent data and similar band intensities. Template DNA concentration should range between 0.5 and 1.0 µg/µl (see Tables 11 and 12). The amount of template DNA used in the reaction is based on the size of the DNA sequence between the two primers. If lower yields are obtained, concentrate the DNA by resuspending the final pellet in a smaller volume of buffer (see Exercise 2).

**TABLE 11**
**Template Amount Used in Simultaneous Bidirectional Sequencing (SBS)**

| Size (bp) | Template (fmoles) |
|---|---|
| 300 – 600 | 50 – 100 |
| 600 – 1200 | 125 – 225 |
| 1300 – 1800 | 250 – 300 |
| > 1800 | 300 – 500 |

**TABLE 12**
**Concentration of Template DNA Recommended for Labeled Primer Cycle Sequencing**

| Template | Amount (fmoles) |
|---|---|
| Plasmid | 200 – 500 |
| PCR products | 20 – 50 |
| M13 | 100 – 200 |
| Cosmids | 50 (1.5 µg) |

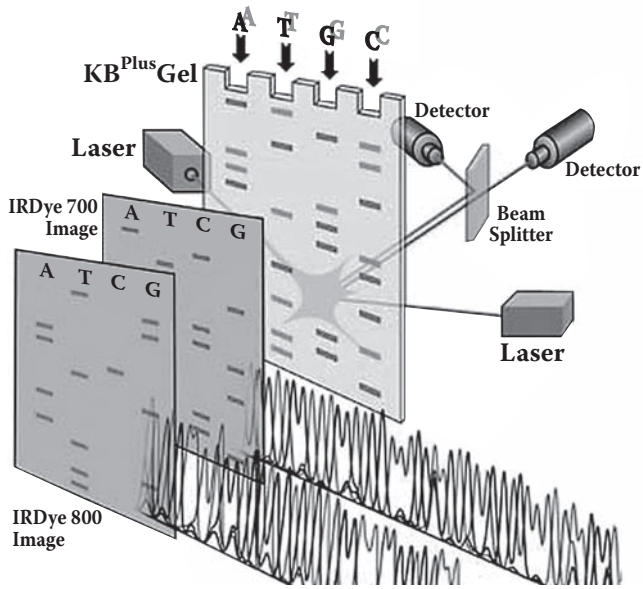



**FIGURE 19**
The LI-COR Infrared DNA Analyzer (Model 4300). **Note:** Samples are loaded on the polyacrylamide gel and separated by electrophoresis. As the samples pass in front of the scanning laser or microscope, two photodiodes (e.g., the detectors) detect fluorescence. Each detector measures fluorescence from only one of the infrared dyes. A separate image (similar to an autoradiogram) for each IR dye is collected in real time and can be displayed in an Internet browser or in LI-COR application software (e.g., e-Seq or Saga). *Source*: Used with permission from LI-COR Biosciences.

## B. Primers

Successful sequencing reactions depend on many factors; however, primer design (i.e., having 40–50% GC content, containing a G or C at the 3´ end, and avoiding base repeats greater than 3 bases), primer purity and concentration are the most critical factors. Many primers are commercially available from several distributors and are, in general, of a high quality. If not commercially available, it is essential that the main impurities (e.g., salts or organic groups) that can affect the sequencing reaction have been removed.

## C. Labeled Primer Sequencing

Simultaneous bidirectional sequencing (SBS) uses two labeled primers (e.g., forward and reverse primer pairs labeled with different IR dyes) on a single DNA template in a single reaction. An SBS reaction uses equal amounts of labeled primers (IRDye™ 700 and IRDye™ 800) to obtain equal signal strength in both channels. **Note:** The Model 4300 detection system uses two separate lasers and detection that maximizes sequence accuracy (see Figure 19).

## D. Setting Up the PCR Amplification

1. Label four 0.5 ml microcentrifuge tubes as follows: A, T, G, and C.
2. Prepare the following "Template–Primer Master Mix" (see Table 13). Add the largest volume first, and then add solutions in descending order based on volume. Mix the components by pipetting.

**TABLE 13**
**Template–Primer Master Mix**

| Template/Primer Master Mix Components | Volume |
|---|---|
| Template DNA | 0.3 µl (300 ng) |
| IRDye™ 700 Forward Primer (1.0 pmol/µl) | 1.5 µl |
| IRDye™ 800 Reverse Primer (1.0 pmol/µl) | 1.5 µl |
| Thermo sequenase reaction buffer | 2.0 µl |
| 2.5 mM dNTP nucleotide mix | 1.0 µl |
| Thermo sequenase DNA polymerase | 2.0 µl |
| $ddH_2O$ to bring final volume to 17.0 µl | __ µl |
| **Total volume** | 17.0 µl |

3. Add 4 µl of the template–primer master mix to each tube labeled A, T, G, and C.
4. Add 4 µl of the A reagent to the tube labeled A, 4 µl of the T reagent to the tube labeled T, and so on (reagents supplied with Sequenase Cycle Sequencing Kit).
5. Add 1 drop of mineral oil to each microcentrifuge tube to prevent evaporation. This step is required for thermal cyclers without heated lids. Close the tubes, and centrifuge briefly (5 sec).
6. Place the reaction tubes into a thermal cycler programmed to run at least 30 cycles with the following parameters:

| | | | |
|---|---|---|---|
| Step 1 | 2.0 min | 92°C | Denaturation |
| Step 2 | 30 sec | 92°C | Denaturation |
| Step 3 | 30 sec | 54°C | Annealing |
| Step 4 | 1.0 min | 70°C | Extension |
| Step 5 | Repeat Steps 2–4 for a total of 30 cycles. | | |
| Step 6 | Soak or hold | 4°C | |

**Note:** The parameters outlined above may vary according to the thermal cycler used for the PCR amplification.

7. Start or "run" the PCR incubation reaction.
8. At the completion of the cycling program, add 4 µl of the IR$^2$ stop solution to each tube.
9. If mineral oil was used, remove the oil from each sample. Denature the samples at 92°C for 3 min. Then place the samples on ice.

## *E. Gel Electrophoresis*

### Assembling the Gel Apparatus

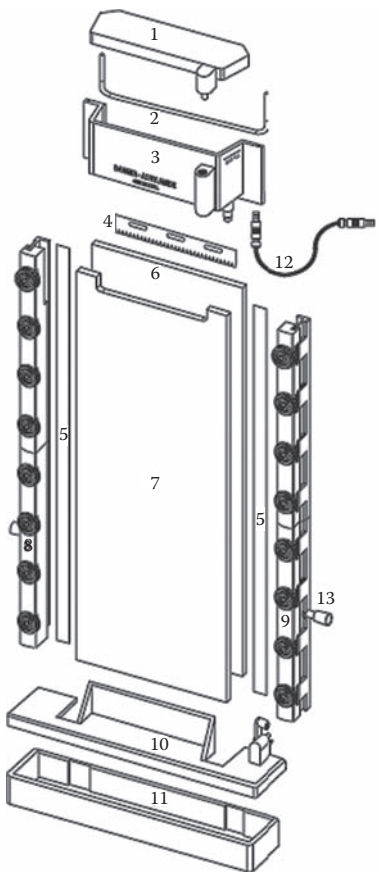
Follow the manufacturer's manual and protocol for specific instructions on assembling the electrophoresis apparatus, preparing the gel, pouring the gel, pre-electrophoresis preparation, starting the run, using the

e-Seq software, and disassembling and cleaning up the gel apparatus. The protocol outlined below highlights the major steps in the mtDNA sequencing analysis.

1. Assemble the gel sandwich by laying the back plate (Figure 20, #6) down on the bench (gel side up) and placing two spacers (Figure 20, #5) along the edges of the long axis of the glass plate.
2. Place the front plate (Figure 20, #7, gel side down) on top of the bottom plate containing the spacers, making sure the plates are aligned at the bottom.
3. Place the left and right rail assemblies over the long axis of the plate edges (Figure 20, #8 and #9). Tighten the glass clamp knobs on each rail "finger tight."

## Gel Preparation

1. The gel and running buffer solutions are prepared from a 10X TBE buffer. Empty the contents of the KB$^{Plus}$10X TBE package (supplied with the sequencing kit) in a 1 L beaker, and add distilled water to bring the volume up to 800 ml. Stir the solution until all of the solids have gone into the solution.
2. Bring the final volume to 1 L with distilled water. Store at room temperature.




| # | Description |
|---|---|
| 1 | Upper buffer tank lid |
| 2 | Silicone tubing gasket |
| 3 | Upper buffer tank |
| 4 | Comb |
| 5 | Spacers |
| 6 | Back plate |
| 7 | Front plate |
| 8 | Left rail assembly |
| 9 | Right rail assembly |
| 10 | Lower buffer tank lid |
| 11 | Lower buffer tank |
| 12 | High voltage cable |
| 13 | Rail support pin |

**FIGURE 20**
Expanded View and List of Parts for the Gel Apparatus. **Note:** The KB$^{Plus}$ Gel Matrix is a "ready-to-use solution" containing polyacrylamide. The final gel concentration is 3.7%, that is 66 cm long and 0.2 mm thick. There are other commercial acrylamides available that can be used in the LI-COR system. *Source*: Used with permission from LI-COR Biosciences.

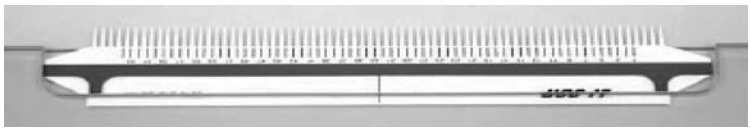


FIGURE 21
Sharkstooth Comb. *Source*: Used with permission by LI-COR Biosciences.

3. Prepare the running buffer (0.8X) by adding 80 ml of the 10X TBE to 920 ml of distilled water, and mix well.
4. For polymerization of the gel, an ammonium persulfate solution (APS) is prepared by adding 0.1 g ammonium persulfate to 1.0 ml of deionized water. The APS should be prepared fresh.
5. Bring 40 ml of the KB$^{Plus}$ Gel Matrix to room temperature.
6. Add 175 µl of the 10% APS and 17.5 µl of the TEMED to the 40 ml KB$^{Plus}$ Gel Matrix, and mix thoroughly.
7. Using a 60 cc Hamilton syringe with a 14 gauge needle, "draw" the gel solution (from Step 6, above) into the syringe, and "inject" the solution into the gel cassette.
8. After pouring the gel, invert the sharkstooth comb (Figure 20, #4), and insert it "upside down" at the top of the gel cassette between the front and back plates (Figure 20, #6 and #7).
9. The sharkstooth comb (Figure 21) is inverted prior to polymerization of the gel to make a trough. After polymerization, the comb is removed, inverted "teeth down," and inserted into the gel, forming the wells for the samples.
10. Place the casting plate (part not shown) at the top of the gel cassette and on the front plate. The casting plate will secure the comb until polymerization has occurred.
11. Allow at least 1.5 hr for polymerization.

## Electrophoresis Preparation

1. After polymerization, remove the casting plate and the sharkstooth comb.
2. Place the silicone tubing gasket (Figure 20, #2) into the back of the upper buffer tank (Figure 20, #3). Loosen the upper clamp knobs of the rail assembly (Figure 20, #8 and #9), and slide the tank into place; tighten the knobs as before.
3. Open the door of the Model 4300 DNA Analyzer, and place the lower buffer tank (Figure 20, #11) at the base of the unit.
4. Place the gel apparatus on the DNA analyzer (against the heater plate), with the bottom of the gel cassette inside the lower buffer tank. The rail support pins (Figure 20, #13) will hold the gel cassette on the instrument.
5. Fill the upper and lower buffer tanks with the 0.8X TBE running buffer prepared earlier (see "Gel Preparation," Step 3, above). Before adding the running buffer, make sure the drain fitting in the upper buffer tank is closed.
6. Place the upper and lower buffer tank lids (Figure 20, #1 and #10) onto the tanks. Attach the high-voltage cable (Figure 20, #12) to the bottom of the upper buffer tank, and insert the opposing end into the instrument chassis.

### Starting the Run

Follow the manufacturer's manual and protocol for specific instructions on starting a new run using the e-Seq software. The e-Seq software automates almost the entire sequencing process by controlling the pre-electrophoretic and electrophoretic runs, and by identifying the bases and their sequence along the mtDNA. After the pre-electrophoretic run, the e-Seq software will automatically pause the process for the user to load samples for analysis. The protocol outlined below highlights the major steps in the mtDNA sequencing analysis.

1. After the "prerun," open the instrument door of the DNA analyzer, and remove the upper buffer tank lid (Figure 20, #1).
2. Using a 20 cc syringe, flush the wells with buffer to remove any debris that may have settled during the pre-electrophoresis run.
3. Load the samples to be analyzed using a Hamilton syringe or a pipet with a flat 0.2 mm micropipet tip. Position the tip between the glass plates, and slowly release the sample into the wells.
4. After loading the samples, replace the upper buffer tank lid, close the instrument door, and push the "Start Run" button.

### Base Calling

For base calling and editing the data output, refer to the e-Seq User Guide.

## Results

In this exercise, you will be analyzing mtDNA data, specific to a case study, generated using infrared fluorescence detection (Table 14) or data that you have generated using mtDNA isolated from a human liver cell line or from buccal swabs. As the nucleotides are electrophoretically separated, the fluorescently labeled nucleotides (or bases) are excited by the laser light source, the emission is captured by a detection system, and, because of the dual-IR dye capability, the Dual Dye Automated Sequencer permits simultaneous generation of two sequence ladders.

### *A Case Study*

A very respected and dependable young man did not show up for work one day. When the young man did not show up for work on the second day, his employer called his home to see if there was something wrong. When no one answered the phone, the employer tried to contact the young man on his cell phone. The employer was only able to leave a voice mail message. The employer then called the young man's family, only to find out that he had left for work yesterday morning at the usual time.

The family called the police, who, suspecting foul play, launched an investigation. By the end of the week, the young man's body was found in an alley behind the building where he was employed. He had been beaten to death with a blunt-ended object. The victim's body was sent to the medical examiner's office, where his clothing was removed and sent to the state's laboratory for DNA analysis. During the police investigation, a hammer was found in a dumpster in the alley where the body was found. The hammer was placed in a paper bag and sent to the laboratory for analysis. Investigators also determined that the young man was having a relationship with a married woman who also worked for the same employer. When the married woman was questioned, she told the police that her husband had recently learned of the

## TABLE 14
## Mitochondrial DNA Typing Results

| Sample | Hypervariable Region 1 (HV1) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 16131 | 16185 | 16186 | 16191 | 16217 | 16225 | 16226 | 16280 | 16296 | 16313 | 16322 | 16362 | 16370 | 16392 |
| Standard | T | C | C | C | T | C | A | C | C | C | A | T | G | T |
| DD201Q1 | • | • | • | • | • | • | G | • | • | G | T | • | • | • |
| DD201Q2 | A | • | • | • | • | • | G | • | • | G | • | • | • | • |
| DD201Q3 | • | • | • | • | • | • | G | • | • | G | T | • | • | R |
| DD201K1 | A | G | G | G | C | A | • | A | A | G | • | A | C | • |
| DD201K2 | • | • | • | • | • | • | G | • | • | G | T | • | • | • |

| Sample | Hypervariable Region 2 (HV2) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 75 | 148 | 153 | 155 | 184 | 188 | 191 | 197 | 249 | 265 | 309.1 | 315.1 | 318 | |
| Standard | G | A | A | T | G | A | A | A | A | T | - | - | T | |
| DD201Q1 | C | G | • | • | • | • | • | G | • | C | G | G | • | |
| DD201Q2 | C | G | • | • | • | • | • | G | • | C | • | G | • | |
| DD201Q3 | C | G | • | • | • | • | • | G | • | C | • | G | • | |
| DD201K1 | C | • | T | G | A | T | G | • | T | C | G | G | A | |
| DD201K2 | C | G | • | • | • | • | • | G | • | C | G | G | • | |

-: At this position in the published reference sequence referred to as the Anderson sequence ("Standard," top row), there is no nucleotide, and the samples have an insertion.

•:The nucleotide is the same at this position as in the "Standard."

R: Both an A and G were observed at this position.

affair and had vowed to "straighten things out." The police spoke to the husband, who denied any knowledge of the young man's death. Considering all facts, the police arrested the husband and charged him with the murder of the young man. While in custody, the police collected a buccal swab from the husband and sent the sample to the laboratory for analysis. During the autopsy, the medical examiner collected a reference sample from the young man, which was also sent to the laboratory for analysis.

At the laboratory, several strands of hair were discovered on the victim's shirt (designated DD201Q1) and on the "handle" grip of the hammer (designated DD201Q2), and a hair was discovered on the "head" of the hammer (designated DD201Q3). During microscopic examination of the hairs, it was determined that the hair samples were candidates for mtDNA typing. The isolated mtDNA from the hair samples was purified and amplified by PCR, and a complete mtDNA profile obtained comprising the HV1 and HV2 regions. The mtDNA sequence for the evidentiary and known reference samples and the nucleotide substitutions with respect to the standard published reference (i.e., the Anderson sequence) sequence for each sample in this case are presented in Table 14 (Anderson et al., 1981).

## Data Analysis

1. Each of the evidentiary hair samples was analyzed according to standard protocol prior to opening or handling the known reference samples. The known samples were also analyzed according to standard protocol established by the laboratory.
2. All negative controls (i.e., reagent blanks and PCR blanks) remained free of contaminating DNA. All positive controls responded as expected.
3. For each sample, a complete mtDNA profile was obtained comprising nucleotide positions 15995 to 16400 (HV1) and from nucleotide 45 to 405 (HV2).
4. The mtDNA profiles of the 3 hairs (DD201Q1, DD201Q2, and DD201Q3) should be compared to the mtDNA profiles of the husband, who is the primary suspect (DD201K1), and the victim (DD201K2). The resulting mtDNA profiles are shown in Table 14.

## Interpreting Your mtDNA Results

Your group will work together to interpret the mtDNA profiles shown in Table 14. Compare the mtDNA typing results from the suspect's reference sample (DD201K1) to the mtDNA profiles generated from the hair samples. Also, compare the mtDNA results from the victim's known reference sample to the mtDNA typing results from the 3 hair samples. Each mtDNA profile from the known reference and evidentiary samples should be compared to the standard mtDNA sequence, also known as the Anderson sequence. After completing your analysis, answer the following questions:

1. Is the mtDNA profile of DD201Q2, the hair found on the "handle" grip of the hammer, similar to or different from the mtDNA profile of DD201K2, the victim? Why?

2. Are the victim (DD201K2) and his maternal relatives excluded or included as potential contributors of the hair (DD201Q2)? Why?

3. Are the mtDNA profiles of all 3 hair samples different from or similar to the mtDNA profile of DD201K1, the husband or leading suspect? Why?

4. The Federal Bureau of Investigation (FBI) sponsors an mtDNA population database containing sequences from HV1 and HV2 from several racial groups: Caucasians, Africans, Hispanics, and Asians. The database currently contains over 4800 sequences of North American forensic significance. However, the database is updated frequently and has increased over time. When a sequence from an evidentiary sample is searched in the database, a number will be reported that represents the number of observations of that sequence in each racial subgroup. What is the significance of the statement that 5 out of 4800 sequences were observed when a mtDNA sequence was searched in the database?

## Mitochondrial DNA Typing Results

| Sample | Hypervariable Region 1 (HV1) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | |
| Standard | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |

| Sample | Hypervariable Region 2 (HV2) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | |
| Standard | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |

-: At this position in the published reference sequence referred to as the Anderson sequence ("Standard," top row), there is no nucleotide, and the samples have an insertion.

•:The nucleotide is the same at this position as in the "Standard."

R: Both an A and G were observed at this position.

## Questions

1. Why were the hair samples subjected to mtDNA typing when the laboratory in this chapter's case study routinely performed STR analysis?

2. Why were the evidentiary samples analyzed separately from the known referenced samples?

3. When the mtDNA profile from the suspect was searched against profiles in the mtDNA database, it was found that the sequence was observed once in the Caucasian database. Because the database includes over 4800 sequences, what is the forensic significance of this frequency? What is the significance of a frequency reported as "not previously observed" in the current database?

4. When will reliable population frequency estimates for mtDNA types be available?

Chapter 15

# Exercise 12

## *Assessment of Lumigraph or Autoradiograph Data*

## Introduction

There are four major steps in the assessment of lumigraph or autoradiograph (autorad) data: 1) visual examination of the lumigraphs or autoradiographs, 2) computer-assisted band size determination, 3) confirmation of visual matcher, and 4) determination of point estimate value.

## A. Visual Evaluation of Lumigraphs or Autoradiographs

1. Visually examine the lane(s) of the lumigraph or autoradiograph containing the positive control (K562 DNA). There must be either one or two DNA bands for the K562 DNA positive control, depending on which RFLP or STR locus has been probed or amplified, respectively. If the positive control does not exhibit the expected number of bands for the locus under investigation, the lumigraph or autoradiograph should not be assessed.
2. Visually examine the positive control band(s) (i.e., the K562 DNA bands) for their position relative to the adjacent molecular weight markers. Depending on the locus being probed or amplified, the positive control band(s) should be located in an expected position on the lumigraph or autoradiograph. If the positive control band(s) are not located in the visually expected position on the film, the lumigraph or autoradiograph should not be assessed.
3. Visually examine the lanes of the lumigraph or autoradiograph containing the molecular weight markers. The bands in these lanes must be of sufficient intensity to be used as molecular weight references for the positive allelic control (K562 DNA), the known or reference sample(s), and the questioned or

evidentiary DNA bands. If portions of the molecular weight markers (i.e., the ladder lanes) are not visible, the size of the evidentiary DNA bands cannot be determined in these regions.

4. To assess the quality of the DNA bands, visually examine the lanes of the lumigraph or autoradiograph containing the known or evidentiary DNA. DNA band irregularities in these lanes, such as increased band width (extremely broad bands) or "smiles" (pronounced band curvature), usually indicate potential mobility shifts during electrophoresis and will often compromise the interpretation of the data. If any DNA band for the known or reference sample(s) or the questioned or evidentiary DNA is observed at a position that indicates a molecular weight greater than 10,000 bp, the evaluation of that sample at that locus is considered inconclusive.
5. Based on the assessments of the quality and position of the DNA from the lumigraph or autoradiograph, decide which samples will be subjected to the computer-assisted band-sizing procedure.

## B. Computer-Assisted Band Size Determination

The molecular weight determination of each DNA band is carried out using Windows-based or MS DOS–based computer programs (e.g., GenoTyper, GeneScan Analysis, Gel-Pro, and DNA IMAGE ANALYSIS). The forensic DNA analyst is guided through the imaging and sizing procedures by text display on the computer screen. The computer software program enables an objective estimation of the sizes of the DNA fragments in the positive control, the known samples, and each evidentiary sample. The sizing program ends by printing out the calculated fragment sizes or molecular weight, in base pairs, for each of the samples and the allelic control sample. If the DNA fragment sizes for the positive control (HaeIII-digested or amplified K562 DNA) in a particular autoradiograph or a lumigraph are not within the acceptable size range, the lumigraph or autoradiograph in question should not be used for any conclusive match determinations.

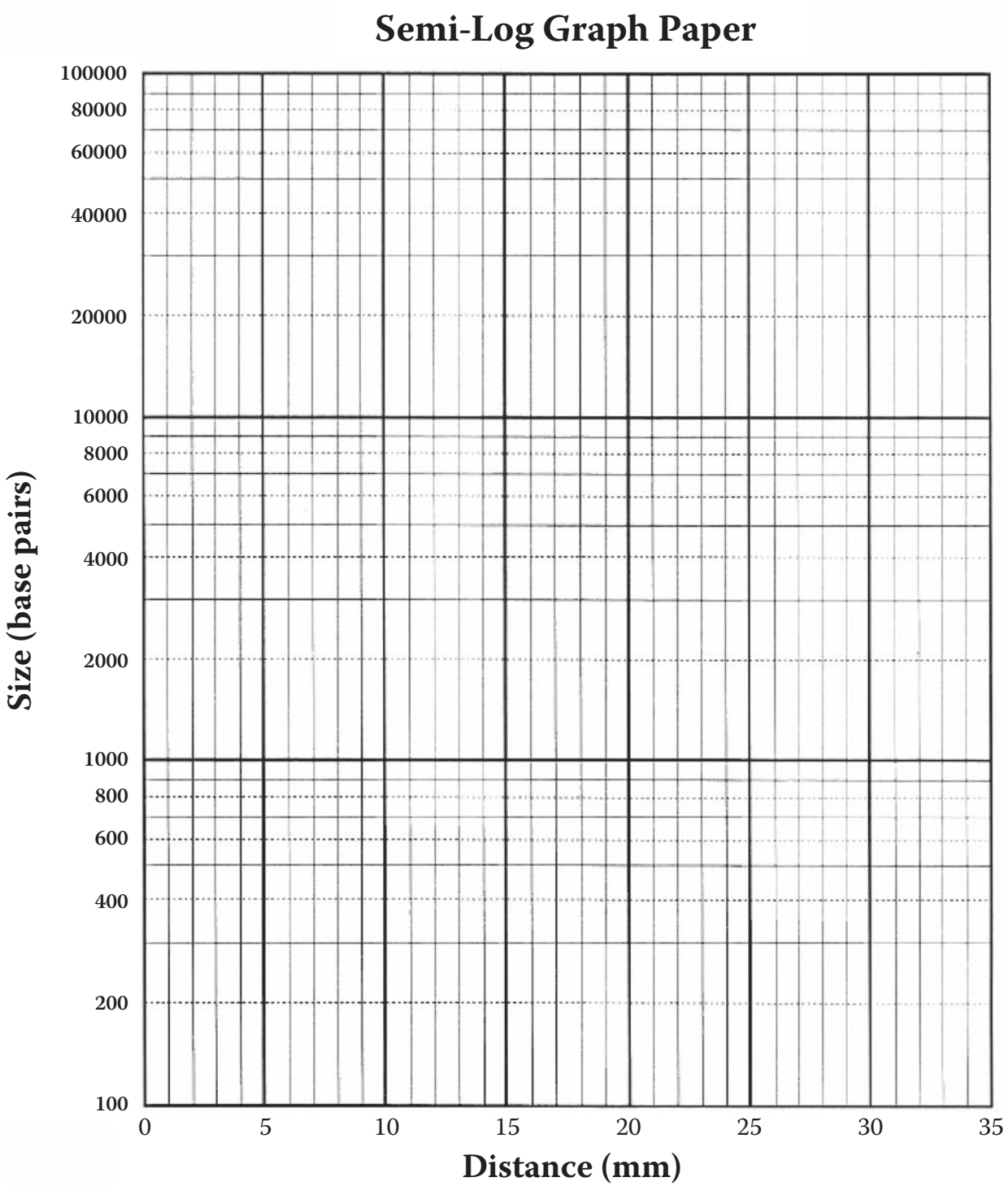
## C. Confirmation of Visual Matcher

For autoradiographs containing RFLP data, visual matches must be confirmed or rejected through application of the appropriate mathematical procedures. To make a comparison between the test samples (or crime scene samples) and the known reference samples, other than a visual comparison, a quantitative measurement of the DNA fragments observed following hybridization or amplification and visualization needs to be created. Therefore, to accomplish this procedure in the absence of a computer program, the following steps can be carried out manually to determine the molecular weight of each DNA fragment.

1. For each DNA band in the known sample, calculate a value that is 2.5% of the base pair size determined by the sizing procedure. Add the calculated value to the base pair size of the DNA fragment. Also, subtract the calculated value from the base pair size of the fragment.
2. For each fragment in a questioned specimen that has been determined to be presumptively equal in size to a fragment in a known specimen, calculate a value that is 2.5% of the base pair size of the questioned fragment determined by the sizing procedure. Add the calculated value to the base pair size of the fragment. Also, subtract the calculated value from the base pair size of the fragment.
3. Compare the calculated ranges of base pair values for the known and questioned sample bands. If these ranges overlap, the presumptive equality has been confirmed. If the ranges do not overlap, the presumptive equality of the fragment sizes is either inconclusive or exclusionary.

## D. Determination of Point Estimate Value

If sample fragment size equalities have been confirmed, the best estimate values may be determined for the fragment bands in the appropriate sample by using the appropriate computer software program.


Semi-Log Graph Paper
Size (base pairs)
100000
80000
60000
40000
20000
10000
8000
6000
4000
2000
1000
800
600
400
200
100
0
5
10
15
20
25
30
35
Distance (mm)

## Questions

1. What are the four major steps in the assessment of a DNA profile on a lumigraph or an autoradiograph?

2. When the positive control fails to exhibit the expected number of DNA fragments (or bands) in a lumigraph or an autoradiograph, are the results of the other samples tested (i.e., known or evidentiary) interpretable? Reliable? Why or why not?

3. If the ladder alleles or molecular weight markers are not visible or the DNA bands in these lanes are very low in intensity, the lumigraph or the autoradiograph cannot be assessed. Why?

Appendix

# Composition of Buffers and Solutions

**ammonium acetate 7 M:** Dissolve anhydrous $NH_4OAc$ in water, and bring the final volume to 100 ml with water (add only 1/2 volume of water to dissolve the $NH_4OAc$). Sterilize using a sterile 0.45 µm filter. (Expiration: 2 months.)

**ammonium persulfate solution (10%):** Add 0.1 g ammonium persulfate to 1.0 ml of deionized water. Prepare fresh before use.

**analytical gel visual markers:** KpnI-digested Adenovirus II DNA. Fragment lengths (in base pairs): 1086, 1699, 2049, 2339, 3648, 5167, 5758, 6478, and 7713.

**blotting pads (autoradiographic detection):** 11 × 12.5 cm blotting pads.

**blotting pads (chemiluminescent detection):** 11 × 16 cm blotting pads.

**Chelex:** Weigh out 1 g of Chelex 100 (100–200 mesh, sodium form). Add 50 mM Tris to the 1.0 g of Chelex to make 10 ml of solution. Adjust the pH to 11 using 4 N NaOH. Store at room temperature. (Expiration: 3 months.)

**denaturation solution (autoradiographic detection):** 0.4 M NaOH. Combine 500 ml of 4 M NaOH and 4.5 l of distilled water.

**denaturation solution (chemiluminescent detection):** 0.5 M NaOH/1.5 M NaCl. Combine 250 ml of 4.0 M NaOH and 600 ml of 5.0 M NaCl. Bring to a volume of 2.0 l with distilled water.

**dithiothreitol (DTT) 1 M:** Dissolve 1.54 g of DTT in 100 µl of sterile sodium acetate, pH 5.2, and bring final volume to 10 ml with sterile deionized water. Store in 100 µl aliquots in 1.5 ml microcentrifuge tubes at –20°C. (Expiration: 1 year.)

**ethidium bromide (5 mg/ml) (WARNING:** mutagenic substance): Dissolve 1.0 g of ethidium bromide in 200 ml of distilled water. Keep bottle wrapped in foil to protect contents from light.

**ethylenediaminetetraacetic acid (EDTA) 0.5 M:** Add 80 ml of water to 18.62 g of disodium EDTA-$2H_2O$. Slowly add NaOH pellets to lower the pH to 8.0. When fully dissolved, add more NaOH to bring the pH to 8.0. Adjust final volume to 100 ml. Autoclave, and store at room temperature. (Expiration: 6 months.)

**1X final wash solution (chemiluminescent detection):** 10 mM Tris-HCl, pH 8.6; and 0.15 M NaCl. Prepare 100 ml of 10X Final Wash Buffer and 900 ml of distilled water. Final wash is a room-temperature wash.

**10X final wash buffer (chemiluminescent detection):** 2.0 10X Final Wash Buffer. Contains 0.1 M Tris-HCl, pH 8.6, and 1.5 M sodium chloride.

**HaeIII:** Restriction endonuclease used during RFLP analysis. HaeIII recognizes the four base sequence GGCC cleaving between the G and C to create a "blunt"-ended DNA strand.

**herring sperm DNA (10 mg/ml):** Dissolve 0.1 g of herring sperm DNA in distilled water, and bring the final volume to 10 ml with water. (Expiration: 6 months.)

**high-stringency wash solution (autoradiographic detection):** 0.1X SSC and 0.1% SDS. Prepare 5 ml of 20X SSC and 5 ml of 20% SDS, and bring to a final volume of 1.0 l with distilled water. Heat to 65°C before use.

**hybridization solution (chemiluminescent detection):** Contains 0.5 M sodium phosphate, pH 7.2; 0.5% (v/v) Tween 20; 1% (w/v) Hammersten Casein; and 0.02% (w/v) sodium azide (ACES 2.0 Hybridization Buffer).

**K562 DNA (HaeIII-digested):** HaeIII-digested K562 DNA is available commercially at 25 ng/µl; it is used as cell line control for postrestriction digestion and analytical gels.

**K562 DNA standard (uncut):** The typing grade K562 DNA is available commercially and is diluted to 20 ng/µl prior to use.

**lambda DNA (HindIII-digested):** Commercially available HindIII-digested DNA that is used as a visual marker for yield and postrestriction endonuclease digestion gels. Fragment lengths in base pairs are 125, 564, 2027, 2322, 4361, 6557, 9416, and 23,130.

**lambda DNA (uncut):** Commercially available DNA for preparation of yield gel quantitation standards. Length in base pairs is 48,502.

**loading buffer:** 50% Glycerol (v/v), 0.1% (w/v) Bromophenol blue, and 0.1 M EDTA. Prepare 50 ml of 100% glycerol, 0.1 g of bromophenol blue, and 20 ml of 0.5 M EDTA, and bring to a final volume of 100 ml with TE.

**loading buffer CH:** 10 mM Tris-Cl, pH 7.5; 10% Glycerol (v/v); 0.02% (w/v) bromophenol blue; and 20 mM EDTA. Prepare 400 µl of 2 M Tris-Cl, pH 7.5; 5 ml of 100% glycerol; 10 mg of bromophenol blue; and 2 ml of 0.5 M EDTA, and bring to a final volume of 50 ml with distilled water. *Note*: Loading buffer CH is used only for dilution of molecular weight markers for chemiluminescent detection.

**low-stringency wash solution (autoradiographic detection):** 2X SSC and 0.1% SDS. Prepare 100 ml of 20X SSC and 5 ml of 20% SDS, and bring to a final volume of 1.0 l with distilled water.

**Lumiphos Plus:** Chemiluminescent substrate, prepared ready for use.

**membrane rinse solution:** 0.2 M Tris, pH 7.5; and 2X SSC. Prepare 100 ml of 2 M Tris-Cl, pH 7.5, and 100 ml of 20X SSC, and bring to a final volume of 1.0 l with distilled water.

**molecular weight marker probes (chemiluminescent detection):** Contains 100 µl molecular weight marker probes specific for the DNA Analysis Marker and 125 ml Lumiphos Plus (ACES 2.0 Marker Probe).

**molecular weight markers (autoradiographic detection):** DNA sizing standards that are commercially available; fragments range from 640 base pairs to 23,408 base pairs.

**molecular weight markers (chemiluminescent detection):** DNA sizing markers that are available commercially; these contain a tube with the molecular weight markers in solution and a tube of loading buffer.

**neutralization solution (chemiluminescent detection):** 1.0 M Tris-Cl, pH 7.5; and 1.5 M NaCl. Prepare 1.0 l of 2.0 M Tris-Cl, pH 7.5, and 600 ml of 5.0 M NaCl, and bring the final volume to 2.0 l with distilled water.

**PEG (50%):** Dissolve 50 g of polyethylene glycol (MW 8000) in distilled water, and bring the final volume to 100 ml. PEG dissolves very slowly; allow sufficient time to prepare solution.

**phenol/chloroform/isoamyl alcohol (100/100/4):** Melt 100 g of phenol at 65°C, and pour into a Bellco glass bottle. Add 200 mg 8-hydroxy-quinoline, and mix the solution thoroughly. Add an equal volume of 1.0 M Tris, pH 7.5; transfer to a separatory funnel; mix; and let the phases separate. Drain the lower phenol layer into the Bellco bottle. Drain the upper aqueous phase into a waste beaker. Add an equal volume of 0.01 M Tris, pH 7.5, to the phenol; transfer to the separatory funnel; and mix. Capture the lower phase in the bottle. Capture the upper phase, and determine its pH. If the upper-

phase pH is 7.5, cease equilibration procedures. If the pH is less than 7.5, repeat the extraction(s) with 0.01 M Tris until the pH of the upper phase is 7.5. Combine the equilibrated phenol with a solution composed of 100 ml of chloroform and 4 ml of isoamyl alcohol. Cover the solution with 0.01 M Tris, and store at 4°C. This solution (in the ratio of 25:24:1) is also available commercially from various sources.

**phosphate-buffered saline (PBS):** An isotonic salt solution frequently used to wash residual growth medium from a cell culture monolayer. 5X PBS (per liter) = 40 g NaCl, 1.0 g KCl, 5.75 g $Na_2HPO_4$, and 19 g $KH_2PO_4$; autoclave; dilute aseptically to 1X with sterile $H_2O$ prior to use.

**proteinase k (20 mg/ml):** Dissolve 500 mg of Proteinase K in a small volume of distilled water, and bring to a final volume of 25 ml with distilled water. Dispense into convenient-size aliquots, and freeze.

**sarkosyl (20%):** Dissolve 20 g of N-lauroylsarcosine in distilled water, and bring to a final volume of 100 ml. Sterilize by filtration using a sterile 0.45 µm filter.

**sodium acetate (2 M):** Dissolve 41.02 g of sodium acetate in distilled water, and bring to 200 ml with distilled water. Adjust the pH to 7.0 with concentrated HCl, and adjust final volume to 250 ml with distilled water. Autoclave, and store at room temperature.

**sodium chloride (5 M):** Dissolve 292.2 g of sodium chloride in distilled water, and adjust final volume to 1.0 l with distilled water. Autoclave and store at room temperature. (Expiration date: 6 months.)

**sodium dodecyl sulfate (SDS) (20% [w/v]):** Add 200 g of sodium dodecyl sulfate to 700 ml water, and heat to 65°C to dissolve. Bring to a final volume of 1.0 l with distilled water.

**sodium hydroxide (0.2 M):** Prepare 10 ml of 4 M NaOH and 190 ml of distilled water.

**sodium hydroxide (4 M):** Dissolve 800 g of sodium hydroxide pellets in about 4.2 l distilled water. **CAUTION**: Heat is generated when adding NaOH pellets. Bring to a final volume of 5.0 l with distilled water. Store at room temperature.

**20X SSC:** 3 M NaCl and 0.3 M NaCitrate, pH 7.0. Dissolve 175.3 g of NaCl and 88.2 g of $Na_3Citrate\text{-}2H_20$ in 800 ml of distilled water. Adjust the solution to pH 7.0 by the gradual addition of HCl. Bring to a final volume of 1.0 l with distilled water.

For 5.0 l, weigh 876.5 g of NaCl and 441 g of $Na_3Citrate\text{-}2H_20$, and dissolve in 4.0 l of distilled water. Adjust the solution to pH 7.0 by the gradual addition of HCl. Bring to a final volume of 5.0 l with distilled water and autoclave.

*Note*: 2OX SSC is also commercially available as a ready-to-use solution; 2OX SSC has been validated for use in these protocols.

**20X SSPE, pH 7.0:** 3.6 M NaCl, 0.2 M $NaH_2PO_4$, and 20 mM EDTA. Dissolve 210.4 g of NaCl and 24.0 g of $NaH_2PO_4$ (anhydrous) in 900 ml of distilled water. Titrate to pH 7.0 with NaOH (approximately 40 ml of 4 M NaOH). Add 40 ml of 0.5 M $Na_2EDTA\text{-}2H_20$. Bring to a final volume of 1.0 l with distilled water, and autoclave.

**stain extraction buffer:** 10 mM Tris-Cl, 0.1 M NaCl, 2% SDS, 10 mM EDTA, and 39 mM DTT. Dissolve 1.21 g of Tris and 5.84 g of NaCl in 500 ml of distilled water. Add 100 ml of 20% SDS and 20 ml of 0.5 M $Na_2EDTA\text{-}2H_20$, and adjust pH to 8.0 with HCl. Bring to a final volume of 1.0 l with distilled water.

Supplement with DTT before use. To 100 ml of the above solution, add 0.6 g of powdered DTT, and stir until dissolved. Store at room temperature. (Expiration date: The final solution is good for no more than 2 weeks.)

**1X strip solution (chemiluminescent detection):** 10 mM Tris-Cl, pH 7.5; 1 mM EDTA; and 0.5% Tween 20. Prepare 50 ml of 10X Strip Solution and 450 ml of distilled water.

**10X strip solution (chemiluminescent detection):** 0.1 M Tris-Cl, pH 7.5; 10 mM EDTA; and 5% Tween 20. Prepare 100 ml of 2.0 M Tris-Cl, pH 7.5; 40 ml of 0.5 M EDTA; and 100 ml of 100% Tween 20. Bring to a final volume of 2.0 l with distilled water.

**1X TAE:** 40 mM Tris-acetate, pH 8.3; and 1 mM EDTA. Prepare 50 ml of 2OX TAE and 950 ml of distilled water.

**20X TAE:** 0.8 M Tris-acetate, pH 8.3; and 20 mM EDTA. Prepare 96.6 g of Tris base, 22.8 ml of glacial acetic acid, and 40.0 ml of 0.5 M EDTA, pH 8.0. Bring to a final volume of 1.0 l with distilled water, and autoclave.

For 5.0 l, weigh 483 g of Tris base, 114 ml of glacial acetic acid, and 200 ml of 0.5 M EDTA, pH 8.0. Bring to a final volume of 5.0 l with distilled water.

**TE buffer:** 10 mM Tris-Cl, pH 7.5; and 0.1 mM EDTA. Prepare 1.21 g of Tris base and 0.037 g of $Na_2EDTA$. Dissolve Tris in 800 ml of distilled water, and adjust the pH to 7.5 with HCl. Add EDTA, check the pH, and adjust to 7.5 if required. Bring the final volume to 1.0 l with distilled water, and autoclave.

**TE-9 buffer:** 0.5 M Tris, pH 9.0; 20 mM EDTA; and 10mM NaCl.

**TNE (pH 8.0):** 10 mM Tris-Cl, pH 7.5; 0.1 M NaCl; and 1 mM EDTA. Prepare 2.5 ml of 2 M Tris-Cl, pH 7.5; 10 ml 5 M NaCl; and 1.0 ml 0.5 M EDTA, pH 8.0. Add distilled water to 400 ml. Titrate to pH 8.0 with 0.1 N NaOH. Bring to a final volume of 500 ml with distilled water and autoclave.

**transfer membrane (autoradiographic detection):** Biodyne B (Pall Biosupport, Port Washington, New York).

**transfer membrane (chemiluminescent detection):** Biodyne A (Pall Biosupport, Port Washington, New York).

**transfer solution (chemiluminescent detection):** 10X SSC. Prepare 1 liter of 20X SSC and 1 liter of distilled water.

**transfer sponges (chemiluminescent detection):** Lifecodes Corporation, Stamford, CT.

**Tris (2 M):** Dissolve 242.2 g of Tris base in 800 ml distilled water. Adjust to pH 7.5 with concentrated HCl. Bring the final volume to 1.0 l with distilled water, and autoclave.

**Tris-acetate EDTA buffer (TAE buffer):** Common electrolyte reagent for the electrophoresis buffer for large (> 12 kb molecular weight) DNA. 50X TAE (per liter) = 242.0 g Tris base; 100 ml 0.5M $Na_2EDTA$, pH 8.0; and 57.1 ml glacial acetic acid; autoclave. Working concentration is 1X TAE.

**Tris borate-EDTA buffer (TBE buffer):** Common electrolyte reagent for the electrophoresis buffer for low (< 1 kb) molecular weight DNA. 20X TBE (per liter) = 121 g Tris base, 61.7 g sodium borate, and 7.44 g $Na_2EDTA$. Working concentration is 1X TBE.

**VNTR locus oligonucleotide probes (chemiluminescent detection):**

| | |
|---|---|
| D2S44 | Lifecodes Corporation NICE format |
| D2S44 | Promega GenePrint Light (#DK5411) |
| D10S28 | Lifecodes Corporation NICE format |
| D10S28 | Promega GenePrint Light TBQ7 (#DK632A) |
| D17S79 | Promega GenePrint Light D17S79 (#DK5431) |
| D5S110 | BRL ACES™ Probe LH1 (#14232-011)* |
| D4SI39 | BRL ACES™ Probe pH30 (#24230-013)* |
| D1S7 | BRL ACES™ Probe MS1 (#14231-013)* |
| D1S7 | Lifecodes Corporation NICE format |

* Life Technologies, Inc. (Gibco BRL), Rockville, MD.

**Wash I Concentrate (chemiluminescent detection):** 2.0 Wash Buffer I Concentrate. Contains 0.5 M sodium phosphate, pH 7.2; and 5% (v/v) Tween 20.

**0.5X Wash I solution (chemiluminescent detection):** 25 mM sodium phosphate, pH 7.2; and 0.25% Tween 20. Prepare 50 ml of Wash I Concentrate and 950 ml of distilled water. Heat solution to 55°C prior to use.

**1X Wash I solution (chemiluminescent detection):** 50 mM sodium phosphate, pH 7.2; and 0.5% Tween 20. Prepare 100 ml Wash I Concentrate and 900 ml of distilled water. Heat solution to 55°C prior to use.

# Appendix B

# Using a Micropipet

## Introduction

Most chemical reactions in forensic DNA analysis are performed in small volumes of liquid, partly because DNA is available only in small quantities and because reagents and enzymes are expensive. The various reactions are performed in small microcentrifuge tubes (0.2–1.5 ml) in volumes as small as 0.5 µl. Consequently, the forensic DNA analyst must be able to dispense such small volumes correctly and accurately. Dispensing such volumes of liquids is accomplished using micropipets. The series of steps below provides an overview on the structure and use of the micropipet.

### *Structure of the Micropipet*

Take a micropipet in your hand, and become familiar with the various parts (see Figure 22). On top of the micropipet is the plunger button for filling with and dispensing liquids. The second button, the tip ejector button, allows the user to eject the disposable tip, thus eliminating the need to touch the tip and/or liquid. An inset wheel or knob, the volume adjustment dial, permits the user to adjust the volume; whereas the dial with numbers (digital volume indicator) indicates the volume that has been selected. The plastic shaft at the base of the micropipet holds the disposable tip.

The micropipets are available in different ranges or capacities:

- P20: up to 20 µl (up to 0.02 ml)
- P200: 20 to 200 µl (up to 0.2 ml)
- P1000: 200 to 1000 µl (up to 1.0 ml)

Micropipets are expensive and can be easily damaged if not handled properly. When using the micropipet always adhere to the follow rules:

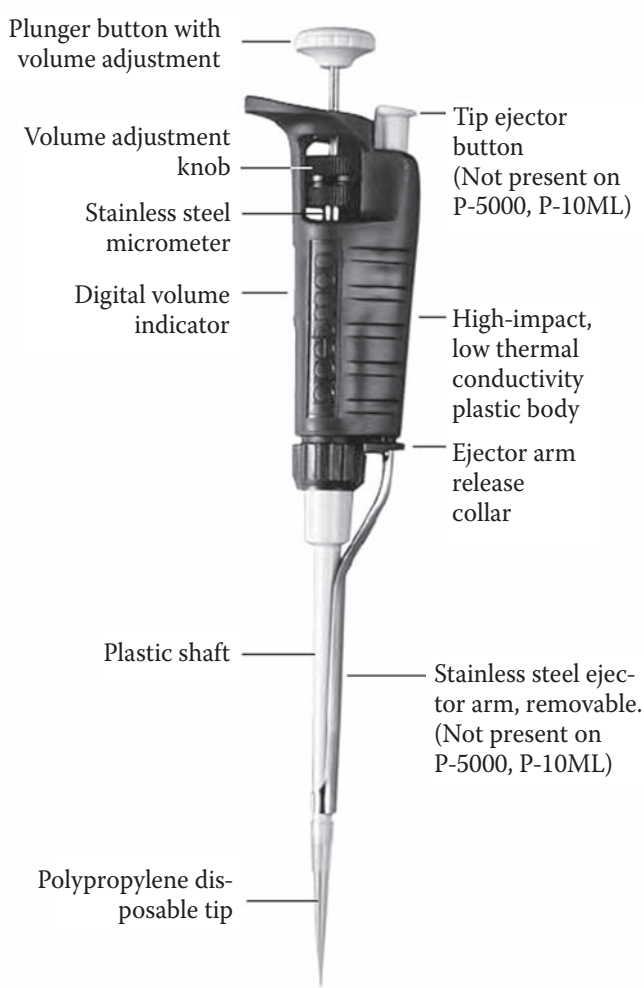



**FIGURE 22**
A Rainin Classic Pipetman. *Source*: Image used with permission of Rainin Instrument, LLC.

- Never rotate the volume adjustment knob below the lower-volume limit or above the upper-volume limit.
- Never lay the micropipet on the bench; always replace in the stand when not in use. This prevents liquid from entering the pipet and causing damage.
- Never immerse the plastic shaft of the pipet into fluid without a tip in place. Always use a new tip for each different reagent. Use the proper size tip for each pipettor.
- Always slowly release the plunger when withdrawing or dispensing liquids.

Before using the pipet, familiarize yourself with the feel of the pipet. Hold the pipet in your writing hand. With your thumb, slowly lower and raise the plunger. As you press down on the plunger you will feel resistance at the "first stop," but you can continue to press until the plunger stops. The pipet is filled by pressing the plunger down to the first stop and slowly releasing the plunger. The first stop plus the second stop, or end, will empty the pipet.

## Using the Pipet

1. Check the top of the micropipet's plunger button to select the pipet that you will need. Use a pipet with a volume greater than the amount to be pipetted. Refer to the range of sizes above.
2. To select the volume of liquid to be pipetted, rotate the volume adjustment knob until the digital indicator reaches the desired volume.
3. Place a disposable tip on the plastic shaft of the micropipet. Press firmly to ensure that the tip is in place.
4. Press the plunger down to the first stop. Hold the micropipet vertically, and place the disposable tip into the liquid to be pipetted. It is only necessary to place the tip in the liquid to a depth of several millimeters.
5. Slowly release the plunger button to its original position. Make sure the liquid is drawn into the tip.
6. Withdraw the tip from the liquid.
7. To dispense the liquid sample, place the tip against the wall of the receiving tube, and press the plunger down to the first stop, then to the final stop to dispense any remaining liquid.
8. While the plunger is still pushed down, remove the tip from the tube, and allow the plunger to slowly return to its original position.
9. Discard the disposable tip into a waste container by pressing the tip ejector button.

# Appendix C

# Glossary

**allele:** An alternative form of a gene occupying a given location on a chromosome that determines alternative characteristics in inheritance.

**allele-specific oligonucleotides (ASO):** A short, specific DNA sequence that is used as a probe in the AmpliType PM/DQA1 test to detect a unique sequence.

**amino acids:** Building blocks of proteins. Each protein consists of a specific sequence of amino acids. There are 20 common amino acid molecules that can make up proteins.

**AmpliTaq:** Recombinant form of the naturally occurring thermostable DNA polymerase from the organism *Thermus aquaticus*.

**anneal:** The base pairing of complementary polynucleotides to form a double-stranded molecule.

**antiparallel:** The manner in which two complementary polynucleotides base-pair to one another; the 5′ and 3′ ends of each molecule are reversed in relation to each other, so that the 5′ end of one strand is aligned with the 3′ end of the other strand. Antiparallel base pairing accompanies the formation of double-stranded DNA (DNA:DNA), double-stranded RNA (RNA:RNA), and DNA–RNA hybrids (DNA:RNA).

**autoradiograph (*also* autoradiogram):** A photographic record of the spatial distribution of radiation in an object or specimen. It is made by placing the object very close to a photographic film or emulsion.

**autoradiography:** The process by which an autoradiograph is made.

**autosome:** Chromosomes that are in the same number and kind in males and females; chromosomes other than the sex chromosomes.

**avidin (streptavidin) enzyme conjugate (HRP-SA):** A nonisotopic detection system used with the AmpliType PM/DQA1 typing system. Biotin is covalently attached to each primer pair. The biotin-labeled amplified products are allowed to hybridize to the DNA probes immobilized on the nylon test strips. The strips are reacted with the enzyme horseradish peroxidase (HRP) covalently bound to streptavidin (SA). This HRP–SA conjugate can bind only to hybridized or double-stranded targets. If hybridization occurs, the HRP–SA conjugate will react with the colorless substrate, causing a blue color to develop. The spot remains colorless if no hybridization occurred, hence a negative response.

**bacteriophage lambda:** A virus that infects and is propagated in a bacterial host, often for cloning purposes. Among the best characterized and most widely exploited are derivatives of the A bacteriophage.

**base:** One of five molecules that make up the informational content of DNA and RNA. In DNA, bases pair across the two chains of the double helix: adenine (A) with thymine (T), and guanine (G) with cytosine (C). RNA is single stranded and contains uracil (U) instead of thymine.

**base pair:** Two complementary nucleotides bonded together at the matching bases (A and T or C and G) to form a double-stranded complex; the length of the DNA is often described in base pairs (bp).

**base pairing:** The formation of hydrogen bonds between the nitrogenous bases of two nucleic acid molecules.

**beta particle:** An elementary particle emitted from a nucleus during radioactive decay. It has a single electrical charge. A negatively charged B particle is identical to an electron. A positively charged B particle is known as a positron.

**biotechnology:** The set of biological techniques developed through basic research and now applied to research and product development; in particular, the use of microorganisms and plant and animal cells to produce useful materials, such as food, medicine, and other chemicals.

**biotin:** A small vitamin used to label nucleic acids for a variety of purposes, including nonisotopic hybridization by chemiluminescence or chromogenic techniques.

**buoyant density:** A measure of the ability of a substance to float in a standard fluid. For example, differences in the buoyant densities of RNA and DNA allow them to be separated in a gradient of cesium chloride or cesium trifluoroacetate.

**capillary electrophoresis (CE):** A technique for separating DNA from a fluid substrate; the sample is injected into a capillary tube, which is then subjected to a high-voltage current that separates its chemical constituents based on charge and size.

**cDNA (complementary DNA):** DNA synthesized from an RNA template. The single-stranded form of cDNA is an important laboratory tool (e.g., as a probe) for isolating and studying the expression of individual genes.

**chemiluminescence:** A nonisotopic hybridization detection technique. Chemiluminescence is the production of visible light by a chemical reaction.

**chromatin:** The complex of genomic DNA and protein found in the nucleus of a cell in interphase.

**chromosome jumping:** A technique very similar to chromosome walking with the exception that very large fragments of DNA (> 100 kb) under investigation are purified by pulse field gel electrophoresis. Thus, moving from one end of this DNA fragment, for subcloning purposes, constitutes more of a "jump" than a "walk." *See* **chromosome walking**.

**chromosome walking:** The systematic isolation of a set of clones containing overlapping DNA fragments that collectively makes up a specific genomic region. The process is initiated by identification of a unique site recognized by a sequence-specific probe. When a clone is retrieved from a library, the process of chromosome walking continues by subcloning and rehybridization to the library. In each successive hybridization, the probe corresponds to the 3′ or 5′ end of the clone previously recovered from the library. Thus, the resulting series of overlapping clones permits locus characterization within, upstream of, and downstream of a particular locus. This approach can be exploited to "walk" either toward or away from a locus of interest.

**clone (noun):** A collection of genetically equivalent cells or molecules.

**clone (verb):** A series of manipulations designed to isolate and propagate a specific nucleic acid sequence or cell for characterization, storage, or further amplification.

**clone bank:** Older terminology for what are currently known as cDNA libraries and genomic DNA libraries.

**cloning:** 1. A collection of genetically equivalent cells or molecules. 2. Asexually producing multiple copies of genetically identical cells or organisms descended from a common ancestor. *Compare with* **gene cloning**.

**coding strand:** In double-stranded DNA, the strand that has the same sequence as the resulting RNA (except for the substitution of uracil for thymine).

**codon:** A triplet of nucleotides in an RNA molecule that specifies the placement of an amino acid during protein synthesis.

**competitive PCR:** A very sensitive method for quantification of transcript abundance by the inclusion of a new DNA sequence, which competes for primers and deoxyribonucleotide triphosphates (dNTPs), in the same reaction tube as the experimental sample. By varying the amount of the competitor DNA (also known as a DNA mimic), a dilution will be identified in which the concentrations of target and mimic are equivalent, allowing for very accurate quantification.

**complementary DNA (cDNA):** DNA enzymatically synthesized in vitro from an RNA template by reverse transcription. cDNA may be single-stranded or double stranded, as required by the parameters governing a particular assay. The synthesis of cDNA represents a permanent biochemical record of the cellular biochemistry and also provides a means by which that record can be propagated.

**constitutive gene expression:** Interaction of RNA polymerase with the promoters of specific genes not subjected to additional regulation. Such genes are frequently expressed continually at low or basal levels, and are sometimes referred to as genes with housekeeping functions.

**curie (Ci):** A basic unit for measuring radioactivity in a sample. One curie is equivalent to $3.7 \times 10^{10}$ becquerel (i.e., disintegrations per second).

**cytoplasm:** The cellular contents found between the plasma membrane and the nuclear membrane.

**denaturation (of nucleic acids):** Conversion of DNA or RNA from a double-stranded form to a single-stranded form. This can mean dissociation of a double-stranded molecule into its two constituent single strands, or the elimination of intramolecular base pairing.

**deoxyribonucleic acid (DNA):** The substance of heredity; a large linear molecule that consists of deoxyribose sugar, phosphate groups, and the bases adenine, thymine, guanine, and cytosine, and that carries the genetic information that cells need to replicate and to produce proteins. A polymer of deoxyribonucleoside monophosphates, assembled by a DNA polymerase. In vivo, DNA is produced by the process known as replication. DNA can also be synthesized using a variety of in vitro methods, such as the polymerase chain reaction.

**differential display PCR (DD-PCR):** A method for identification of uniquely transcribed sequences among two or more RNA populations. DD-PCR is a PCR-based method that utilizes large combinations of relatively short primers to ensure amplification of all transcribed RNAs in the form of cDNA. Electrophoretic comparison of the products of each reaction shows products of identical molecular weight when a transcript is common to the biological samples under investigation; a band in only one lane is observed if gene expression has been induced or repressed.

**differentiation:** The process of biochemical and structural changes by which cells become specialized in form and function.

**diploid:** Having two complete sets of chromosomes (two of each chromosome). *Compare to* **haploid** *and* **triploid**.

**directional cloning:** Unidirectional insertion of a DNA molecule into a vector accomplished by placement of different sequences or restriction enzyme sites at the ends of double-stranded cDNA or genomic DNA molecules.

**direct repeats:** Identical or closely related sequences present in two or more copies in the same orientation on the same molecule of DNA; they are not necessarily adjacent.

**DNA (deoxyribonucleic acid):** *See* **deoxyribonucleic acid**.

**DNA polymerase I:** A prokaryotic enzyme capable of synthesizing DNA from a DNA template. The native DNA polymerase I, also known as the holoenzyme or Kornberg enzyme, manifests three distinct activities: 5′ to 3′ polymerase, 5′ to 3′ exonuclease, and 3′ to 5′ exonuclease. *See also* Klenow fragment.

**DNA probe:** A specific sequence of single-stranded labeled DNA that is used to determine the presence of complementary nucleic acid sequences.

**DNA profile:** The pattern of band lengths on an autoradiograph (*see* autoradiography) representing all of the tests to link DNA samples with probes.

**DNA sequencing:** A technology for determining the order of nucleotides in a specific DNA molecule.

**dominant:** Pertaining to the form of a gene that exerts its effect when present in the individual in just a single copy; an expressed trait.

**dot blot analysis:** A rapid, quantitative assay for determining the prevalence of a DNA or RNA sequence in a sample. Denatured samples are applied directly to a filter without prior electrophoretic separation. Results are based on signal intensity within the "dot." Dot blot analysis lacks the qualitative component associated with gel electrophoresis. *See also* **slot blot analysis**.

**downstream: A** term that refers to sequences proceeding further in the direction of expression (i.e., in the 3′ direction); for example, the coding region is downstream from the initiation codon.

**duplex:** The formation of a double-stranded molecule or portion thereof by the base pairing of two complementary polynucleotides.

**electropherogram:** A recording of the separated DNA components of a sample produced by gel or capillary electrophoresis. A photograph or printout of a gel or capillary separation made after electrophoresis, which records the spatial distribution of macromolecules within the gel or capillary.

**electrophoresis:** A type of chromatography in which macromolecules (i.e., proteins and nucleic acids) are resolved through a matrix based on their charge.

**ethidium bromide (EtBr):** A planar, intercalating agent used to visualize nucleic acids, both DNA and RNA. This dye emits a bright orange fluorescence when UV irradiated; thus, gels that contain samples can be photographed for future reference. Standard ethidium bromide stock solution is 10mg/ml in water; standard staining concentration is 0.5–1.0 mg/ml.

**ethylenediamine tetraacetic acid (EDTA):** A chemical preservative added to biological samples to inhibit the activity of enzymes that are responsible for degrading DNA.

**exon:** A portion of a eukaryotic gene represented in the mature mRNA molecule. Exons may or may not be translated.

**formaldehyde (HCHO):** A commonly used denaturant of RNA.

**FTA collection card:** An absorbent cellulose-based paper that contains chemical substances to inhibit bacterial growth and to protect the DNA from enzymatic degradation. Liquid samples such as blood and saliva are often collected and "spotted" onto the card for short- or long-term storage at room temperature. FTA is a registered trademark of Flinders Technologies, Pty. Ltd.

**gel electrophoresis:** In RFLP analysis, the process of separating DNA (cut or uncut) by size in an electrical field; the different-sized fragments move at different rates through the gel.

**gene:** The fundamental physical and functional unit of heredity. A gene is an ordered sequence of nucleotides located in a particular position on a chromosome that ultimately encodes for the synthesis of a polypeptide.

**gene cloning:** Isolation and propagation of a gene or gene fragment by inserting it into a suitable vector or host and allowing it to multiply.

**gene expression:** The manifestation of the genetic material of an organism in the form of a functional polypeptide.

**gene library:** A collection of DNA fragments (introduced into a virus or plasmid) that, when taken together, represents the total DNA of a certain cell type or organism. An older term for gene library is "clone bank."

**gene mapping:** Determining the relative locations of different genes on chromosomes.

**gene regulation:** The processes controlling the synthesis or suppression of gene products.

**gene splicing:** Joining pieces of DNA from different sources using recombinant DNA technology.

**gene therapy:** A set of experimental techniques for the introduction of a normal functioning gene into a cell in which the gene is defective.

**genetic code:** The language in which the instructions of DNA are written. It consists of triplets of nucleotides (codons), with each corresponding to an amino acid or a signal to start or stop protein synthesis.

**genome:** The entire chromosomal DNA found in a cell; its size is generally given in the total number of base pairs. In some applications, it may be useful to distinguish nuclear genomic DNA from the mitochondrial genome.

**genomic DNA:** Chromosomal DNA.

**genotype:** The genetic composition of an individual cell or organism; the total of all the genes present in an individual.

**haploid:** Having one complete set of chromosomes (one of each chromosome, as found in gametes). *Compare to* **diploid** *and* **triploid**.

**haplotype:** Refers to the genetic constitution of an individual chromosome. Haplotype may refer to only one locus or to an entire genome. In the case of humans, a genome-wide haplotype comprises one member of the pair of alleles for each.

**heterozygous:** Both alleles at a given locus on each of a pair of homologous chromosomes are different; one is inherited from each parent.

**histones:** Proteins that help to organize the chromosomes by their association with DNA. Histone proteins are among the most highly conserved among all eukaryotic genes.

**homologous chromosome:** Chromosomes that share an identical sequence of genes, but may carry similar or different alleles at the same loci; they associate in pairs.

**homozygous:** Both alleles are the same at a given locus; one is inherited from each parent.

**housekeeping gene:** A gene that is expressed, at least theoretically, at constant levels in all cells, the products of which are required to maintain cellular viability. Because of their purported invariance, assay of transcription of these sequences is often performed to demonstrate that an *overall* change in gene expression has *not* occurred, in the context of an experimental manipulation.

**human leukocyte antigen (HLA):** Antigen (foreign body that stimulates an immune response) located on the surface of most cells (sperm and red blood cells excluded) and different among individuals. HLA DQA1 is a particular class of HLA whose locus has been completely identified (i.e., sequenced) and used in forensic typing.

**hybridization:** The formation of hydrogen bonds between two nucleic acid molecules that demonstrate some degree of complementarity. The specificity of hybridization is a direct function of the stringency of the system in which the hybridization is being conducted.

**hydrogen bonding:** The highly directional attraction of an electropositive hydrogen atom to an electronegative atom such as oxygen or nitrogen. This is the manner of interaction between complementary bases during nucleic acid hybridization. *See also* **base pairing**.

**image:** The document that image analysis software works upon. "Image" may also refer to the original artwork, graphics, or photograph that is scanned or imported into image analysis software.

**image analysis:** An electronic method for the digital capture and storage of an image, accompanied by the automated measurement of parameters such as molecular weight, mass, relative abundance, and optical density of various objects in the image (e.g., bands on a gel).

**intron:** A DNA sequence that interrupts the coding sequences (exons) for a gene product. After information from the genes is transcribed into new strands of heterogeneous nuclear RNA (hnRNA), the introns are spliced out of the RNA molecule, and are not represented in the mature mRNA. Although the functions of introns are unknown, it has been postulated that some introns have a role in regulating gene expression.

**isotope:** One of two or more atoms with the same atomic number but different atomic weights.

**Klenow fragment:** The large fragment of *E. coli* DNA polymerase I, generated by cleavage of the holoenzyme with subtilisin, or obtained by cloning. The Klenow fragment manifests the 5′ to 3′ polymerase and 3′ to 5′ exonuclease activities but lacks the often troublesome 5′ to 3′ exonuclease activity associated with the intact enzyme. *See also* **DNA Polymerase I**.

**library:** A collection of clones that partially or completely represent the complexity of genomic DNA or cDNA from a defined biological source, one or several of which are of immediate interest to the investigator. Members of the library, which consists of cDNA or genomic DNA sequences ligated into a suitable vector, may be selected or retrieved from the library by nucleic acid hybridization or, in the case of expression vectors, by antibody recognition.

**ligase:** An enzyme that catalyzes the formation of the phosphodiester bond between the termini of two DNA molecules.

**locus:** The precise position of a particular gene, and any possible allele, on a chromosome.

**marker:** A very generic term that can refer to any allele of interest in an experiment. Also, marker can refer to a molecular standard.

**melting temperature:** *See* $\mathbf{T_m}$.

**messenger RNA (mRNA):** The mature product of RNA polymerase II transcription. In eukaryotic cells, mRNA is derived from heterogeneous nuclear RNA (hnRNA) and, in conjunction with the protein translation apparatus, is capable of directing the translation of the encoded polypeptide.

**mismatch:** One or more nucleotides in a double-stranded molecule that do not base pair. In order for mismatches to be tolerated, the temperature of annealing must be sufficiently below the melting temperature, $T_m$; at the $T_m$, only perfectly matched duplexes are stable. The location and context of mismatching have profound ramifications with respect to primer annealing in the polymerase chain reaction.

**multiplex PCR:** Simultaneous amplification of two or more targets in the same PCR reaction.

**Northern analysis (also Northern blotting):** A technique for transferring electrophoretically chromatographed RNA from an agarose gel matrix onto a filter paper, for subsequent immobilization and hybridization. The information gained from Northern analysis is used to qualitatively and quantitatively assess the expression of specific genes.

**nucleotide:** A subunit or molecule of DNA or RNA consisting of a 5-carbon sugar (ribose or deoxyribose), a nitrogenous base (adenine, cytosine, guanine, thymine, or uracil), and a phosphate group. Nucleotides are the building blocks used to assemble both DNA and RNA.

**oligonucleotide:** A short, artificially synthesized, single-stranded DNA molecule that can function as a nucleic acid probe or a molecular primer. Oligonucleotide can also refer to a short fragment of RNA.

**palindrome:** A segment of duplex DNA in which the base sequences of the two strands exhibit a twofold rotational symmetry about the central axis. Restriction endonucleases often recognize and cut the DNA at a variety of such palindromic sites.

**PCR (polymerase chain reaction):** A systematic, primer-mediated enzymatic process for the geometrical amplification of a target DNA sequence. PCR product can be generated from as little as one molecule of target material (DNA or RNA) under optimal conditions.

**phenotype:** The observable characteristics of an organism or individual.

**photodocumentation:** A method for preserving the image of a gel immediately after electrophoresis, or after hybridization with a labeled probe. Media that support photodocumentation include Polaroid film, X-ray film, thermal paper, and digital storage. *See* **image analysis**.

**plasmid:** A covalently closed, double-stranded DNA molecule capable of autonomous replication in a prokaryotic host (eukaryotic plasmids have also been developed). Plasmids can accept foreign DNA inserts, usually less than 10 kb, and often contain a variety of selectable markers and ancillary sequences for characterization of the insert DNA.

**polymerase chain reaction (PCR):** A primer-mediated enzymatic process for the systematic amplification of minute quantities of specific genomic or cDNA sequences. This technique, which has revolution-

ized molecular biology, mimics DNA replication. It has the advantage of being a very sensitive technique that can be performed in a short time frame, amplifying a targeted sequence hundreds of millions to billions of times.

**polymorphism (see RFLP):** The quality or character occurring in more than one form.

**posttranscriptional regulation:** Any event that occurs after transcription and that influences any of the subsequent steps involved in the ultimate expression of that gene. Reference to posttranscriptional regulation usually refers to events between the termination of transcription and just prior to assembly of the translation apparatus.

**posttranslational regulation:** Any event that occurs after synthesis of the primary peptide that influences any of the subsequent steps involved in the ultimate expression of the gene. Reference to posttranslational regulation usually refers to the efficiency of the events that modify a peptide, including, but not limited to, glycosylation, methylation, and hydroxylation.

**precursor RNA:** An unspliced RNA molecule; the primary product of transcription.

**primer:** A short nucleic acid molecule that, upon base pairing with a complementary sequence, provides a free 3´-OH for any of a variety of primer extension-dependent reactions.

**probe:** Usually, labeled nucleic acid molecules, either DNA or RNA, that are used to hybridize to complementary sequences in a library, or that are among the complexity of different target sequences present in a nucleic acid sample, as in the Northern analysis, Southern analysis, or nuclease protection analysis. In forensics, a short segment of DNA is used to detect certain alleles. The probe hybridizes, or matches up to, a specific complementary sequence, allowing for the visualization of the DNA complex by either a radioactive "tag" (RFLP) or biochemical tag (HLA DQA1). A single-locus probe marks a specific site (locus), whereas a multilocus probe marks multiple sites.

**prokaryote:** A microorganism (cell) that has no nucleus (the DNA is not enclosed within a membrane) and lacks other organelles found in the cells of higher organisms.

**promoter:** A DNA sequence associated with a particular locus at which RNA polymerase binds at the onset of transcription. Promoters typically consist of several regulatory elements involved in the initiation, regulation, and efficiency of transcription.

**protein:** A molecule composed of amino acids arranged in a specific order determined by the genetic code.

**radiochemical:** A chemical containing one or more radioactive atoms.

**recessive:** A gene or allele that is "masked" by another, dominant allele.

**recombinant DNA:** The hybrid DNA produced in the laboratory by joining pieces of DNA, frequently from different organisms.

**renaturation:** The reassociation of denatured, complementary strands of DNA or RNA.

**replication:** The formation of an exact copy. DNA replication occurs when each strand acts as a template for a new, complementary strand, formed according to base-pairing rules.

**restriction endonuclease:** A class of enzymes that recognizes a specific base sequence (usually four to six base pairs in length) in a double-stranded DNA molecule and cuts both strands of the DNA at every site where this sequence occurs.

**restriction endonuclease recognition site:** The site where a specific restriction endonuclease cuts the DNA molecule.

**restriction fragment length polymorphism (RFLP):** The presence of variants in the size of DNA fragments produced upon restriction enzyme digestion due to a change in bases. These different-sized fragments may result from an inherited variation in the distribution of restriction endonuclease sites. RFLPs are used in the laboratory for human identification or parentage determination.

**reverse transcriptase (RT):** A class of enzymes that catalyze the formation of DNA strands from RNA templates. The name "reverse transcriptase" is from its ability to "reverse" the normal first step of gene expression, that is, the formation of an RNA strand from a DNA template. Once the first DNA

strand has been synthesized, it serves as the template for the enzymatic synthesis of the second complementary DNA strand.

**RFLP analysis:** A technique that uses single-locus or multilocus probes to detect variation in a DNA sequence according to differences in the length of segments created by cutting DNA with a restriction enzyme.

**ribonuclease (RNase):** A family of resilient enzymes that rapidly degrade RNA molecules. Control of RNase activity is a key consideration in all manipulations involving RNA.

**ribonucleic acid (RNA):** A chemical found in the nucleus and cytoplasm of cells. A polymer of ribonucleoside monophosphates, synthesized by an RNA polymerase. RNA is the product of transcription and plays an important role in protein synthesis.

**ribosomal RNA (rRNA):** The predominant class of RNA in the cell. The highly abundant nature of rRNA makes it a useful indicator of sample integrity, quality, and probable utility. The low complexity of this RNA species also makes it useful as a molecular weight marker for RNA electrophoresis.

**ribozyme:** An RNA molecule with the capacity to act as an enzyme.

**RNA polymerase:** An enzyme responsible for the synthesis of RNA polynucleotides by the process of transcription, using DNA as a template.

**saline sodium phosphate-EDTA (SSPE):** A salt solution frequently used for blotting of nucleic acids. It is also an essential component of various hybridization buffers and posthybridization filter washes. The phosphate in this buffer mimics the phosphodiester backbone of nucleic acids, thereby providing enhanced blocking, lower background, and higher signal-to-noise ratio on membranes during Northern and Southern analyses.

**sequence:** The order of nucleotides (A, C, G, and T) in a nucleic acid or DNA molecule.

**single nucleotide polymorphism (SNP):** A change in the DNA in which a single base or nucleotide differs from the usual base at that position.

**slot blot analysis:** A membrane-based technique for the quantitation of specific RNA or DNA sequences in a sample. The sample is usually slot-configured onto a filter by vacuum filtration through a manifold. Slot blots lack the qualitative component associated with electrophoretic assays. *See also* **dot blot analysis**.

**sodium dodecyl sulfate (SDS):** An ionic detergent commonly used to disrupt biological membranes and to inhibit RNase.

**Southern analysis:** A technique for transferring electrophoretically chromatographed DNA from an agarose gel matrix onto a filter paper for subsequent immobilization and hybridization. The information gained from Southern analysis is used to qualitatively and quantitatively assess the organization of specific genes or other loci.

**specific activity:** The amount of radioactivity per unit mass of a radioactive material. It is most frequently expressed in curies per millimole of material (Ci/mmol).

**splicing:** Ligating two fragments of DNA or RNA end to end to create a new molecule.

**Stoffel fragment (AmpliTaq DNA polymerase):** A thermostable recombinant DNA polymerase that is smaller (by 289 amino acids) than the full-length AmpliTaq polymerase. The Stoffel fragment of AmpliTaq is more thermostable, has activity over a broader range of Mg++ concentrations, and lacks a 5′ to 3′ exonuclease activity. It is commonly used in multiplex PCR applications.

**stringency:** A measure of the likelihood that double-stranded nucleic acid molecules will dissociate into their constituent single strands; it is also a measure of the ability of single-stranded nucleic acid molecules to discriminate between other molecules that have a high degree of complementarity and those that have a low degree of complementarity. High-stringency conditions favor stable hybridization only between nucleic acid molecules with a high degree of complementarity. As stringency is lowered, a proportional increase in nonspecific hybridization is favored.

**SYBR Green:** One member of a new family of dyes for staining nucleic acids. Commonly prepared as a 10,000X stock solution in DMSO, SYBR Green is diluted to a working concentration of 1X in Tris buffer, such as 1X TAE. Among the advantages of using SYBR Green are greatly reduced background fluorescence, higher sensitivity, and reduced mutagenicity when compared with ethidium bromide. SYBR Green I is used to stain DNA, whereas SYBR Green II is used to stain RNA.

**Taq DNA polymerase:** Thermostable DNA polymerase from the organism *Thermus aquaticus*. Taq is one of several enzymes that can be used to support the polymerase chain reaction.

**target:** Single-stranded DNA or RNA sequences that are complementary to a nucleic acid probe. Target sequences may be immobilized on a solid support or may be available for hybridization in solution.

**template:** A macromolecular informational blueprint for the synthesis of another macromolecule. All polymerization reactions, including replication, transcription, and PCR, require templates; these dictate the precise order of nucleotides in the nascent strand. Primer extension-type reactions cannot proceed in the absence of template material.

**termination codon:** Codons (e.g., UAG, UGA) that signal termination of the synthesis of a polypeptide chain (UAG, UGA).

**$T_m$:** Melting temperature; that temperature at which 50% of all possible duplexes are dissociated into their constituent single strands. To facilitate formation of all possible duplexes, hybridization is conducted below the $T_m$ of the duplex; the lower the temperature, the greater the likelihood that duplexes, including those with mismatches, will form.

**transcription:** The transfer of information from various parts of the DNA molecule to new strands of rRNA, tRNA, or mRNA, which then carry this information from the nucleus (in eukaryotes) into the cytoplasm.

**transduction:** Transfer of genetic material from one cell to another by means of a viral vector.

**transfer RNA (tRNA):** A moderately abundant class of RNA molecules that shuttle amino acids to the aminocyl site of the ribosome during protein synthesis. The total mass of tRNA in the cell is occasionally assayed as a housekeeping indicator of transcription, in order to show that a particular experimental manipulation has not resulted in a change in overall transcription in the cell.

**transformation:** Introduction of exogenous DNA into a cell, causing it to acquire a new phenotype, as in bacterial transformation.

**translation:** The process by which peptides are synthesized from the instructions encoded within an RNA template. Translation occurs as mRNA is deciphered by the ribosomes.

**triploid:** Having three complete sets of chromosomes (three of each chromosome). *Compare to* **diploid** *and* **haploid**.

**tRNA:** *See* transfer RNA.

**upstream:** Sequences in the 5′ direction (away from the direction of expression) from some reference point. For example, the 5′ cap in eukaryotic mRNA is located *upstream* from the initiation codon.

**UV light (ultraviolet light):** Short-wave, high-energy portion of the electromagnetic spectrum. Because nucleic acids absorb light maximally in the ultraviolet range (260 nm), samples of nucleic acids are stained with dyes (e.g., EtBr) and irradiated with UV light for visualization. **CAUTION**: UV light is mutagenic and can severely damage the skin and the retina of the eye. Be certain to wear proper eye and skin protection at all times.

**variable number of tandem repeats (VNTR):** Multiple copies of virtually identical base pair sequences, arranged in succession at a specific locus on a chromosome. The number of repeats varies from individual to individual, thus providing a basis for individual recognition.

**vector:** A nucleic acid molecule such as a plasmid, bacteriophage, or phagemid into which another nucleic acid molecule (the so-called insert or foreign DNA) has been ligated. Vectors contain sequences that, in a suitable host, permit propagation of the vector and the DNA that it carries.

**Western analysis:** A technique for transferring electrophoretically chromatographed protein from a polyacrylamide gel matrix onto a filter paper for subsequent characterization by antigen–antibody recognition. The information gained from Western analysis is used to qualitatively and quantitatively assess the prevalence of specific polypeptides.

# References and Suggested Reading


Anderson, S., A. T. Bankier, B. G. Barrell, M. H. de Bruijn, A. R. Coulson, J. Drouin, I. C. Eperon, D. P. Nierlich, B. A. Roe, F. Sanger, P. H. Schreier, A. J. Smith, R. Staden, and I. G. Young. 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457-465.

Andrews, R. M., I. Kubacka, P. F. Chinnery, R. N. Lightowlers, D. M. Turnbull, and N. Howell. 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA [letter]. Nat. Genet. 23:147.

Butler, J. M., and B. C. Levin. 1998. Forensic applications of mitochondrial DNA. Trends in Biotech. 16:158-162.

Edwards, A., A. Civitello, H. A. Hammond, and C. T. Caskey. 1991. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am. J. Hum. Genet. 49:746-756.

Edwards, A., H. A. Hammond, L. Jin, C. T. Caskey, and R. Chakraborty. 1992. Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 12:242-253.

Gilbert, D. A., Y. A. Reid, M. H. Gail, D. Pee, C. White, R. J. Hay, and S. J. O'Brien. 1990. Application of DNA fingerprint for cell-line individualization. Am. J. Hum. Genet. 47:499-514.

Gill, P., A. J. Jeffreys, and D. J. Werrett. 1985. Forensic application of DNA "fingerprints." Nature 318:577-579.

Jeffreys, A. J., V. Wilson, R. Neumann, and J. Keyte. 1988. Amplification of human minisatellites by polymerase chain reaction: Towards DNA fingerprint of single cells. Nucleic Acid Res. 16:10953-10971.

Jeffreys, A. J., V. Wilson, and S. L. Thein. 1985. Individual-specific fingerprint of human DNA. Nature 316:76-79.

Kasai, K., Y. Nakamura, and R. White. 1990. Amplification of a variable number of tandem repeat (VNTR) locus (pMCT118) by the polymerase chain reaction (PCR) and its application to forensic science. J. Forensic Sci. 35:1196-1200.

Miller, S. A., D. D. Dykes, and H. F. D. Polesky. 1988. A simple salting out procedure for extracting DNAs from human nucleated cells. Nucleic Acid Res. 16:1215.

Nakamura, Y., M. Carlston, V. Krapcho, and R. White. 1988. Isolation and mapping of a polymorphic DNA sequence (pMCT118) on chromosome 1p (D1S80). Nucleic Acid Res. 16:9364.

Nakamura, Y., S. Gillilan, P. O'Connell, M. Leppert, G. M. Lathrop, J-M. Lalouel, and R. White. 1987b. Isolating and mapping of a polymorphic DNA sequence PYNH24 on chromosome 2 (D2S44). Nucleic Acid. Res. 15:10073.

Nakamura, Y., M. Leppert, P. O'Connell, R. Wolfe, T. Holm, M. Culver, C. Martin, E. Fujimoto, M. Hoff, E. Kumlin, and R. White. 1987a. Variable number of tandem repeat (VNTR) markers for human gene mapping. Science 235:1616-1622.

Sanger, F. S., S. Nilken, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. PNAS 74:5463-5467.

Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–527.

Wahls, W. P., L. J. Wallace, and P. D. Moore. 1990. Hypervariable minisatellite DNA is hotspot for homologous recombination in human cells. Cell 60:95-103.

Warne, D., C. Watkins, P. Bodfish, K. Nyberg, and N. K. Spurr. 1991. Tetranucleotide repeat polymorphism at the human β-actin related psuedogene 2 (actbp2) detected using the polymerase chain reaction. Nucl. Acids Res. 19:6980.

# Index

## F



## G



## H



## I



## L



## M



## O



## P

## Q



## R



## S



## T



## U



## V



## W



## Y

FORENSICS AND CRIMINAL JUSTICE

# Forensic DNA Analysis

## A Laboratory Manual

In its short but active history, the use of DNA typing has revolutionized criminal investigations. It is almost inconceivable to bring a case to trial without positive identification through what is now our most accurate means. Proficiency with the methodology, principles, and interpretation of DNA evidence is crucial for today's criminalist.

An introductory forensic science text, ***Forensic DNA Analysis: A Laboratory Manual*** presents a contextual history and overview of the science and use of DNA typing methods. Logically organized, with clear, concise language, this manual provides a fundamental understanding of forensic DNA analysis and a thorough background in the molecular techniques used to determine an individual's identity or parental lineage. Students are provided with a sound working knowledge of the investigative methodology, scientific principles, and the analysis and interpretation of the resulting data.

- Covers the rules of the laboratory and basic scientific principles
- Describes all types of biological materials, such as hair, blood, and bone
- Outlines techniques commonly used in DNA typing
- Provides exercises designed to be performed in a common laboratory
- Demonstrates DNA extraction, concentration, and assessment; DNA analysis using restriction fragment length polymorphisms; polymerase chain reaction and PCR-based typing tests; short tandem repeat analysis; and mitochondrial DNA analysis
- Adapts procedures from methods and protocols used in federal, state, and private forensic laboratories
- Adapts experiments to a wide range of applications for genetic analysis
- Includes an extensive glossary for DNA typing terminology as well as basic terms used in molecular biology

Instilling confidence, analytical clarity, and a sense of curiosity, this comprehensive introduction is the perfect tool for grasping the techniques and applications of forensic DNA analysis and exploring the questions and issues involved in forensic science investigations.

63294

ISBN 1-4200-6329-4

9 781420 063295 90000

CRC Press
Taylor & Francis Group
an **informa** business
www.taylorandfrancisgroup.com

6000 Broken Sound Parkway, NW
Suite 300, Boca Raton, FL 33487
270 Madison Avenue
New York, NY 10016
2 Park Square, Milton Park
Abingdon, Oxon OX14 4RN, UK

www.crcpress.com