AmpFℓSTR® Profiler Plus®

PCR Amplification Kit

User's Manual

Applied Biosystems

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1 Introduction

Short Tandem Repeat Loci

Human Identification with STRs

Short tandem repeat (STR) markers are polymorphic DNA loci that contain a repeated nucleotide sequence. The STR repeat unit can be from two to seven nucleotides in length. The number of nucleotides per repeat unit is the same for a majority of repeats within an STR locus. The number of repeat units at an STR locus may differ, so alleles of many different lengths are possible. Polymorphic STR loci are therefore very useful for human identification purposes (Edwards *et al.*, 1992).

STR loci can be amplified using the polymerase chain reaction (PCR) process and the PCR products are then analyzed by electrophoresis to separate the alleles according to size. PCR-amplified STR alleles can be detected using various methods, such as fluorescent dye labeling, silver staining, or fluorescent dye staining.

The analysis of short tandem repeat loci is an important complement to the length- and sequence-based DNA typing systems already in use for human identification. A majority of the STRs that have been evaluated by the forensic community are composed of four-nucleotide repeat units (Frégeau and Fourney, 1993; Kimpton *et al.*, 1993; Urquhart *et al.*, 1995).

Advantages of STR Analysis

PCR-based STR analysis has the following advantages over conventional methods of DNA analysis such as Restriction Fragment Length Polymorphism (RFLP):

- The small size of STR loci improves the chance of obtaining a result, particularly for samples containing minute amounts of DNA and/or degraded DNA.
- The small size range of STR loci makes them ideal candidates for co-amplification while keeping all amplified alleles smaller than 350 base pairs. Many STR loci can therefore be typed from a single PCR.
- STR alleles have discrete sizes, allowing for simplified interpretation of results.
- PCR-based tests are rapid, giving results in 24 hours or less.
- PCR-based tests are easy to standardize and automate, ensuring reproducible results.

Multicolor Detection

Applied Biosystems Fluorescent Dye Technology

Applied Biosystems fluorescent multicolor dye technology allows multiple loci, including loci that have alleles with overlapping size ranges, to be analyzed in a single gel lane or capillary injection. Alleles for overlapping loci are distinguished by labeling locus-specific primers with different color dyes.

Because only one primer of each pair is labeled, the ABI PRISM® instruments detect only one strand for each amplified DNA fragment. The detection of only one strand eliminates doublets arising from the different mobilities of complementary strands that are often observed when using gel staining detection methods.

Automated Sizing and Genotyping

Amplified samples can be analyzed in a slab gel format on the ABI PRISM® 377 DNA Sequencer and ABI PRISM 377 DNA Sequencer with XL Upgrade or can be injected into a capillary on the ABI PRISM® 310 Genetic Analyzer. An internal lane size standard is loaded with each sample to allow for automatic sizing of the PCR products and to normalize differences in electrophoretic mobility between gel lanes or injections (Ziegle *et al.*, 1992). GeneScan® software automatically analyzes the collected data, which can then be imported into Genotyper® software for automatic genotyping of alleles.

High Throughput

Laboratories can analyze hundreds of loci in a single day using four-dye fluorescent labeling. This is a dramatic increase in productivity compared with gel staining techniques, which visualize all PCR products in the same color, or with other systems that are limited to one or two colors.

Additionally, the use of an internal size standard eliminates the need to run multiple lanes of the allelic ladder, thus allowing for the analysis of more samples.

AmpFℓSTR Profiler Plus PCR Amplification Kit

AmpFℓSTR Profiler Plus User's Manual

The *AmpFℓSTR® Profiler Plus™ PCR Amplification Kit User's Manual* provides supporting documentation for the AmpFℓSTR Profiler Plus PCR Amplification Kit (P/N 4303326). This manual contains sections describing the following:

- background information on the AmpFℓSTR Profiler Plus kit
- guidelines for setting up a laboratory for PCR DNA analysis
- recommended protocols for DNA extraction
- the importance of DNA quantitation prior to STR analysis
- protocols for PCR amplification of the AmpFℓSTR Profiler Plus loci
- information on the multicomponent analysis of fluorescence data
- protocols for detection and analysis of PCR products on the ABI PRISM 377 DNA Sequencer, ABI PRISM 377 DNA Sequencer with XL Upgrade, and ABI PRISM 310 Genetic Analyzer
- guidelines for interpretation of results
- the use of Genotyper software for automated genotyping of alleles
- guidelines for troubleshooting results
- a summary of the validation work for the AmpFℓSTR Profiler Plus kit according to the guidelines established by the Technical Working Group on DNA Analysis Methods (TWGDAM)
- population genetics data for the AmpFℓSTR Profiler Plus loci

The sections detailing how to set up a laboratory for PCR DNA analysis and how to perform the recommended protocols for DNA extraction can be used by all laboratories that perform PCR analysis. These sections are updated from the Applied Biosystems *AmpliType® User's Guide* (Versions 1 and 2).

Note AmpFℓSTR Profiler Plus detection is not supported on the ABI™ 373 DNA Sequencer or ABI 373 DNA Sequencer with XL Upgrade platforms, as the appropriate band-pass glass filter for the NED dye is not available.

AmpFℓSTR Profiler Plus Loci

The AmpFℓSTR Profiler Plus PCR Amplification Kit co-amplifies the repeat regions of the following nine short tandem repeat loci: D3S1358,[1] vWA,[2] FGA,[3] D8S1179,[4] D21S11,[5] D18S51,[6] D5S818,[7] D13S317,[7] and D7S820.[8] A segment of the X-Y homologous gene amelogenin is also amplified. Amplifying a segment of the amelogenin gene with a single primer pair can be used for gender identification because different length products from the X and Y chromosomes are generated.[9]

One primer of each locus-specific primer pair is labeled with either the 5-FAM, JOE, or NED NHS-ester dye, which is detected as blue, green, and yellow, respectively, on the ABI PRISM instruments. The loci amplified by these primers are summarized in Table 1-1 on page 1-6.

1. Li *et al.*,1993.
2. Kimpton *et al.*, 1992.
3. Mills *et al.*, 1992.
4. Oldroyd *et al.*, 1995. Note: D8S1179 is designated in this paper as D6S502.
5. Sharma and Litt, 1992.
6. Urquhart *et al.*, 1995.
7. Hudson *et al.,* 1995.
8. Green *et al.*, 1991.
9. Sullivan *et al.*,1993.

Table 1-1 AmpFℓSTR Profiler Plus loci

Locus Designation	Chromosome Location	Common Sequence Motif	Size Range (bp)[a]	Dye Label
D3S1358	3p	TCTA $(TCTG)_{1-3}$ $(TCTA)_n$	114–142	5-FAM
vWA	12p12-pter	TCTA $(TCTG)_{3-4}$ $(TCTA)_n$	157–197	5-FAM
FGA	4q28	$(TTTC)_3$ TTTT TTCT $(CTTT)_n$ CTCC $(TTCC)_2$	219–267	5-FAM
Amelogenin	X: p22.1–22.3 Y: p11.2	– –	107 113	JOE
D8S1179[b]	8	$(TCTR)_n$[c]	128–168	JOE
D21S11	21	$(TCTA)_n$ $(TCTG)_n$ [$(TCTA)_3$ TA $(TCTA)_3$ TCA $(TCTA)_2$ TCCA TA] $(TCTA)_n$	189–243	JOE
D18S51	18q21.3	$(AGAA)_n$	273–341	JOE
D5S818	5q21–31	$(AGAT)_n$	135–171	NED
D13S317	13q22–31	$(GATA)_n$	206–234	NED
D7S820	7q11.21–22	$(GATA)_n$	258–294	NED

a. The size range is the actual base pair size of sequenced alleles contained in the AmpFℓSTR Allelic Ladders. The sizes in the table include the 3´ A nucleotide addition.

b. In some literature references, this locus is designated as D6S502.

c. R can represent either an A or G nucleotide.

AmpFℓSTR Profiler Plus PCR Amplification Kit Contents

The AmpFℓSTR Profiler Plus PCR Amplification Kit contains the PCR reagents necessary to co-amplify the ten AmpFℓSTR Profiler Plus loci. The kit components are shown in Table 1-2. The recommended storage temperature for each component is listed in the Product Insert contained in each AmpFℓSTR Profiler Plus kit.

Table 1-2 AmpFℓSTR Profiler Plus PCR Amplification Kit Components

Kit Component	Volume	Description
AmpFℓSTR® PCR Reaction Mix	1.1 mL/tube	Two tubes each containing $MgCl_2$, deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP), bovine serum albumin (BSA), and 0.05% sodium azide (NaN_3) in buffer and salt
AmpFℓSTR Profiler Plus Primer Set	1.1 mL	One tube of locus-specific 5-FAM-, JOE-, and NED-labeled and unlabeled primers in buffer to amplify the STR loci D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820, and the gender marker amelogenin
AmpliTaq Gold® DNA Polymerase	50 µL/tube	Two tubes of enzyme with an activity of 5 U/µL
AmpFℓSTR® Control DNA 9947A	0.3 mL	One tube containing 0.10 ng/µL human cell line DNA in 0.05% NaN_3 and buffer. The genotype of this female DNA is D3S1358 14, 15; vWA 17, 18; FGA 23, 24; D8S1179 13, 13; D21S11 30, 30; D18S51 15, 19; D5S818 11, 11; D13S317 11, 11; D7S820 10, 11
Mineral oil	5 mL	One dropper bottle
AmpFℓSTR® Blue Allelic Ladder	25 µL	One tube of AmpFℓSTR Blue Allelic Ladder containing the following amplified 5-FAM-labeled alleles: D3S1358 12–19, vWA 11–21, and FGA 18–30 (including 26.2)
AmpFℓSTR® Green II Allelic Ladder	25 µL	One tube of AmpFℓSTR Green II Allelic Ladder containing the following amplified JOE-labeled alleles: amelogenin X and Y, D8S1179 8–19, D21S11 24.2–38 (including 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, 35.2), D18S51 9–26 (including 10.2, 13.2, 14.2)
AmpFℓSTR® Yellow Allelic Ladder	25 µL	One tube of AmpFℓSTR Yellow Allelic Ladder containing the following amplified NED-labeled alleles: D5S818 7–16, D13S317 8–15, and D7S820 6–15

The AmpFℓSTR® Allelic Ladders are used to genotype the analyzed samples. The alleles contained in the allelic ladders and the genotype of the AmpFℓSTR Control DNA 9947A are listed in Table 1-3.

Table 1-3 AmpFℓSTR Profiler Plus allele information

STR locus	AmpFℓSTR Allelic Ladder alleles	Other known alleles	Control DNA 9947A genotype
D3S1358	12, 13, 14, 15, 16, 17, 18, 19	(9, 11, 15.2, 20)[a]	14, 15
vWA	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21	(15.2, 22)[a]	17, 18
FGA	18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30	(15, 16, 16.2, 17, 18.2, 19.2, 20.2, 21.2, 22.2, 22.3, 23.2, 24.2, 25.2, 27.2, 28.2, 30.2, 34.2, 46.2)[a]	23, 24
Amelogenin	X, Y	–	X, X
D8S1179	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	–	13, 13
D21S11	24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 38	(24.3, 25.3, 28.3, 29.1),[b] (29.3, 30.1, 30.3, 31.1, 32.1, 33.1),[a] (33.3, 34.1),[b] 36.2[c]	30, 30
D18S51	9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26	9.2,[d] 15.2,[e] (17.2, 19.2)[d]	15, 19
D5S818	7, 8, 9, 10, 11, 12, 13, 14, 15, 16	–	11, 11
D13S317	8, 9, 10, 11, 12, 13, 14, 15	5[a]	11, 11
D7S820	6, 7, 8, 9, 10, 11, 12, 13, 14, 15	6.3[a]	10, 11

a. Applied Biosystems Human Identification Group.

b. Advanced DNA Technologies Workshop, 1997.

c. Zhou *et al.*, 1997.

d. Gill *et al.*, 1996.

e. Barber and Parkin, 1996.

AmpFℓSTR Profiler Plus Kit Performance Characteristics

The AmpFℓSTR Profiler Plus PCR Amplification Kit reagents and protocols have been optimized to give the sensitivity and specificity necessary for forensic analysis. Two nanograms of the AmpFℓSTR Control DNA 9947A provided in the kit will reliably type when the protocols described in this manual are followed. The recommended range of input sample DNA is 1.0–2.5 ng, so it is important that the DNA be quantitated prior to amplification. In the laboratories of Applied Biosystems, the kit components have been used successfully to type samples containing less than 1 ng of DNA.

PCR amplification component concentrations and thermal cycler parameters have been determined to produce specific amplification of the AmpFℓSTR Profiler Plus loci. The STR loci in the AmpFℓSTR Profiler Plus PCR Amplification Kit are specific for primate DNA. The primers used to amplify the amelogenin locus are known to amplify a ~103-bp monomorphic band in some animals (see page 12-9).

The PCR amplification parameters have also been optimized to produce similar peak heights within and between loci. The peak height generated at a locus for a heterozygous individual should be similar between the two alleles. The kit is also designed to generate similar peak heights between loci so that each locus will have approximately the same sensitivity.

For court support purposes, a Certificate of Analysis is available upon request. The certificate confirms that the specific combination of components that comprise a given kit lot number perform together to meet the stated performance.

Getting Started

- Confirm that the Filter Set F module files are installed on the Macintosh computer connected to the ABI PRISM 377 DNA Sequencer or ABI PRISM 310 Genetic Analyzer. The Filter Set F module files must be located in the Modules folder, located in the ABI PRISM 377 or ABI PRISM 310 folder. See Chapter 6, “Multicomponent Analysis,” for more information on Filter Set F module files.
- Make a matrix file using the 5-FAM, JOE, NED and ROX matrix standard samples and the Filter Set F module files. See Chapter 6 for protocols on how to make a matrix file. Be sure to verify the accuracy of the matrix file (Chapter 6).
- Determine the quantity of DNA in samples to be amplified. See Chapter 4 for more details on DNA quantitation.

- Amplify DNA samples using the AmpF*ℓ*STR Profiler Plus kit reagents (see Chapter 5). The recommended range of input DNA is 1.0–2.5 ng.

Note A useful initial experiment is to amplify a range of input DNA for each of several samples in order to establish the range of input DNA (as determined by your laboratory's quantitation system) that provides optimal results. For example, amplify 0.5, 1.0, 1.5, 2.0, and 2.5 ng of input DNA for each sample.

- Run AmpF*ℓ*STR Profiler Plus PCR products on the ABI PRISM 377 DNA Sequencer or ABI PRISM 377 DNA Sequencer with XL Upgrade (see Chapter 7) or on the ABI PRISM 310 Genetic Analyzer (see Chapter 8).

IMPORTANT Use the GeneScan-500 [ROX] Internal Lane Size Standard.

- Analyze the samples using GeneScan Analysis software.
- Examine the electropherograms as described in Chapter 9, "Results and Interpretation," and establish the amount of input DNA that produces signals within the linear dynamic detection range of the instrument.

  See "Off-scale Data" on page 6-15 and "Effect of DNA Quantity on Results" on page 9-33 for more details.

Note Valuable information and laboratory support data can be generated by analyzing a small database of samples. The percent stutter and peak height ratio within a locus can be calculated from these samples. We also recommend that the sizing precision obtained for several alleles within a gel or within a set of capillary injections is calculated using the database samples. Examples and explanations of such experiments are described in Chapter 9.

- Use the Genotyper 2.0 AmpF*ℓ*STR Profiler Plus template for automatic genotyping of samples (see Chapter 10).

2 *Laboratory Setup*

Overview

About This Section

This section provides guidelines for laboratories preparing to implement PCR technology. These important recommendations should also be reviewed by laboratories already using PCR-based typing tests. Careful planning and design of the laboratory and training of all laboratory personnel are necessary to ensure that exogenous DNA and PCR products are confined to designated areas.

Rationale for Lab Organization

Sensitivity of PCR

The sensitivity of the AmpFℓSTR® Profiler Plus™ PCR Amplification Kit (and other PCR-based tests) permits amplification of minute quantities of DNA. This necessitates precautions to avoid contamination of samples yet to be amplified (Kwok and Higuchi, 1989).

While contamination of amplified DNA with unamplified DNA (genomic DNA) does not pose a problem, ordinary precautions, such as changing pipet tips between samples, should be taken when handling and analyzing PCR product. This action should effectively prevent cross-contamination between samples of amplified DNA.

There are three potential sources of laboratory contamination, which are described below:

Contamination by Human Genomic DNA from Equipment or the Work Environment

Because of the specificity of AmpFℓSTR Profiler Plus PCR amplification, contamination of samples with non-primate DNA will not affect results. However, care should be taken while handling and processing samples to prevent chance contamination by human DNA. Gloves should be worn at all times and changed frequently. Sample tubes should be closed when not in use. Dispersal of aerosols should be limited through careful handling of sample tubes and reagents.

Cross-contamination During Sample Preparation

Extra precautions and care should be taken during DNA extraction and PCR setup to prevent transfer of DNA from one sample to another. Use a new, filter-plugged pipet tip for each sample, open tubes carefully, and keep sample tubes closed when not using them.

PCR Product Carryover

PCR product carryover occurs when amplified DNA contaminates a sample which has not yet been amplified. It is important to isolate and contain amplified PCR product to prevent it from coming into contact with unamplified samples.

Carryover is a concern because PCR product serves as an ideal template for subsequent amplifications of that same target. A single PCR amplification produces an enormous number of copies (as many as 10^{13}) that can potentially contaminate samples yet to be amplified.

Since the number of copies of amplified DNA in a completed PCR amplification is so high, inadvertent transfer of even a minute volume to a yet-to-be-amplified sample by splashing or aerosol may result in the amplification and detection of the “contaminating” sequence.

For example, if reuse of a pipet tip transfers 0.1 µL of a completed PCR amplification, then as many as 10^{10} copies of amplifiable sequence will be added to the unamplified sample. By comparison, a nanogram of human genomic DNA contains only about 10^2 copies of a single-copy locus like FGA.

Laboratory Design and Organization

Isolating Work Areas

Special consideration should be given to the design and organization of the laboratory. The laboratory must be organized so that the area where amplified DNA is handled is physically isolated from the work areas for DNA extraction and PCR setup. Strict physical isolation must be maintained between the area designated for handling amplified DNA and the other areas to avoid transfer of amplified DNA out of the designated work area.

Amplified DNA or equipment and supplies used to handle amplified DNA should not be taken out of the designated work area. If the work area for amplified DNA is in a separate but contiguous room, the user should make sure that air flows toward the amplified DNA area. In addition, it is helpful if there is a separate exit from the amplified DNA work area that does not exit into the pre-PCR work areas.

The laboratory should have four designated Work Areas, each ideally with dedicated equipment and supplies, to minimize the potential for contamination (see Figure 2-1 on page 2-5):

- Evidence Handling Work Area. Pieces of evidence are examined, photographed, and divided for analysis.
- DNA Extraction Work Area. This work space is for performing the extraction steps.
- PCR Setup Work Area. PCR reagent and DNA sample additions are made here.
- Amplified DNA Work Area. This area is dedicated to PCR amplification and detection, and other activities that require handling of amplified DNA.

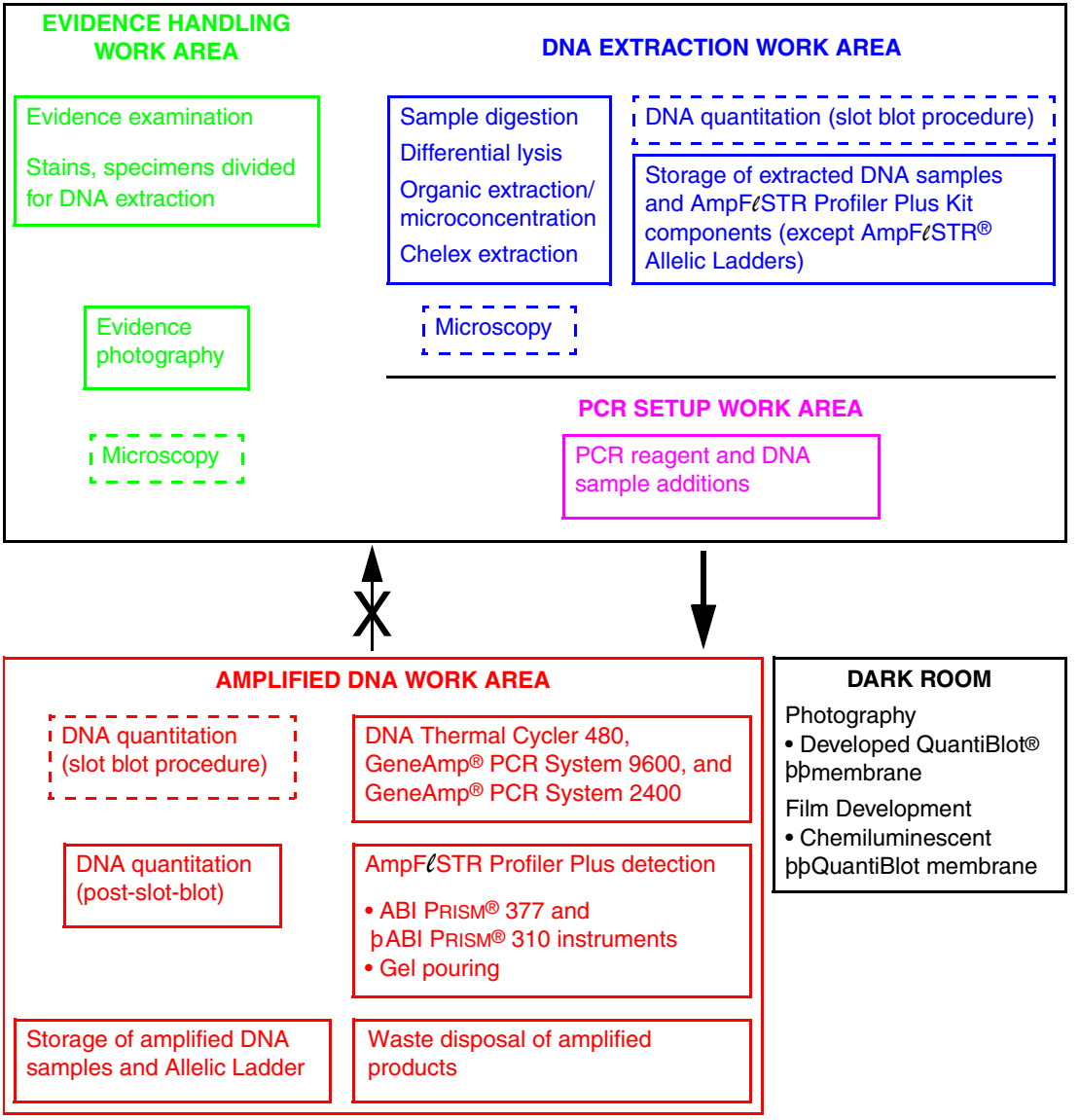


Figure 2-1 Designated Work Areas and activities. The activities performed within each Work Area are designated in the boxes. Certain activities may be performed in more than one Work Area (dashed line border). However, DNA quantitation supplies and reagents should never be moved from the Amplified DNA Work Area to the Evidence Handling, DNA Extraction, or PCR Setup Work Areas.

General Precautions

The DNA Extraction Work Area should not overlap the space designated for Evidence Handling. If it is not possible to handle evidence in a separate area, then all remaining evidence and/or reference samples must be removed from the area and the space must be thoroughly cleaned prior to beginning the DNA Extraction process.

If possible, the DNA Extraction and PCR Setup Work Areas should be separate from each other to prevent potential transfer of exogenous human DNA into the PCR Setup Work Area. If the DNA Extraction and PCR Setup Work Areas are in the same room, they should be clearly delineated. Bench-top biological safety cabinets may serve to isolate areas within a room.

The pipettors and other equipment used for DNA Extraction are routinely exposed to relatively high concentrations of human genomic DNA and should not be used for PCR Setup. Dedicated pipettors and plugged pipet tips should be used for setting up and adding DNA to the PCR reaction tubes.

If possible, use a dedicated area such as a biological safety cabinet with ultraviolet (UV) source for PCR Setup. The UV germicidal lamps in most biological safety cabinets quickly damage any DNA left on exposed surfaces, making it unsuitable for subsequent amplification (Cone and Fairfax, 1993; Kwok and Higuchi, 1989; Prince and Andrus, 1992). All equipment and supplies used for PCR Setup should be kept in this cabinet or a dedicated “clean” area at all times. Do not use these items to handle amplified DNA.

Because of the equipment used in the Amplified DNA Work Area, a relatively large space is required. This space requirement exceeds the space requirement for DNA Extraction and PCR Setup. A common mistake is to allocate more space for DNA Extraction and PCR Setup than for PCR amplification and PCR product detection.

The Amplified DNA Work Area should be in a separate room. The Amplified DNA Work Area must have a dedicated sink. Using color coded tape to identify the supplies and reagents used for handling PCR product and posting signs to indicate PCR product usage in the Amplified DNA Work Area may make it easier to contain PCR product within this laboratory area.

Evidence Handling Work Area

Overview

This work area should be used for examining and photographing evidence and for selecting samples to be analyzed by PCR-based tests. Portions of the samples to be tested are transferred from this area to the DNA Extraction Work Area for processing.

Microscopy of samples during the differential lysis procedure (see “Differential lysis procedure” on page 3-10) may be performed in this area, provided the sample is already spotted on the microscope slide.

The work surface, equipment, and supplies used in this area must be clean and free of PCR product.

Special Precautions

- Examine and sample from pieces of evidence at a separate time from the handling and sampling from reference samples. This precaution will help to minimize the potential for cross-contamination between evidence and reference samples.
- Use disposable gloves at all times. Change gloves frequently to avoid sample-to-sample contamination. Change them whenever they might have been contaminated with DNA and whenever exiting the work area.
- Clean scissors thoroughly with ethanol and water or use fresh scalpel blades after cutting from each sample.
- Use a clean cutting surface (*e.g.*, writing tablet paper) for each sample.
- When possible, avoid consuming an entire evidence sample for a single DNA extraction. This will minimize exposure of the work areas to large (microgram) quantities of human genomic DNA.

  Retaining a portion of the original sample also permits repeat analysis. Since PCR consumes only a small amount of most samples, repeat analyses may be performed at a later time, if desired. The probability of a sporadic contamination event occurring in replicate samples is very low.

DNA Extraction Work Area

Overview

This work area should be used for extraction and isolation of DNA, including sample digestion, organic or Chelex extraction, and Centricon microconcentration. Reagents used for sample extraction should be prepared in this work area.

Microscopy may be performed in this area. Slot blot quantitation of extracted DNA samples may be performed in this area, or in the Amplified DNA Work Area (remove only an aliquot of extracted DNA for analysis if this step is to be performed in the Amplified DNA Work Area).

Dedicated Equipment and Supplies

See "Required Chemicals and Enzymes" on page 3-34 for a list of dedicated equipment and supplies. These items should not leave the DNA Extraction Work Area.

Special Precautions

- To minimize the potential for sample-to-sample contamination, perform DNA extraction from samples containing high levels of DNA (*e.g.*, whole blood) separately from samples containing low levels of DNA (*e.g.*, single hairs and small bloodstains).
- Perform the DNA extraction of evidence samples at a separate time from the DNA extraction of reference samples. This precaution will help to minimize the potential for cross-contamination between evidence samples and reference samples.
- Limit the number of samples processed at the same time to a manageable number. This precaution will reduce the risk of sample mix-up and the potential for sample-to-sample contamination.
- Use disposable gloves at all times. Change gloves frequently to avoid sample-to-sample contamination. Change gloves whenever they might have been contaminated with DNA and whenever exiting the work area.
- Steam sterilize those solutions that can be heated in an autoclave without affecting their performance (*e.g.*, EDTA, TE Buffer, PBS, sodium acetate, sodium chloride, Tris-HCl, and deionized water). Steam sterilization, under bacterial decontamination conditions, degrades DNA to very small fragments, rendering it unamplifiable (Kwok and Higuchi, 1989). Solutions such as SDS and Digest Buffer cannot be autoclaved and should be sterilized by filtration. See "Preparation of Reagents" on page 3-38.

- Use sterile, disposable, hydrophobic filter-plugged pipet tips and microcentrifuge tubes.

IMPORTANT Do not use gamma irradiation to sterilize microcentrifuge tubes, as this may inhibit PCR subsequently carried out in these tubes.

- Always change pipet tips between handling each sample.
- Store reagents as small aliquots to minimize the number of times a given tube of reagent is opened. Record the lot numbers of reagents used in each set of samples so that if contamination occurs, it can be more readily traced.
- Avoid splashes. Some types of sample tubes have tightly fitting caps which may cause splashing when they are forced open. Centrifuge all liquid to the bottom of the closed tube before opening it. Use a tube decapper device to open tubes more easily. Clean the tube decapper device often.
- Include reagent blank controls with each set of DNA extractions to check for the presence of contaminating DNA in the reagents.

IMPORTANT Never “blow out” the last bit of sample from a pipettor. Blowing out increases the potential for aerosols, which may contaminate a sample with DNA from other samples. The accuracy of liquid volume delivered is not critical enough to justify blowing out.

- Use disposable bench paper (for example Benchkote sheets) on permanent work surfaces to prevent the accumulation of human DNA.
- Wear a labcoat dedicated to pre-amplification sample handling when working in the DNA Extraction Work Area.

PCR Setup Work Area

Overview

This work area is used for combining PCR reagents and adding mineral oil (DNA Thermal Cycler 480 reactions only) and extracted DNA to the appropriate reaction tubes.

Dedicated Equipment and Supplies

See Chapter 5 for a list of dedicated equipment and supplies. These items should never leave the PCR Setup Work Area.

Special Precautions

- Use a dedicated 20–200 µL or 2–20 µL pipettor with sterile, disposable, hydrophobic filter-plugged pipet tips for adding DNA sample to the PCR reaction mixture.
- Use a new sterile, disposable, hydrophobic filter-plugged pipet tip for each DNA sample addition to a PCR reaction tube. Discard used pipet tips.

IMPORTANT Never "blow out" the last bit of sample from the pipettor. Blowing out increases the potential for aerosols, which may contaminate a sample with DNA from other samples. The accuracy of liquid volume delivered is not critical enough to justify blowing out.

- Cap all tubes before beginning the addition of DNA. Only open the tube to which DNA is being added.
- To minimize cross-contamination, always add the DNA last to the PCR tubes (after the addition of mineral oil for the DNA Thermal Cycler 480).
- Make additions to the negative control (no DNA added) tube last. This control will provide a check for contamination occurring during PCR setup.
- Avoid handling the inside surface of the tube caps.
- Change gloves frequently whenever they may have been contaminated with DNA or were used to handle anything outside of the PCR Setup Work Area.
- Store the DNA amplification reagents in a refrigerator that is located in the DNA Extraction Work Area. Do not store the reagents close to samples containing high levels of DNA.

- Two microtube racks should be designated for specific tasks:
  - One rack is used for holding the tubes during PCR setup, and should be kept in the PCR Setup Work Area at all times.
  - The second rack, designated the “carrier rack,” should be used exclusively for carrying the PCR tubes to the thermal cycler located in the Amplified DNA Work Area. The carrier rack should be stored in the PCR Setup Work Area, and should never touch any surface in the Amplified DNA Work Area.

    Using a rack of a unique color will make it easier to identify as the carrier rack. The carrier rack should be sterilized frequently by autoclaving or by exposure to UV light.
- If using two tube decapper devices, designate one device exclusively for opening the PCR tubes when setting up reactions. Designate the second tube decapper exclusively for opening microtubes containing sample DNA. These devices should be sterilized frequently by autoclaving or exposure to UV light.
- Avoid exposing mineral oil to UV light. Exposure to UV light causes the mineral oil to inhibit PCR.

Amplified DNA Work Area

Overview

This work area should be a physically separate area used only for those activities that involve the handling of amplified DNA. These activities include the following:

- pouring of gels
- electrophoresis of amplified DNA
- handling of the AmpFℓSTR Allelic Ladders
- waste disposal of amplified DNA solutions
- storage of amplified DNA, the AmpFℓSTR Allelic Ladders, GeneScan-500 [ROX] Internal Lane Size Standard and Dye Primer Matrix Standards

Dedicated Equipment and Supplies

Amplified DNA or equipment and supplies used to handle amplified DNA should not be taken out of the Amplified DNA Work Area. Samples that have not yet been amplified should never come into contact with this equipment.

See Chapters 7 and 8 for lists of dedicated equipment and supplies.

Special Precautions

Even in the Amplified DNA Work Area, amplified DNA should be handled carefully to avoid dispersal around the room. Reducing the dispersal of amplified DNA within this work area will reduce the potential for transfer of amplified DNA to other work areas.

- Always remove gloves and lab coat when leaving the Amplified DNA Work Area to avoid the transfer of amplified DNA into other work areas.
- Reduce dispersal of DNA around the work area by changing gloves whenever they may have become contaminated with amplified DNA.
- Avoid splashing by opening tubes containing amplified DNA carefully. It may be helpful to spin down the contents of the amplified DNA tubes before opening. A tube decapper device makes it easier to open the tubes.
- Use disposable bench paper to cover the work area used to prepare samples for electrophoresis. This prevents the accumulation of amplified DNA on permanent work surfaces. Ten percent bleach solution should be used periodically to wash

exposed work surfaces. Soap and water can also be used to clean work surfaces.

- Use the thermal cycler only for amplification and for the denaturation of amplified DNA for typing. Never use the thermal cycler for incubation of tubes containing unamplified DNA.
- The slot blot step for DNA quantitation may be performed in this room, provided that only an aliquot of the extracted DNA was brought into this room. No DNA sample should be taken out of this area.
- Store tubes of amplified DNA in the Amplified DNA Work Area.
- Store the AmpFℓSTR Allelic Ladders in this area because they contain amplified PCR product.

DNA Extraction Protocols

3

Introduction

Overview

DNA for AmpF*ℓ*STR® Profiler Plus™ PCR amplification and analysis may be extracted from fresh or frozen whole blood, peripheral blood lymphocytes, blood stains, sperm cells, hair, tissue, bone, and other biological samples. Slightly different extraction procedures are required for each type of specimen. Because of the varied nature of evidence samples, you may need to modify the recommended procedures.

Protocols in this section outline a phenol-chloroform and a Chelex method for DNA extraction. The phenol-chloroform method removes proteins and other cellular components from nucleic acids, resulting in relatively purified DNA preparations. This method results in double-stranded DNA that is suitable for AmpF*ℓ*STR Profiler Plus amplifications. DNA extracted by the phenol-chloroform method is also suitable for RFLP analysis provided it is not significantly degraded. This method is also recommended when extracting DNA from relatively large samples (*i.e.*, when the amount of DNA in a sample is expected to be greater than 100 ng).

The Chelex method of DNA extraction is more rapid than the phenol-chloroform method. It involves fewer steps, resulting in fewer opportunities for sample-to-sample contamination. This method produces single-stranded DNA that is suitable for AmpF*ℓ*STR Profiler Plus amplification. DNA extracted with Chelex cannot be used for RFLP analysis.

Regardless of the method used for DNA extraction, all samples must be handled carefully to prevent sample-to-sample contamination or contamination by extraneous DNA. Also, we recommend that evidence samples be processed at a separate time from the reference samples.

Warnings To Users

The equipment and reagents needed for both extraction protocols are listed in “Reagents, Equipment and Supplies for Sample Preparation” on page 3-34. Read the Material Safety Data Sheet (MSDS) and label warning furnished by the supplier of each chemical or reagent used for guidelines on correct handling and the proper use of protective equipment.

- Biological samples have the potential to transmit infectious diseases, and should be handled with precautions published in *Biosafety in Microbiological and Biomedical Laboratories* (U.S. Department of Health and Human Services, 1993) and OSHA Bloodborne Pathogen Standard 29 CFR, part 1910.1030 (U.S. Department of Health and Human Services).
- Never pipet by mouth. Instead, use mechanical pipetting devices.
- Wear gloves whenever handling blood samples.
- To avoid generation of aerosols, we recommend that steps involved in the mixing of blood or cells derived from blood be performed in a biological safety cabinet.
- Decontaminate work surfaces daily. Contaminated items should be autoclaved before disposal.
- Phenol is extremely corrosive to skin and eyes and can cause severe burns. Wear safety glasses and chemical-resistant gloves when working with phenol. Procedures involving phenol should be performed in a chemical fume hood and the subsequent waste stored in the hood.
- Chloroform is a carcinogen and is toxic by inhalation, skin absorption, and ingestion. Procedures involving chloroform should be performed in a chemical fume hood and the subsequent waste stored in the hood.
- Acrylamide is a potent neurotoxin and is absorbed through the skin. Effects are cumulative. Wear chemical-resistant gloves and safety glasses when handling powders, solutions, and gels. Unpolymerized acrylamide sublimes; use in a well-ventilated area and clean up spills immediately.

Collection and Storage of Samples for DNA Extraction

Blood

Collect 5–10 mL of whole blood in EDTA tubes (lavender top), ACD tubes (yellow top, acid citrate dextrose) or heparinized tubes (green top). Whole blood specimens received by the lab may be stored in the refrigerator up to 1 month. Whole blood may be spotted on untreated, autoclaved cotton cloth, dried at room temperature, and stored frozen at –15 to –25 °C.

For reference samples, we recommend that a drop of blood be applied to the cotton cloth to produce a spot about 1 cm^2 in area. Aliquots of autopsy blood should be spotted on cotton cloth, dried and frozen at –15 to –25 °C as soon as possible after receipt. Several drops from each sample should be spotted to permit replicate experiments. Ten microliters (~7000–8000 white blood cells/µL) of whole blood will yield approximately 500 ng of DNA. The actual yield varies with the number of white blood cells present in the sample and the efficiency of DNA extraction.

If the specimen consists of a buffy coat, store it frozen at –15 to –25 °C and thaw at room temperature before use. Blood stain specimens should be stored dried and frozen at –15 to –25 °C.

Swabs and Stains

Sexual assault kit swabs and oral (buccal cell) reference sample swabs should be dissected into two or three pieces of equal size using a scalpel equipped with a fresh disposable blade. Use a clean cutting surface for each sample. Sections that are not to be analyzed immediately should be stored frozen at –15 to –25 °C.

When possible, stains deposited on fabric or other substrates that can be easily cut should be divided in half to preserve a section for repeat or referee analysis. Scissors used to cut stains should be washed with 95% ethanol and deionized water and dried with a fresh Kimwipe tissue before use and between each cutting. We recommend immersing scissors in 95% ethanol and deionized water between uses. Use a clean cutting surface for each sample.

Stains deposited on substrates that cannot be cut (*e.g.*, glass, metal) may be removed with a fresh swab moistened with autoclaved deionized water. After drying, the swab can be dissected and stored as described above. Alternatively, some dried fluids on these substrates can be carefully scraped off with a fresh scalpel blade. The scrapings can be stored at –15 to –25 °C.

Hair Collect loose hairs and store them at –15 to –25 °C in folded paper, envelopes, or plastic evidence bags.

Phenol-Chloroform (Organic) DNA Extraction

Before Proceeding Read "Collection and Storage of Samples for DNA Extraction" on page 3-3 before proceeding.

Blood The following DNA extraction protocol can be used with whole blood, buffy coats, bloodstains, and dried blood scrapings.

Reagents to be prepared for extraction

See "Preparation of Reagents" on page 3-38.

- Buffered Phenol-Chloroform-Isoamyl Alcohol Solution
- Digest Buffer
- Proteinase K Solution
- TE Buffer

To extract DNA from blood samples:

Step	Action
1	Pipet 0.5 mL Digest Buffer into an autoclaved 1.5-mL microcentrifuge tube. Add one of the following and mix: ♦ 10– 50 µL whole blood, or ♦ 2–10 µL buffy coat (approximately 10^5 WBC), or ♦ 1 cm^2 blood stain **Note** These amounts are recommended for reference samples. Evidence samples may be in limiting supply. Add as much evidential sample to the tube as possible, up to the quantities specified above, retaining sufficient sample for replicate analysis when possible.
2	Add 15 µL of 10 mg/mL Proteinase K Solution (to a final concentration of 0.3 mg/mL). Mix gently.
3	Incubate at 56 °C for at least 1 hour. For evidence samples, digestion should continue for a minimum of 6 hours. Digestion may be performed overnight, but more than 24 hours is not recommended.
4	After digestion, remove any bloodstain substrate with a fresh autoclaved toothpick or autoclaved, disposable pipette tip. Discard substrate.

To extract DNA from blood samples: *(continued)*

Step	Action
5	Separate the DNA from protein fragments with phenol-chloroform: **! WARNING ! CHEMICAL HAZARD. Wear chemical-resistant gloves and eye protection when handling phenol-chloroform solution. Use in a well-ventilated area.** a. To the 0.5 mL lysed and digested cells, add 0.5 mL Buffered Phenol-Chloroform-Isoamyl Alcohol Solution. Cap the tube and vortex for 15 seconds or until an emulsion forms. b. Spin in a microcentrifuge for 3–5 minutes at 10,000–15,000 × *g* (maximum speed) at room temperature to separate the two phases. c. Transfer the aqueous (upper) phase to a new autoclaved 1.5-mL microcentrifuge tube. A white layer of protein may be visible between the upper and lower phases. Be careful not to transfer protein to the new tube.
6	Repeat step 5 an additional two or three times until nothing is visible at the interface and the aqueous phase is clear. For these additional extractions, the lower phenol-chloroform layer may be removed and discarded, eliminating the need for a fresh microcentrifuge tube after the first extraction.

Concentration by Centricon-100 ultrafiltration is recommended for samples containing less than 500 ng DNA (approximately 10 µL of whole blood). Samples with larger amounts of DNA may be concentrated by ethanol precipitation (Sambrook *et al.*, 1989).

To wash and concentrate the DNA solution (aqueous phase):

Step	Action
1	Assemble a Centricon-100 concentration unit according to the manufacturer's directions and label the unit.
2	Add 1.5 mL TE Buffer to the upper Centricon-100 reservoir.
3	Transfer the entire (approximately 0.5 mL) aqueous (upper) phase containing extracted DNA to the TE Buffer in the Centricon-100 unit. Cover the unit with Parafilm. Use a toothpick or pipette tip to punch a pinhole in the Parafilm. Be careful not to touch the solution.

To wash and concentrate the DNA solution (aqueous phase): *(continued)*

Step	Action
4	Centrifuge in a fixed-angle rotor such as the Beckman JA20 or Sorvall SS34 (see Centricon manufacturer's instructions) at 1000 × *g* at room temperature for 20 minutes. The DNA sample will remain concentrated in about 15–50 µL of TE Buffer in the bottom of the upper Centricon reservoir, while molecules with molecular weights of less than about 100,000 daltons will pass through the filter.
5	Discard the effluent in the lower reservoir. **Note** The Centricon units are sensitive to rotor force. Do not centrifuge above 1,000 × *g*. Centrifugation time can be increased if the volume does not reduce to 15–50 µL within 20 minutes.
6	Add 2 mL of TE Buffer to the concentrated DNA solution in the upper Centricon reservoir.
7	Repeat the centrifugation and washing in steps step 4–6 twice for a total of three washes.
8	After the last wash, collect the approximately 15–50 µL concentrated DNA sample (as per Centricon manufacturer's instructions) by inverting the upper reservoir into the provided retentate cup and centrifuging at 500 × *g* for 2 minutes to transfer the concentrate into the cup.
9	Label the retentate cup. The sample is now ready for DNA quantitation and the PCR amplification process.
10	Store the sample at 2–6 °C or freeze at –15 to –25 °C until ready to perform PCR.

DNA extracted from bloodstain evidence is sometimes refractory to amplification by PCR. Lack of amplification is usually due either to insufficient quantity of DNA or to the presence of certain red blood cell components that inhibit PCR, such as heme. Discoloration of the DNA extract is generally predictive of inhibition. See Chapter 9, "Results and Interpretation," for more information about PCR inhibition.

Sexual Assault Swabs and Stains

The following protocols for differential lysis and DNA extraction can be used with the following types of sexual assault samples: vaginal, rectal, oral, and penile swabs, as well as stains and other swabbings that may contain sperm.

Note The following protocol for DNA extraction from sexual assault samples will not retain the ability to assay protein markers. If protein studies are to be performed on these samples, first perform the protocol in "Sexual Assault Swabs and Stains With Retained Ability to Assay Protein Markers" on page 3-14. Then complete the extraction starting at step 2 of "To prepare the cell pellet:" on page 3-8, instead of step 1.

Reagents to be prepared for lysis and extraction

See "Preparation of Reagents" on page 3-38.

- Autoclaved deionized water
- Buffered Phenol-Chloroform-Isoamyl Alcohol Solution
- Digest Buffer
- 1 M Dithiothreitol
- Proteinase K Solution
- TE Buffer

To prepare the cell pellet:

Step	Action
1	Suspend the swab or stained fabric sample in 1 mL of autoclaved distilled or deionized water in an autoclaved 1.5-mL microcentrifuge tube.
2	Incubate at room temperature for 30 minutes to rehydrate the sample.
3	Twirl the swab or fabric substrate vigorously for 2 minutes with an autoclaved toothpick to release the cells.
4	Remove the substrate and toothpick. Do not discard the substrate until microscopic analysis (see "To examine the resuspended cell pellet for the presence of sperm:" on page 3-9) is performed to detect sperm. Store the substrate in an autoclaved tube. If sperm cells are not visible microscopically, agitate the substrate more vigorously (*i.e.,* repeat step 1).
5	Centrifuge the sample in a microcentrifuge for 1 minute at 10,000–15,000 $\times$ *g* (maximum speed) at room temperature.

To prepare the cell pellet: *(continued)*

Step	Action
6	Without disturbing the pellet, remove and discard all but 50 µL (or twice the volume of the pellet, whichever is greater) of the supernatant.
7	Resuspend the pellet in the remaining 50 µL by stirring it with an autoclaved pipette tip. **Note** This pellet contains epithelial cells and sperm cells and is called the cell pellet.

If microscopic examination of the resuspended sample will not be performed, proceed to “Differential lysis procedure” on page 3-10.

To examine the resuspended cell pellet for the presence of sperm:

Step	Action
1	Spot approximately 3 µL of the resuspended sample on a glass microscope slide and fix cells to the slide by incubating in a 56 °C oven for 5 minutes.
2	Perform a Gram-modified Christmas tree stain as described in “Gram-modified Christmas Tree Stain” on page 3-42.
3	If epithelial cells are detected on the stained slide, proceed to “Differential lysis procedure” on page 3-10.
4	If no epithelial cells are observed, the differential lysis procedure may be omitted and the sample processed beginning with “To lyse sperm cells:” on page 3-11.

Differential lysis procedure

To lyse epithelial cells:

Step	Action
1	To the approximately 50 µL resuspended cell pellet, add 0.5 mL Digest Buffer.
2	Add 15 µL of 10 mg/mL Proteinase K Solution (to a final concentration of 0.3 mg/mL). Mix gently.
3	Incubate at 56 °C for at least 1 hour to lyse epithelial cells. **IMPORTANT** Do not incubate more than 2 hours to minimize lysis of sperm.
4	Spin the sample in a microcentrifuge for 5 minutes at 10,000–15,000 × *g* (maximum speed) at room temperature.
5	Transfer the supernatant to a new microcentrifuge tube. This epithelial cell material can be set aside and extracted in parallel with the lysed sperm fraction (see “To lyse sperm cells:” on page 3-11).

To resuspend and wash the sperm cell pellet:

Step	Action
1	Resuspend the pellet in 0.5–1.0 mL Digest Buffer by vortexing briefly.
2	Spin the sample in a microcentrifuge for 5 minutes at 10,000–15,000 × *g* at room temperature. If there are very few sperm in the sample, recovery of sperm in the pellet may be improved by use of a horizontal rotor instead of a fixed-angle rotor.
3	Remove and discard all but 50 µL of the supernatant.
4	Wash the sperm cell pellet according to steps1-3 an additional two to four times to remove residual epithelial cell material. **Note** Additional wash steps are recommended when the ratio of epithelial cells to sperm is high and the number of sperm cells is not limiting.
5	After washing the sperm cell pellet with Digest Buffer, resuspend the pellet in 0.5–1.0 mL autoclaved deionized water by vortexing briefly.
6	Spin the sample in a microcentrifuge for 5 minutes at 10,000–15,000 × *g* at room temperature.
7	Remove and discard all but 50 µL of the supernatant.

To resuspend and wash the sperm cell pellet: *(continued)*

Step	Action
8	Resuspend the pellet in the remaining 50 µL of autoclaved water by stirring it with an autoclaved pipette tip. **Note** The resuspended sample should be examined to verify the digestion of the epithelial cells and the recovery of sperm. Follow the procedure in "To examine the resuspended cell pellet for the presence of sperm:" on page 3-9. If epithelial cells are still observed, the "Differential lysis procedure" on page 3-10 can be repeated, but reduce the incubation at 56 °C to 30 minutes. Then repeat steps1–8 of this resuspension and wash procedure.

To lyse sperm cells:

Step	Action
1	Add 0.5 mL Digest Buffer to the approximately 50 µL resuspended sperm cell pellet.
2	Add 20 µL of 1 M dithiothreitol (to a final concentration of about 40 mM).
3	Add 15 µL of 10 mg/mL Proteinase K Solution (to a final concentration of about 0.3 mg/mL).
4	Incubate at 56 °C for at least 1 hour. For evidential material, digestion should continue for a minimum of 6 hours. For convenience, the digestion can be carried out overnight.

To extract DNA:

Step	Action
1	Separate the DNA in the sperm and epithelial cell fractions from protein fragments with phenol-chloroform: **! WARNING ! CHEMICAL HAZARD. Wear chemical-resistant gloves and eye protection when handling phenol-chloroform solution. Use in a well-ventilated area.** a. To the 0.5 mL lysed and digested sperm or epithelial cells, add 0.5 mL Buffered Phenol-Chloroform-Isoamyl Alcohol Solution. Cap the tube and vortex for 15 seconds or until an emulsion forms. b. Spin in a microcentrifuge for 3– 5 minutes at 10,000–15,000 × *g* (maximum speed) at room temperature to separate the two phases. c. Transfer the aqueous (upper) phase to a new autoclaved 1.5-ml microcentrifuge tube.
2	Repeat step 1 an additional 2–3 times until nothing is visible at the interface and the aqueous phase is clear. For these additional extractions the lower phenol-chloroform layer may be removed and discarded, eliminating the need for a fresh microcentrifuge tube after the first extraction.

Concentration by Centricon-100 ultrafiltration is recommended for samples containing less than 500 ng DNA. Samples with larger amounts of DNA may be concentrated by ethanol precipitation.

To wash and concentrate the DNA solution (aqueous phase):

Step	Action
1	Assemble a Centricon-100 concentration unit according to the manufacturer's directions and label the unit.
2	Add 1.5 mL TE Buffer to the upper Centricon-100 reservoir.
3	Transfer the entire (approximately 0.5 mL) aqueous (upper) phase containing extracted DNA to the TE Buffer in the Centricon-100 unit. Cover the unit with Parafilm. Use a toothpick or pipette tip to punch a pinhole in the Parafilm. Be careful not to touch the solution.

To wash and concentrate the DNA solution (aqueous phase): *(continued)*

Step	Action
4	Centrifuge in a fixed-angle rotor such as the Beckman JA20 or Sorvall SS34 (see Centricon manufacturer's instructions) at 1000 × *g* at room temperature for 20 minutes. The DNA sample will remain concentrated in about 15–50 µL of TE Buffer in the bottom of the upper Centricon reservoir, while molecules with molecular weights of less than about 100,000 daltons will pass through the filter.
5	Discard the effluent in the lower reservoir. **Note** The Centricon units are sensitive to rotor force. Do not centrifuge above 1,000 × *g*. Centrifugation time can be increased if the volume does not reduce to 15–50 µL within 20 minutes.
6	Add 2 mL of TE Buffer to the concentrated DNA solution in the upper Centricon reservoir.
7	Repeat the centrifugation and wash steps in steps 4 through 6 twice for a total of three washes.
8	After the last wash, collect the approximately 15–50 µL concentrated DNA sample (as per Centricon manufacturer's instructions) by inverting the upper reservoir into the provided retentate cup and centrifuging at 500 × *g* for 2 minutes to transfer the concentrate into the cup.
9	Label the retentate cup. The sample is now ready for DNA quantitation and the PCR amplification process.
10	Store the sample at 2–6 °C or freeze at –15 to –25 °C until ready to perform PCR.

Sexual Assault Swabs and Stains With Retained Ability to Assay Protein Markers

If protein studies are to be performed on the same sexual assault samples on which PCR DNA analysis will be performed, substitute the following procedure for step 1 of "To prepare the cell pellet:" on page 3-8.

Reagents to be prepared for extraction

See "Preparation of Reagents" on page 3-38.

- Autoclaved deionized water
- 1X PBS Buffer

To retain protein markers:

Step	Action
1	Suspend the swab or stained fabric sample in 200 µL of 1X PBS Buffer in an autoclaved 1.5-mL microcentrifuge tube.
2	Incubate 1 hour at 2–6 °C.
3	Twirl the swab or fabric substrate vigorously for 2 minutes with an autoclaved toothpick to release the cells.
4	Remove the substrate and toothpick. You should not discard the substrate until microscopic analysis is performed (see"To examine the resuspended cell pellet for the presence of sperm:" on page 3-9).
5	Centrifuge the sample in the microcentrifuge for 1 minute at 10,000–15,000 × *g* at 2–6 °C to pellet the cells.
6	Carefully pipet off all but 50 µL of the supernatant, containing the enzyme fraction, to a fresh microcentrifuge tube and store at –15 to –25 °C.
7	To the cell pellet, add back the fabric or swab substrate and 1 mL autoclaved deionized water. Proceed with the DNA extraction in "Sexual Assault Swabs and Stains" on page 3-8 (organic), starting with step 2, or page 3-24 (Chelex), starting with step 2.

Oral Swabs Collected for Reference Samples

The following protocol is to be used with oral swabs that have been collected for reference samples only. If the oral swab may contain sperm in addition to buccal cells, follow the protocols in “Sexual Assault Swabs and Stains” on page 3-8.

Reagents to be prepared for extraction

See “Preparation of Reagents” on page 3-38.

- Autoclaved deionized water
- Buffered Phenol-Chloroform-Isoamyl Alcohol Solution
- Digest Buffer
- Proteinase K Solution
- TE Buffer

To prepare oral swabs collected as reference samples:

Step	Action
1	Suspend the swab cutting in 1 mL of deionized water in an autoclaved 1.5-mL microcentrifuge tube.
2	Incubate at room temperature for 30 minutes to rehydrate sample.
3	Use an autoclaved toothpick to tease the fibers apart on the inside of the tube. Twirl the swab and the toothpick vigorously for 2 minutes to release the cells from the swab.
4	Remove the substrate and toothpick. Centrifuge the sample in a microcentrifuge for 1 minute at 10,000–15,000 $\times$ *g* (maximum speed) at room temperature.
5	Without disturbing the pellet, remove and discard all but 50 µL of the supernatant. Resuspend the pellet in the remaining 50 µL by stirring it with an autoclaved pipette tip.
6	To the approximately 50 µL resuspended pellet, add 0.5 mL Digest Buffer.
7	Add 15 µL 10 mg/mL Proteinase K Solution (to a final concentration of 0.3 mg/mL). Mix gently.
8	Incubate at 56 °C for 1– 2 hours.
9	Follow the procedure in “To extract DNA:” on page 3-12 to extract the DNA.

Hair Samples

This method may be used on all hairs submitted as evidence.

Reagents to be prepared for extraction

See “Preparation of Reagents” on page 3-38.

- Autoclaved deionized water
- Buffered Phenol-Chloroform-Isoamyl Alcohol Solution
- Digest Buffer
- 1 M Dithiothreitol
- Proteinase K Solution
- TE Buffer

To extract DNA from hair samples:

Step	Action
1	Transfer the hair with clean forceps to a dissecting microscope and examine the hair for the presence of sheath material. The hair may be placed on a clean piece of white paper. Note the possible presence of body fluids on hair.
2	Wash the hair to reduce surface dirt and contaminants as follows:
	For shed hairs: a. Fill a clean 50 mL beaker with autoclaved deionized water. b. Pick up a single hair with a pair of forceps that has been cleaned previously by flaming. c. Wash each hair to be analyzed separately by immersing in fresh deionized water.
	For mounted hairs: a. Freeze the slide in a –15 to –25 °C freezer for 20 minutes. b. Remove the cover slip by prying it off using a scalpel. Alternatively, the coverslip may be removed by soaking the slide in xylene for several hours after cracking the coverslip with a diamond scribe. c. Using a Pasteur pipette, wash away the mounting medium by squirting the slide with xylene. d. Pick up the hair with clean forceps and wash in a 1.5-mL microcentrifuge tube containing 100% ethanol. e. Then wash the hair in a 1.5-mL microcentrifuge tube containing autoclaved deionized water.

To extract DNA from hair samples: *(continued)*

Step	Action
3	Return the hair to the dissecting microscope using a new piece of paper. Use a clean scalpel to cut a 1 cm portion from the root end of the hair. **Note** Because hair may contain cellular material on the surface which may or may not originate from the hair donor, cut off about 5–10 mm of the shaft adjacent to the root portion for separate analysis as a control.
4	To an autoclaved 1.5-mL microcentrifuge tube, add the following: ♦ 0.5 mL of Digest Buffer ♦ 20 µL of 1 M dithiothreitol (to a final concentration of about 40 mM) ♦ 15 µL of 10 mg/mL Proteinase K Solution (to a final concentration of about 0.3 mg/mL)
5	Add the hair sample, root end at the bottom, to the tube and check that it is completely immersed in the solution. Repeat for the hair shaft.
6	Incubate at 56 °C for 6–8 hours. Hair will usually soften but not dissolve after this initial incubation.
7	Vortex for 30 seconds.
8	Add to the sample an additional 20 µL of 1 M dithiothreitol and 15 µL of 10 mg/mL Proteinase K Solution.
9	Incubate at 56 °C for 6–8 hours or overnight until the hair is completely dissolved.
10	Vortex for 30 seconds.
11	Spin the sample in a microcentrifuge for 1 minute at 10,000–15,000 × *g* (maximum speed) at room temperature to remove any pigment and particles.
12	Transfer the supernatant to a new autoclaved microcentrifuge tube.

To separate DNA from protein fragments with phenol-chloroform:

! WARNING ! CHEMICAL HAZARD. Wear chemical-resistant gloves and eye protection when handling phenol-chloroform solution. Use in a well-ventilated area.

Step	Action
1	To the approximately 0.6 mL lysed and digested hair sample, add 0.6 mL Buffered Phenol-Chloroform-Isoamyl Alcohol Solution. Cap the tube and vortex for 15 seconds or until an emulsion forms.
2	Spin in a microcentrifuge for 3–5 minutes at 10,000–15,000 × *g* (maximum speed) at room temperature to separate the two phases.
3	Transfer the upper aqueous phase to a new autoclaved microcentrifuge tube.

Concentration by Centricon-100 ultrafiltration is recommended for samples containing less than 500 ng DNA. Samples with larger amounts of DNA may be concentrated by ethanol precipitation.

To wash and concentrate the DNA solution (aqueous phase):

Step	Action
1	Assemble a Centricon-100 concentration unit according to the manufacturer's directions and label the unit.
2	Add 1.5 mL TE Buffer to the upper Centricon-100 reservoir.
3	Transfer the entire (approximately 0.5 mL) aqueous (upper) phase containing extracted DNA to the TE Buffer in the Centricon-100 unit. Cover the unit with Parafilm. Use a toothpick or pipette tip to punch a pinhole in the Parafilm. Be careful not to touch the solution.
4	Centrifuge in a fixed-angle rotor such as the Beckman JA20 or Sorvall SS34 (see Centricon manufacturer's instructions) at 1000 × *g* at room temperature for 20 minutes. The DNA sample will remain concentrated in about 15–50 µL of TE Buffer in the bottom of the upper Centricon reservoir, while molecules with molecular weights of less than about 100,000 daltons will pass through the filter.
5	Discard the effluent in the lower reservoir. **Note** The Centricon units are sensitive to rotor force. Do not centrifuge above 1,000 × *g*. Centrifugation time can be increased if the volume does not reduce to 15–50 µL within 20 minutes.
6	Add 2 mL of TE Buffer to the concentrated DNA solution in the upper Centricon reservoir.

To wash and concentrate the DNA solution (aqueous phase): *(continued)*

Step	Action
7	Repeat the centrifugation and wash steps in steps 4–6 twice for a total of three washes.
8	After the last wash, collect the approximately 15–50 µL concentrated DNA sample (as per Centricon manufacturer's instructions) by inverting the upper reservoir into the provided retentate cup and centrifuging at 500 × *g* for 2 minutes to transfer the concentrate into the cup.
9	Label the retentate cup. The sample is now ready for DNA quantitation and the PCR amplification process.
10	Store the sample at 2–6 °C or freeze at –15 to –25 °C until ready to perform PCR.

Chelex DNA Extraction

Overview

Chelex is a chelating resin that has a high affinity for polyvalent metal ions. The Chelex resin is composed of styrene divinylbenzene copolymers containing paired iminodiacetate ions, which act as chelating groups.

It has been postulated that the presence of Chelex during boiling prevents the degradation of DNA by chelating metal ions that may catalyze the breakdown of DNA subjected to high temperatures in low ionic strength solutions (Singer-Sam and Tanguay, 1989; Walsh *et al.*, 1991).

The basic Chelex procedure consists of boiling the sample in a 5% Chelex solution, and then adding a fraction of the supernatant directly to the PCR. This procedure results in denatured (*i.e.*, single stranded) sample DNA.

Preparing Chelex Mixtures

To prepare and handle Chelex mixtures:

Step	Action
1	Make 20% and 5% (w/v) stock mixtures in autoclaved, distilled water to a final volume of 100– 300 mL.
2	Check that the mixture has a pH value of 9–11 before using. The pH of the Chelex mixture is a critical determinant of extraction efficiency.
3	Mix gently with a stir bar in a beaker to distribute the resin beads evenly while pipetting.
4	Add Chelex to individual samples as follows: a. For each set of samples, pour approximately 15 mL of Chelex stock solution into an autoclaved 50 mL beaker containing an autoclaved stir bar. b. Pipet the volume needed for each sample directly from the beaker while the stir bar is rotating. The tip used must have a relatively large bore (for example, the tip for a Rainin P-1000 pipettor) to ensure that sufficient Chelex resin beads are transferred to the sample tube. **Note** Read “Collection and Storage of Samples for DNA Extraction” on page 3-3 before proceeding.
5	Chelex-extracted samples can be stored at 2–6 °C for short periods of time (less than 2 months). For longer periods, the extracts should be stored at –15 to –25 °C either with or without the Chelex beads present.

Blood

The following protocol can be used with whole blood and bloodstains.

See page 3-32 for extraction of buffy coat samples.

Reagents to be prepared for extraction

See “Preparation of Reagents” on page 3-38.

- Autoclaved deionized water
- 5% Chelex
- TE buffer

To extract DNA from blood samples:

Step	Action
1	**If using whole blood:**
	Pipet 1 mL of TE buffer into an autoclaved 1.5-mL microcentrifuge tube.
	If using bloodstains:
	Pipet 1 mL of autoclaved deionized water into an autoclaved 1.5-mL microcentrifuge tube. **Note** Some laboratories substitute 1 mL of 1X PBS for H_2O at this step, but only for extraction of bloodstains. Do not use 1X PBS for extraction of whole blood samples (1X PBS will not result in adequate red cell lysis when used for fresh whole blood samples).
2	Add one of the following and mix gently: ♦ 3 µL whole blood (use of a higher volume is likely to result in PCR inhibition), or ♦ portion of bloodstain approximately 3 mm × 3 mm (use of a larger portion is likely to result in PCR inhibition) **Note** These amounts are recommended for both reference and evidential samples. Do not use greater amounts, even when the sample is not in limiting supply.
3	Incubate at room temperature for 15–30 minutes. Mix occasionally by inversion or gentle vortexing.
4	Spin the sample in a microcentrifuge for 2–3 minutes at 10,000–15,000 × *g* (maximum speed) at room temperature.

To extract DNA from blood samples: *(continued)*

Step	Action
5	Carefully remove and discard as much of the supernatant as possible. Leave no more than 20 µL of residual supernatant in the tube. If the sample is a bloodstain, leave the fabric substrate in the tube with the pellet.
6	Add 5% Chelex to a final volume of 200 µL. Vortex the sample briefly to mix.
7	Incubate the sample at 56 °C for 15–30 minutes.
8	Vortex the sample at high speed for 5–10 seconds.
9	Incubate the sample in a boiling water bath for 8 minutes. Use either a floating rack or the water bath/ice rack (see “Equipment” on page 3-35).
10	Vortex the sample at high speed for 5–10 seconds.
11	Spin the sample in a microcentrifuge for 2–3 minutes at 10,000–15,000 × *g* (maximum speed) at room temperature. The sample is now ready for DNA quantitation and the PCR amplification process.
12	Store the remainder of the sample at 2–6 °C or –15 to –25 °C. To reuse, thaw at room temperature and repeat steps 10–11.

Sexual Assault Swabs and Stains

The protocols for differential lysis and DNA extraction can be used for the following types of sexual assault samples: vaginal, rectal, oral, and penile swabs as well as stains and other swabbings that may contain sperm.

The protocol also may be used for liquid semen samples. Start at step 1 of “To lyse sperm cells:” on page 3-26 and add 3 µL semen to 200 µL 5% Chelex instead of 50 µL resuspended sperm pellet to 175 µL 5% Chelex. All other additions and incubations are the same.

Note The following protocol for DNA extraction from sexual assault samples will not retain the ability to assay protein markers. If protein studies are to be performed on these samples, first perform the protocol in “Sexual Assault Swabs and Stains With Retained Ability to Assay Protein Markers” on page 3-14. Then complete the extraction starting at step 2 of “To prepare the cell pellet:” on page 3-24.

Reagents to be prepared for extraction

See “Preparation of Reagents” on page 3-38.

- Autoclaved deionized water
- 5% Chelex
- 20% Chelex
- Digest Buffer
- 1 M Dithiothreitol
- Proteinase K Solution
- TE Buffer

To prepare the cell pellet:

Step	Action
1	Suspend the swab or stained fabric sample in 1 mL of autoclaved deionized water in an autoclaved 1.5-mL microcentrifuge tube.
2	Incubate at room temperature for 30 minutes to rehydrate the sample.
3	Twirl the swab or fabric substrate vigorously for 2 minutes with an autoclaved toothpick to release the cells.
4	Remove the substrate and toothpick. Do not discard the substrate until microscopic analysis (see “To examine the resuspended cell pellet for the presence of sperm:” on page 3-25) is performed to detect sperm.
5	Store the substrate in an autoclaved tube. If sperm cells are not visible microscopically, agitate the substrate more vigorously (*i.e.,* repeat step 3).
6	Centrifuge the sample in a microcentrifuge for 1 minute at 10,000–15,000 × *g* (maximum speed) at room temperature.
7	Without disturbing the pellet, remove and discard all but 50 µL (or twice the volume of the pellet, whichever is greater) of the supernatant.
8	Resuspend the pellet in the remaining 50 µL by stirring it with an autoclaved pipette tip. **Note** This pellet contains epithelial cells and sperm cells and is called the cell pellet.

If microscopic examination of the resuspended sample will not be performed, proceed to “Differential lysis procedure” on page 3-25.

To examine the resuspended cell pellet for the presence of sperm:

Step	Action
1	Spot approximately 3 µL of the resuspended sample on a glass microscope slide and fix cells to the slide by incubating in a 56 °C oven for 15–30 minutes.
2	Perform a Gram-modified Christmas tree stain as described in “Gram-modified Christmas Tree Stain” on page 3-42.
3	If epithelial cells are detected on the stained slide, proceed to “Differential lysis procedure” on page 3-25.
4	If no epithelial cells are observed, the differential lysis procedure may be omitted and the sample processed beginning with “To lyse sperm cells:” on page 3-26.

Differential lysis procedure

To lyse epithelial cells:

Step	Action
1	To the approximately 50 µL resuspended cell pellet, add TE Buffer to a final volume of 200 µL.
2	Add 2–6 µL of 10 mg/mL Proteinase K Solution. Mix gently. **Note** Use 2 µL of Proteinase K solution if the number of epithelial cells is low to moderate (1–20 cells per field). Use up to 6 µL of Proteinase K solution if the ratio of epithelial cells to sperm is high (10:1).
3	Incubate at 56 °C for at least 1 hour to lyse epithelial cells. **IMPORTANT** Do not incubate more than 2 hours to minimize lysis of sperm.
4	Spin the sample in a microcentrifuge for 5 minutes at 10,000–15,000 × *g* (maximum speed) at room temperature.
5	Add 150 µL of the supernatant to 50 µL of 20% Chelex in a new, autoclaved 1.5-mL microcentrifuge tube. This epithelial cell material can be set aside and then extracted in parallel with the lysed sperm fraction (see “To extract DNA:” on page 3-26, step 1).
6	Resuspend the pellet in 0.5–1.0 mL Digest Buffer by vortexing briefly.

To lyse epithelial cells: *(continued)*

Step	Action
7	Spin the sample in a microcentrifuge for 5 minutes at 10,000–15,000 × *g* at room temperature. If there are very few sperm in the sample, recovery of sperm in the pellet may be improved by use of a horizontal rotor instead of a fixed-angle rotor.
8	Remove and discard all but 25 µL of the supernatant.
9	Wash the sperm cell pellet according to steps 6–8 an additional two to four times to remove residual epithelial cell material. **Note** Additional wash steps are recommended when the ratio of epithelial cells to sperm is high and the number of sperm cells is not limiting.
10	After washing the sperm cell pellet with Digest Buffer, resuspend the pellet in 1.0 mL autoclaved deionized water by vortexing briefly.
11	Spin the sample in a microcentrifuge for 5 minutes at 10,000–15,000 × *g* at room temperature. Remove and discard all but 25 µL of the supernatant. Resuspend the pellet in the remaining 25 µL of autoclaved water by stirring it with the autoclaved pipette tip. **Note** We recommend that the resuspended samples be examined to verify the digestion of the epithelial cells and the recovery of the sperm. Follow the procedure in “To examine the resuspended cell pellet for the presence of sperm:” on page 3-25.

To lyse sperm cells:

Step	Action
1	Add 175 µL of 5% Chelex to the approximately 25 µL resuspended sperm cell pellet (final volume should be about 200 µL).
2	Add 2 µL of 10 mg/mL Proteinase K Solution and 7 µL of 1 M dithiothreitol. Mix gently.
3	Incubate the sample at 56 °C for 30–60 minutes.

To extract DNA:

Step	Action
1	Vortex the sperm and epithelial cell fractions at high speed for 10 seconds.
2	Spin both sperm and epithelial cell fractions in a microcentrifuge for 10–20 seconds at 10,000–15,000 × *g* (maximum speed) at room temperature.

To extract DNA:

Step	Action
3	Incubate the samples in a boiling water bath for 8 minutes.
4	Vortex the samples at high speed for 5–10 seconds.
5	Spin the samples in a microcentrifuge for 2–3 minutes at 10,000–15,000 × *g* (maximum speed) at room temperature. The samples are now ready for DNA quantitation and the PCR amplification process.
6	Store the remainder of the sample either at 2–6 °C or –15 to –25 °C.
7	To reuse, thaw the samples at room temperature and repeat steps 4–6.

Oral Swabs Collected for Reference Samples

Reagents to be prepared for extraction

See “Preparation of Reagents” on page 3-38.

- Autoclaved deionized water
- 5% Chelex
- 10 mg/mL Proteinase K

To prepare the cell pellet:

Step	Action
1	Suspend the swab sample in 1 mL of autoclaved deionized water in an autoclaved 1.5-mL microcentrifuge tube.
2	Incubate at room temperature for 30 minutes to rehydrate the sample.
3	Use an autoclaved toothpick to tease the fibers apart on the inside of the tube. Twirl the swab and toothpick for 2 minutes to release the cells from the swab.
4	Remove the substrate and toothpick. Spin in a microcentrifuge for 2 minutes at 10,000–15,000 × *g* (maximum speed) at room temperature.
5	Without disturbing the pellet, remove and discard all but 25 µL (or twice the volume of the pellet, whichever is greater) of the supernatant.
6	Resuspend the pellet in the remaining 25 µL by stirring with an autoclaved pipette tip.

To extract DNA:

Step	Action
1	Add 5% Chelex to a final volume of 200 µL.
2	Add 2 µL of 10 mg/mL Proteinase K. Mix gently.
3	Incubate the sample at 56 °C for 15–30 minutes.
4	Vortex the sample at high speed for 5–10 seconds.
5	Spin the sample in a microcentrifuge for 10–20 seconds at 10,000–15,000 × *g* (maximum speed) at room temperature.
6	Incubate the sample in a boiling water bath for 8 minutes.
7	Vortex the sample at high speed for 5–10 seconds.
8	Spin the sample in a microcentrifuge for 2–3 minutes at 10,000–15000 × *g* (maximum speed) at room temperature. The sample is now ready for DNA quantitation and the PCR amplification process.
9	Store the remainder of the sample at either 2–6 °C or –15 to –25 °C.
10	To reuse, thaw the sample at room temperature and repeat steps 7–8.

Buccal Scrapings Collected for Reference Samples

Reagents to be prepared for extraction

See “Preparation of Reagents” on page 3-38.

- Autoclaved deionized water
- 5% Chelex
- 10 mg/mL Proteinase K

To prepare the cell pellet:

Step	Action
1	Scrape the inside of the cheek with an autoclaved toothpick. Do not allow the toothpick to dry.
2	Twirl the toothpick directly into 200 µL of 5% Chelex in an autoclaved 1.5-mL microcentrifuge tube.
3	Remove the toothpick. Add 2 µL of 10 mg/mL Proteinase K. Mix gently.
4	Follow the protocol for oral swabs in “Oral Swabs Collected for Reference Samples” beginning with step 3 of “To extract DNA:” on page 3-28.

Saliva Stains

Reagents to be prepared for extraction

See “Preparation of Reagents” on page 3-38.

- 5% Chelex
- 10 mg/mL Proteinase K

To prepare the stain for DNA extraction:

Step	Action
1	Add a portion of saliva stain (about 3 mm × 3 mm) to 200 µL of 5% Chelex in an autoclaved 1.5-mL microcentrifuge tube.
2	Add 2 µL of 10 mg/mL Proteinase K. Mix gently.
3	Follow the protocol for oral swabs in “Oral Swabs Collected for Reference Samples” beginning with step 3 of “To extract DNA:” on page 3-28.

Hair

Reagents to be prepared for extraction

See “Preparation of Reagents” on page 3-38.

- Autoclaved deionized water
- 5% Chelex
- 10 mg/mL Proteinase K

To extract DNA:

Step	Action
1	Transfer the hair with clean forceps to a dissecting microscope and examine the hair for the presence of sheath material. The hair may be placed on a clean piece of white paper. Note the possible presence of body fluids on hair.
2	Wash the hair to reduce surface dirt and contaminants as follows:
	For shed hairs: a. Fill a clean 50 mL beaker with autoclaved deionized water. b. Pick up a single hair with a pair of forceps that has been cleaned previously by flaming. c. Wash each hair to be analyzed separately by immersing in fresh deionized water.
	For mounted hairs: a. Freeze the slide in a –15 to –25 °C freezer for 20 minutes. b. Remove the cover slip by prying it off using a scalpel. Alternatively, the coverslip may be removed by soaking the slide in xylene for several hours after cracking the coverslip with a diamond scribe. c. Using a Pasteur pipette, wash away the mounting medium by squirting the slide with xylene. d. Pick up the hair with clean forceps and wash in a 1.5-mL microcentrifuge tube containing 100% ethanol. e. Then wash the hair in a 1.5-mL microcentrifuge tube containing autoclaved deionized water.
3	Return the hair to the dissecting microscope using a new piece of paper. Use a clean scalpel to cut a 1-cm portion from the root end of the hair. **Note** Because hair may contain cellular material on the surface which may or may not originate from the hair donor, cut off about 5–10 mm of the shaft adjacent to the root portion for separate analysis as a control.

To extract DNA: *(continued)*

Step	Action
4	Add the hair sample, root end at the bottom, to 200 µL of 5% Chelex in a 1.5-mL microcentrifuge tube.
5	Add 2 µL of 10 mg/mL Proteinase K. Mix gently.
6	Incubate the sample at 56 °C for at least 6–8 hours or overnight.
7	Vortex the sample at high speed for 5–10 seconds.
8	Spin the sample in a microcentrifuge for 10–20 seconds at 10,000–15,000 × *g* maximum speed) at room temperature.
9	Incubate the sample in a boiling water bath for 8 minutes. **Note** Check that the hair is completely immersed in the Chelex solution before boiling.
10	Vortex the sample at high speed for 5–10 seconds.
11	Spin the sample in a microcentrifuge for 2–3 minutes at 10,000–15,000 × *g* (maximum speed) at room temperature. The sample is now ready for DNA quantitation and the PCR amplification process.
12	Store the remainder of the sample at either 2–6 °C or –15 to –25 °C.
13	To reuse, thaw sample at room temperature and repeat steps 10–11.

Buffy Coat or Cells in Culture

Reagents to be prepared for extraction

See “Preparation of Reagents” on page 3-38.

- Autoclaved deionized water
- 5% Chelex
- 1X PBS Buffer

To prepare the sample for DNA extraction:

Step	Action
1	Add up to 0.5 mL of cells to an autoclaved 1.5-mL microcentrifuge tube.
2	Add 1 mL of 1X PBS Buffer and centrifuge for 1 minute at 10,000–15,000 × *g* (maximum speed) at room temperature.
3	Remove and discard supernatant.
4	Resuspend the cells in 50 to 100 µL of autoclaved deionized water.
5	Add a volume of cell suspension to 200 µL of 5% Chelex such that the final cell concentration is about 25 cells/µL (5,000 cells total). Mix gently.
6	Incubate the sample at 56 °C for 15–30 minutes.
7	Vortex the sample at high speed for 5–10 seconds.
8	Follow the protocol for “Blood,” beginning with step 9 of “To extract DNA from blood samples:” on page 3-22.

Samples from Human Tissue or Bone

Reagents to be prepared for extraction

See “Preparation of Reagents” on page 3-38.

- 5% Chelex

To extract DNA from tissue or bone:

Step	Action
1	Dissect a piece of tissue (*e.g.*, muscle) approximately 2 mm × 2 mm.
2	Add the tissue to 200 µL of 5% Chelex in an autoclaved 1.5-mL microcentrifuge tube.
3	Incubate the sample at 56 °C for 15–30 minutes.
4	Vortex the sample at high speed for 5–10 seconds.
5	Incubate in a boiling water bath for 8 minutes.
6	Vortex the sample at high speed for 5–10 seconds.
7	Spin the sample in a microcentrifuge for 2 minutes at 10,000–15,000 × *g*. The sample is now ready for DNA quantitation and the PCR amplification process.
8	Store the remainder of the sample at either 2–6 °C or –15 to –25 °C.
9	To reuse, thaw the sample at room temperature and repeat steps 6–7.

Reagents, Equipment and Supplies for Sample Preparation

Chemicals and Enzymes

Chemicals used should be reagent grade.

Required Chemicals and Enzymes

- Acetic acid, glacial
- Aluminum sulfate
- Chelex 100 (100–200 mesh, sodium form, biotech grade)
- Chloroform
- Disodium ethylenediaminetetraacetate, dihydrate (EDTA)
- Dithiothreitol (DTT)
- Ethanol (100%), in glass container
- Hydrochloric acid (HCl), concentrated
- Indigo carmine
- Isoamyl alcohol
- Nuclear Fast Red
- Permount compound
- Phenol
- Picric acid, saturated solution (for slides)
- Proteinase K
- Sodium acetate, trihydrate ($CH_3COONa•3H_2O$)
- Sodium chloride (NaCl)
- Sodium dodecyl sulfate (SDS), ultrapure electrophoresis grade
- Sodium hydroxide pellets (NaOH)
- Tris(hydroxymethyl)aminomethane (Tris base)

Optional Chemicals and Enzymes

- Crystal (methyl) violet, for Gram stain
- Iodine, for Gram stain
- Potassium chloride (KCl), for protein marker extraction
- Potassium iodide (KI), for Gram stain

- Potassium phosphate, monobasic, anhydrous (KH_2PO_4), for protein marker extraction
- Sodium phosphate, dibasic, anhydrous (Na_2HPO_4), for protein marker extraction
- Xylene, for removing coverslips

Equipment

Required Equipment

- Autoclave
- Balance, accurate to 0.1 mg
- Beakers, 1 L Pyrex (Chelex extraction method only)
- Centrifuge with rcf of 1,000 × *g* and fixed-angle rotor with adapters accepting 17×100 mm tubes
- Deionizer column for water
- Freezer, –15 to –25 °C
- Hotplate/stirrer, to 100 °C (Chelex extraction method only)
- Microcentrifuge with rcf at least 10,000 × *g*
- Microscope, to 400X magnification
- Microscope, dissecting (for hairs)
- Oven, to 60 °C
- pH meter that can measure Tris solutions
- Pipettors, adjustable to deliver 1–20 µL, 20–200 µL, and 200–1000 µL
- Refrigerator, 2–6 °C
- Vortex mixer
- Water bath/ice racks (Chelex extraction method only), USA Scientific Plastics (P/N 2016-7001)

Optional Equipment

- Biological safety cabinet with UV source, *e.g.,* Labconco 11000
- Cold room or refrigerated cabinet (for ethanol precipitation of DNA and for protein isolation)
- Microcentrifuge with horizontal rotor (for sample preparation)
- Microcentrifuge with rcf at least 10,000 × *g*, kept at 2–6 °C (for ethanol precipitation of DNA and protein marker extraction)

Supplies

- Absorbent bench paper (*e.g.,* Benchkote)
- Absorbent tissues (*e.g.,* Kimwipes)
- Aluminum foil
- Beakers, 50 mL to 2 L
- Biohazard waste bags
- Bottles, glass
- Centricon-100 microconcentrators
- Cotton cloth, untreated (for preparing stains)
- Evidence bags or envelopes (for storage)
- Filter paper, Whatman 3M, Whatman #1
- Filtering flasks, at least 2 L (need two)
- Flasks, 10 mL to 1 L
- Forceps, metal
- Glasses, safety
- Graduated cylinders, 25 mL to 1 L
- Gloves, disposable latex
- Lab coat
- Lab marker, waterproof ink
- Microcentrifuge tubes, 0.5-mL and 1.5-mL
- Microcentrifuge tube racks
- Microcentrifuge decapping device
- Microscope slides and cover slips
- Paper, white
- Paper towels

- Parafilm
- Pasteur pipettes
- Pipettes, plastic, autoclaved disposable, to deliver 1–10 mL
- Pipette bulbs
- Pipette tips, sterile, disposable, filter-plugged
- Plastic container with tight fitting cover (for slides)
- Racks, to hold 15 mL and 50 mL tubes
- Scalpel with disposable blades
- Scissors
- Stir bars
- Timer, 60-minute
- Toothpicks
- Tube decapper
- Tube storage boxes
- Wash bottles

Preparation of Reagents

General Considerations

- Use the highest grade of reagents available.
- Prepare all solutions using glass-distilled or deionized water.
- Whenever possible, autoclave solutions that are used for sample preparation. Reagents that can be autoclaved are indicated.
- Follow safety recommendations provided by manufacturers for handling chemicals.

Reagent Preparation

0.5% w/v Crystal (methyl) Violet

Dissolve 0.5 g crystal violet in 100 mL glass-distilled or deionized water. Store at 2–6 °C.

Digest Buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 2% SDS, pH 7.5)

Mix together 1 mL of 1 M Tris-HCl, pH 7.5, 2 mL of 0.5 M EDTA, 1 mL of 5 M NaCl, 10 mL of 20% (w/v) SDS, and 86 mL glass-distilled or deionized water. Do not autoclave. Store at room temperature.

1 M Dithiothreitol (DTT), 10 mM Sodium Acetate, pH 5.2

Dissolve 0.77 g DTT in 5 mL glass-distilled or deionized water. Add 50 µL 1 M sodium acetate pH 5.2. Solution may be sterilized by filtration. Do not autoclave. Aliquot (recommended 1 mL) and store at –15 to –25 °C.

0.5 M EDTA, pH 8.0

Slowly add 186.1 g disodium ethylenediaminetetraacetate dihydrate ($Na_2EDTA•2H_2O$) to 800 mL glass-distilled or deionized water. Stir vigorously on a magnetic stirrer. Adjust to pH 8.0 by adding NaOH pellets (approximately 20 g).

Note EDTA will not go into solution without pH adjustment. Adjust final volume to 1 liter with glass-distilled or deionized water. Autoclave the solution or filter it through a 0.2-µm CN filter. Store at room temperature.

Gram's Iodine

Dissolve 1.0 g iodine and 2.0 g potassium iodide in 300 mL glass-distilled or deionized water. Aliquot in dropper bottle if desired. Store at 2–6 °C.

Nuclear Fast Red Stain

Heat 100 mL glass-distilled or deionized water to boiling. Dissolve 2.5 g aluminum sulfate in the 100 mL hot deionized water. Add 50 mg of Nuclear Fast Red. Stir until dissolved. Allow to cool. Filter through a Whatman #1 filter. Store at 2–6 °C.

1X PBS Buffer (Phosphate Buffered Saline), pH 7.4 (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4)

Dissolve 0.2 g KCl, 8.0 g NaCl, 0.2 g KH_2PO_4 and 2.2 g $Na_2HPO_4 \bullet 7H_2O$ (or 1.1 g Na_2HPO_4, anhydrous) in 800 mL glass-distilled or deionized water. Adjust the pH of the solution to 7.4 if necessary. Adjust to final volume of 1 L using glass-distilled or deionized water. Autoclave the solution. Store at 2–6 °C.

Purified Phenol

! WARNING ! CHEMICAL HAZARD. Phenol can cause severe burns. Chloroform is a carcinogen. Safety glasses and gloves should be worn when working with phenol or chloroform. If phenol solutions come in contact with skin or eyes, wash immediately with large volumes of water. Do not wash with ethanol.

Commercial, liquefied phenol can be used without redistillation if the phenol is colorless. Crystalline or liquefied phenol that is pink or yellow must be redistilled prior to use. Liquefied or redistilled phenol should be stored frozen at –15 to –25 °C in aliquots until needed. Crystalline phenol can be used if the crystals are white and the phenol is washed as described below.

To purify phenol:

Step	Action
1	Remove phenol aliquots from freezer as needed and allow to come to room temperature.
2	Liquefy by immersing in a water bath at 68 °C. Alternatively, liquefy crystalline phenol by mixing it with an equal volume of 1.0 M Tris-HCl, pH 8.0, and immersing container in a water bath at 68 °C.
3	Extract the liquefied phenol several times by adding an equal volume of 1.0 M Tris-HCl, pH 8.0.
4	Discard the upper aqueous phase into organic waste containers.
5	Repeat the extraction procedure until the pH of the aqueous phase is between 7.0 and 8.0.

To purify phenol: *(continued)*

Step	Action
6	After the final extraction, add an equal volume of TE Buffer. Store phenol for up to 3 months at 2–6 °C, protected from light in a brown glass bottle.

Buffered Phenol-Chloroform-Isoamyl Alcohol Solution

Mix 25 parts purified phenol, 24 parts chloroform, and one part isoamyl alcohol. Store phenol-chloroform for up to 2 months at 2–6 °C. Protect from light.

Picroindigocarmine Stain

To 100 mL of saturated solution of picric acid (1.3% w/v in water), add 0.33 g indigo carmine. Stir overnight at room temperature. Filter through a Whatman #1 filter. Store at 2–6 °C.

10 mg/mL Proteinase K

Dissolve 100 mg Proteinase K in 10 mL glass-distilled or deionized water. Aliquot solution (0.2 mL recommended) and store 0.2 mL aliquots frozen at –15 to –25 °C. Do not autoclave.

1 M Sodium Acetate, pH 5.2

Dissolve 13.6 g $CH_3COONa•3H_20$ in 80 mL glass-distilled or deionized water. Adjust to pH 5.2 by adding glacial acetic acid (approximately 2 mL). Adjust the final volume to 100 mL. Autoclave the solution. Store at room temperature.

5 M NaCl

Dissolve 292.2 g NaCl in 800 mL glass-distilled or deionized water. Adjust final volume to 1 L. Autoclave the solution. Store at room temperature.

20% (w/v) SDS

! WARNING ! CHEMICAL HAZARD. SDS is an irritant and a strong sensitizer. Avoid skin contact and inhalation. Wear a lab coat, gloves, and protective eyewear when handling SDS. Prepare SDS solutions in a well-ventilated area.

Slowly dissolve 200 g electrophoresis-grade (ultra-pure) sodium dodecyl sulfate in 800 mL glass-distilled or deionized water. To aid

dissolution, solution may be heated. Adjust volume to 1 L. Store at room temperature.

TE Buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)

Mix together 10 mL of 1 M Tris-HCl, pH 8.0, 0.2 mL of 0.5 M EDTA and 990 mL glass-distilled or deionized water. Aliquot (100 mL recommended) and autoclave the solutions. Store at room temperature.

1 M Tris-HCl, pH 7.5

! WARNING ! CHEMICAL HAZARD. Hydrochloric acid (HCl) causes severe burns and is irritating to the eyes. When preparing this reagent, use a fume hood and avoid inhalation and contact with the skin. Wear a lab coat, gloves, mask, and protective eyewear.

Dissolve 121.1 g Tris base in 800 mL glass-distilled or deionized water. Adjust to pH 7.5 at room temperature by adding concentrated HCl (approximately 65 mL).

Note Some types of electrodes do not measure the pH of Tris buffer accurately; be sure to obtain suitable electrodes that have no silver chloride for the Tris buffer pH adjustment.

Adjust the final volume to 1 L with glass-distilled or deionized water. Autoclave the solution. Store at room temperature.

1M Tris-HCl, pH 8.0

Dissolve 121.1 g Tris base in 800 mL glass-distilled or deionized water. Adjust to pH 8.0 at room temperature by adding concentrated HCl (approximately 45 mL). Adjust the final volume to 1 L with glass-distilled or deionized water. Autoclave the solution. Store at room temperature.

20%/5% Chelex Solution

The DNA Extraction Reagent available from Applied Biosystems (P/N N808-0087) is a 20% Chelex Solution that can be diluted to 5% with autoclaved deionized water.

Note When pipetting Chelex stock solutions, the resin beads must be distributed evenly in solutions; this can be achieved by gentle mixing with a stir bar in a beaker. Also, the pipette tip used for transfer must have a large bore. One mL pipette tips are adequate.

Gram-modified Christmas Tree Stain

Reagents for Staining

See “Preparation of Reagents” on page 3-38 (Luna; Oppitz, 1969).

- Crystal (methyl) Violet
- Gram's Iodine
- Nuclear Fast Red Stain
- Picroindigocarmine Stain

Staining Procedure

Steps 1 through 3 are provided for those who wish to perform microscopic detection of bacteria. If bacterial detection is not needed, proceed to step 4.

Step	Action
1	Wet the slide with deionized water.
2	Stain the cells with one drop of aqueous Crystal Violet for 1 minute. Rinse slide with deionized water.
3	Stain the cells with one drop of Gram's Iodine for 1 minute. Rinse slide with acetone and air dry.
4	Stain the cells with 1– 2 drops Nuclear Fast Red Stain for at least 15 minutes.
5	Wash the slide gently with deionized water until the Nuclear Fast Red stain washes off (about 5 seconds).
6	Stain the slide with one drop Picroindigocarmine Stain for 15–30 seconds. Rinse the slide with 100% ethanol (room temperature), then dry the slide in a 56 °C oven for 5 minutes.
7	Add one drop of Permount and cover with a cover slip. Examine the slide immediately at 400X magnification.
8	The epithelial cells will stain green with red nuclei. The sperm cells will stain red with green tails. The sperm head stains differentially such that the acrosomal cap stains pink and the sperm base stains red.

4 DNA Quantitation

Overview

Importance of Quantitation

The efficiency of a PCR amplification is influenced by the quality (degree of degradation), purity, and total quantity of DNA in a sample. Lack of amplification is usually due to highly degraded DNA, the presence of PCR inhibitors, insufficient DNA quantity, or any combination of these factors.

The QuantiBlot® Human DNA Quantitation Kit (P/N N808-0114) is an ideal method for accurate quantitation of human DNA (Walsh *et al.*, 1992). If the QuantiBlot kit determines that sufficient DNA is present in the extracted sample (greater than approximately 0.05 ng/µL concentration), then lack of amplification is most likely due to PCR inhibitors or severe degradation of the DNA.

Quantitation of samples determines if there is a sufficient amount of DNA present for amplification. Also, PCR inhibition can be minimized by adding the smallest volume of DNA extract necessary for successful amplification (volume containing approximately 1.0 ng). Lastly, by using the minimal volume of extracted DNA for PCR, the number of different genetic marker tests or repeat analyses that can be performed is maximized.

DNA quantitation is particularly important for AmpFℓSTR® Profiler Plus™ amplifications, where optimal results are obtained using a range of 1.0–2.5 ng of input DNA. Adding greater than 2.5 ng of DNA can result in too much PCR product, such that the dynamic range of the instrument used to detect and analyze the PCR product is exceeded.

See “Off-scale Data” on page 6-15 and “Effect of DNA Quantity on Results” on page 9-33 for more details.

Analysis by the QuantiBlot Kit Method

How the Kit Works

The QuantiBlot Human DNA Quantitation Kit method of DNA quantitation is based on probe hybridization to the human alpha satellite locus, D17Z1. A biotinylated probe specific for the D17Z1 sequence is hybridized to sample DNA that has been immobilized via slot blot onto a nylon membrane.

The subsequent binding of horseradish peroxidase/streptavidin enzyme conjugate (HRP-SA) to the bound probe allows for either colorimetric or chemiluminescent detection. In the case of colorimetric detection, the oxidation of 3,3´,5,5´-tetramethylbenzidine (TMB) catalyzed by HRP-SA results in the formation of a blue precipitate directly on the nylon membrane.

For chemiluminescent detection, the oxidation of a luminol-based reagent catalyzed by HRP-SA results in the emission of photons that are detected on standard autoradiography film. This process is called enhanced chemiluminescence (ECL).

In both cases, the quantity of sample DNA is determined by comparison of the sample signal intensity to human DNA standards that have been calibrated against two DNA controls of known quantity.

The colorimetric method allows for detection and quantitation down to 150 pg. The chemiluminescent method can detect 150 pg with a 15-minute exposure to film and can detect as little as 20 pg with longer film exposures (3 hours to overnight). An example of QuantiBlot Kit results obtained from various biological samples is shown in Figure 4-1 on page 4-3.

Note For specific procedures, refer to the QuantiBlot Human DNA Quantitation Kit product insert.

Specificity for Primate DNA

One significant advantage of the QuantiBlot Human DNA Quantitation Kit method is that the probe is highly specific for human/primate DNA. When tested, 300 ng quantities of several non-primate DNA samples (*E. coli*, yeast, dog, cat, mouse, rat, pig, cow, chicken, fish, and turkey) were found to give either no signals or signals that were less than or equal to that obtained for 0.15 ng of human DNA. This high degree of specificity for human/primate DNA allows for the accurate quantitation of target human DNA in samples that also contain significant amounts of microbial or other non-primate DNA.

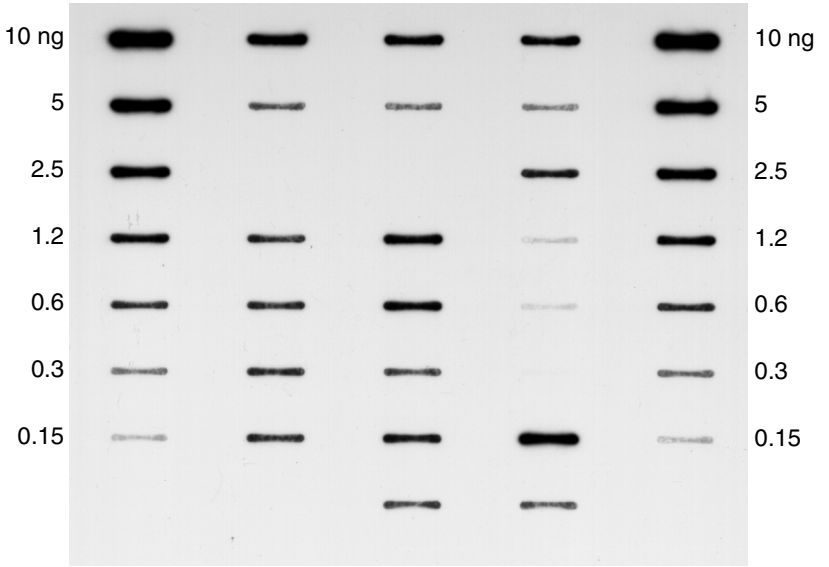


Figure 4-1 QuantiBlot Human DNA Quantitation Kit results (ECL detection)

Single-stranded and Degraded DNAs

Another advantage of the QuantiBlot kit method is that single-stranded and/or non-purified DNA samples can be quantitated. DNA samples extracted using the Chelex method can be quantitated, as can those extracted by other methods, including phenol-chloroform, salting out, and binding to silica particles.

Degraded DNA gives the same results as fully intact DNA over a wide range of average DNA sizes. However, DNA quantity can be underestimated when the DNA is extremely degraded. The results of one experiment, for example, indicated that the signal obtained for DNA degraded to an average size of 500–2000 bp was about half of the expected intensity.

Extremely degraded DNA usually amplifies less efficiently than intact DNA, so a greater quantity of degraded DNA may be required to give the same results as intact DNA. Underestimating DNA quantity should make PCR success more likely.

Commonly Asked Questions about QuantiBlot

How Much DNA?

How much of the DNA extract should be added to the amplification reaction if a sample gives no signal for the QuantiBlot assay?

As an example, assume that 5 µL of the DNA extract is spotted, and the lowest DNA standard tested is 150 pg. So, the DNA concentration in the sample must be less than 150 pg/5 µL or 30 pg/µL. The quantity of DNA in 20 µL of extract, which is the maximum that can be added to an AmpFℓSTR Profiler Plus PCR, would therefore be less than 0.6 ng.

The possible approaches that can be taken for such a sample include the following:

- Attempt amplification using 20 µL of the extract.
- Concentrate the sample to a smaller volume using a Centricon-100 before amplification. See Chapter 3, “DNA Extraction Protocols.”

Multiple Film Exposures

Is it possible to perform multiple film exposures with the ECL detection method?

Yes. In fact, a wise strategy is to perform a 15-minute film exposure first, which gives sensitivity down to at least 150 pg. Then place the film on the membrane for 3 hours or as long as overnight. The longer exposure will give sensitivity down to about 20 pg.

The photon emission kinetics of ECL are such that many exposures can be taken in a relatively short period of time. The light output is the greatest in the first hour, gradually decreasing over the next several hours with a half-life of about 60 minutes. The results of one experiment, for example, indicated that six exposures could be taken in the first 2.5 hours of photon emission, with each exposure detecting 80–150 pg of DNA. A seventh exposure with the film on the membrane overnight was easily able to detect the 80 pg DNA sample.

Sometimes it is beneficial to perform a very short exposure (about 5 minutes) to facilitate quantitation of samples having intense signals in the range of 5–10 ng DNA.

Repeating the Assay

Can the probe be stripped off the membrane so that the QuantiBlot assay can be repeated if a mistake is made during the hybridization/detection steps?

Yes, for the ECL method. This protocol can be used with the TMB method only if no blue precipitate was deposited on the membrane.

The procedure is as follows:

Step	Action
1	Heat 150 mL of the Wash Solution (1.5X SSPE, 0.5% SDS) to approximately 90 °C in a glass bowl.
2	Take the Wash Solution off the heat source and place the nylon QuantiBlot membrane (containing the spotted samples) into the solution.
3	Rotate on an orbital shaker at room temperature for 20 minutes.
4	Remove the membrane from the Wash Solution. **IMPORTANT** Do not let the QuantiBlot membrane dry out at any time.
5	Begin the QuantiBlot protocol starting at the hybridization step (refer to the QuantiBlot Human DNA Quantitation Kit product insert).

Performing Hybridization and Detection Later

Is it possible to spot the samples onto the membrane and then perform the hybridization and detection steps at a later time?

Yes. Proceed as follows:

Step	Action
1	Immediately after spotting the samples onto the membrane, place the membrane in 100 mL of 5X SSPE (without SDS).
2	Store at 2–6 °C protected from light.
3	Resume the protocol beginning with the pre-hybridization step (Section 4.1 in the QuantiBlot Human DNA Quantitation Kit product insert). For best sensitivity, resume the protocol within 24 hours.

AmpFℓSTR Profiler Plus Amplification

5

Amplification Protocol

Overview The AmpFℓSTR® Profiler Plus™ PCR Amplification Kit contains all the reagents necessary to amplify the D3S1358, vWA, FGA, amelogenin, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820 loci in sample DNA, following the protocol described in this section.

Thermal Cycler Parameters The following parameters are used to amplify the AmpFℓSTR Profiler Plus loci on Applied Biosystems thermal cyclers.

Table 5-1 Thermal cycling times and temperatures

GeneAmp® PCR Instrument System	Tube Type	Times & Temperatures for this Kit					
		Initial Incubation Step	Each of 28 Cycles			Final Extension	Final Step
			Melt	Anneal	Extend		
DNA Thermal Cycler 480	GeneAmp Thin-Walled Reaction	STEP CYCLE	STEP CYCLE			TIME DELAY	SOAK
		95 °C 11 min. 1 cycle	94 °C 1 min.	59 °C 1 min.	72 °C 1 min.	60 °C 45 min.	25 °C (forever)
GeneAmp PCR System 9600	MicroAmp® Reaction with Cap	HOLD	CYCLE			HOLD	HOLD
		95 °C 11 min.	94 °C 1 min.	59 °C 1 min.	72 °C 1 min.	60 °C 45 min.	25 °C (forever)
GeneAmp PCR System 2400	MicroAmp Reaction with Cap	HOLD	CYCLE			HOLD	HOLD
		95 °C 11 min.	94 °C 1 min.	59 °C 1 min.	72 °C 1 min.	60 °C 45 min.	25 °C (forever)

Note If the amplified products will be left in the thermal cycler for more than 18 hours, the final step should be a SOAK/HOLD at 10 °C forever.

Amplification

Use the following procedure to amplify the AmpFℓSTR Profiler Plus loci.

IMPORTANT The fluorescent dyes attached to the primers are light-sensitive. Store the AmpFℓSTR Profiler Plus Primer Set protected from light while not in use. Amplified DNAs should also be protected from light.

To perform amplification:

Step	Action
1	Program the appropriate GeneAmp PCR Instrument System with the parameters shown in Table 5-1 on page 5-1. **IMPORTANT** Do not omit the initial incubation or final extension step!
2	Place the required number of PCR tubes into a rack and label them. Be sure to include the positive and negative control tubes. Use 0.5-mL GeneAmp Thin-Walled Reaction Tubes in the DNA Thermal Cycler 480 and 0.2-mL MicroAmp Reaction Tubes with Caps in the GeneAmp PCR Systems 9600 and 2400.
3	Vortex the AmpFℓSTR® PCR Reaction Mix, AmpFℓSTR Profiler Plus Primer Set, and AmpliTaq Gold® DNA Polymerase for 5 seconds. Spin the tubes briefly in a microcentrifuge to remove any liquid from the caps.
4	Prepare the AmpFℓSTR Profiler Plus Master Mix by adding the following volumes of reagents to a 1.5-mL microcentrifuge tube: ♦ Number of samples × 21.0 µL of AmpFℓSTR PCR Reaction Mix ♦ Number of samples × 1.0 µL of AmpliTaq Gold DNA Polymerase ♦ Number of samples × 11.0 µL of AmpFℓSTR Profiler Plus Primer Set **Note** The above formulation provides a slight overfill to allow for volume lost in pipetting. The maximum volume of Master Mix held in a 1.5-mL microcentrifuge tube can be dispensed into 42 PCR tubes. A 2.0-mL tube is recommended when preparing Master Mix for up to 55 samples.
5	Mix thoroughly by vortexing at medium speed for 5 seconds.
6	Spin the tube briefly in a microcentrifuge to remove any liquid from the cap.
7	Dispense 30 µL of Master Mix into each PCR tube.

To perform amplification: *(continued)*

Step	Action	
8	Add mineral oil as follows:	
	IF using the...	**THEN...**
	DNA Thermal Cycler 480	Add one drop mineral oil to the GeneAmp tubes.
	GeneAmp PCR System 9600	Do not use mineral oil.
	GeneAmp PCR System 2400	Do not use mineral oil.
9	Add sample as follows:	
	IF preparing...	**THEN...**
	DNA Test Sample Tubes, AND...	
	sample DNA concentration ð0.125 ng/µL	♦ Add 20 µL of sample to the PCR tube.
	sample DNA concentration >0.125 ng/µL	♦ Dilute a portion of the sample with **TE buffer** (see page 5-6 for preparation) so that only 1.0–2.5 ng of DNA is in a volume of 20 µL (final sample concentration 0.05–0.125 ng/µL). ♦ Add 20 µL of diluted sample to the PCR tube.
	Positive Control Tube	♦ Vortex the AmpFℓSTR® Control DNA 9947A tube (0.10 ng/µL). ♦ Spin the tube briefly in a microcentrifuge to remove any liquid from the cap. ♦ Add 20 µL (2 ng) of AmpFℓSTR Control DNA 9947A to the labeled Positive Control Tube.
	Negative Control Tube	♦ Add 20 µL of **TE buffer** (see page 5-6 for preparation) to the labeled Negative Control Tube.
	Note The final volume in each PCR tube is 50 µL.	

To perform amplification: *(continued)*

Step	Action
10	Place the PCR tubes in the GeneAmp PCR Instrument System and start the program. If performing PCR with the GeneAmp PCR System 9600 or 2400, use the MicroAmp tray and heated cover.
11	After the amplification process, remove the tubes from the instrument block and store the amplified products protected from light. The amplified products can be stored at 2–6 °C for short periods of time (less than 2 weeks). For longer periods, store the tubes at –15 to –25 °C.

Dedicated Equipment and Supplies

Equipment Required

IMPORTANT These items should never leave the PCR Setup Work Area.

- Calculator
- Gloves, disposable
- Marker pen, permanent
- Microcentrifuge
- Microcentrifuge tubes, 1.5-mL (for Master Mix preparation)
- Microcentrifuge tube rack
- Pipet tips, sterile, disposable hydrophobic filter-plugged
- Pipettors, adjustable 0.5–10 µL, 2–20 µL, 20–200 µL, and 200–1000 µL
- Tube decapper, autoclavable
- Vortexer

GeneAmp PCR Instrument Systems and Accessories

IMPORTANT The GeneAmp PCR Instrument Systems should be placed in the Amplified DNA Work Area.

- DNA Thermal Cycler 480 (P/N N801-0100, N801-0101, N801-0102)
- DNA Thermal Cycler 480 Accessories:
  - GeneAmp Autoclaved Thin-Walled Reaction Tubes (P/N N801-0611)
  - GeneAmp Thin-Walled Reaction Tubes (P/N N801-0537)
- GeneAmp PCR System 9600 (P/N N801-0001, N801-0002, N801-0003)
- GeneAmp PCR System 9600 Accessories:
  - MicroAmp Autoclaved Reaction Tubes with Caps (P/N N801-0612)
  - MicroAmp Reaction Tubes with Caps (P/N N801-0540)
  - MicroAmp 9600 Base (P/N N801-0531)
  - MicroAmp 9600 Tray (P/N N801-0532)

- GeneAmp PCR System 2400 (P/N N803-0001, N803-0002, N803-0003)
- GeneAmp PCR System 2400 Accessories:
  - MicroAmp Autoclaved Reaction Tubes with Caps (P/N N801-0612)
  - MicroAmp Reaction Tubes with Caps (P/N N801-0540)
  - MicroAmp 2400 Base (P/N N801-5531)
  - MicroAmp 2400 Tray (P/N N801-5532)

Reagents Required

- AmpF*ℓ*STR Profiler Plus PCR Amplification Kit (P/N 4303326)
- TE Buffer

Preparation of Required Reagents

TE Buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)

Mix together 10 mL of 1 M Tris-HCl, pH 8.0, 0.2 mL of 0.5 M EDTA and 990 mL glass-distilled or deionized water. Aliquot (100 mL recommended) and autoclave the solutions. Store at room temperature.

Multicomponent Analysis

6

Overview

In This Section

This chapter describes the various steps necessary to achieve proper multicomponent analysis with AmpFℓSTR® Profiler Plus™ PCR products:

Topic	See page
Introduction	6-1
Matrix Files	6-2
Using Filter Set F Module Files	6-4
Making a Matrix File on the ABI PRISM 310	6-6
Making a Matrix File on the ABI PRISM 377 and ABI PRISM 377 with XL Upgrade	6-8
Examining Data	6-12
Off-scale Data	6-15

Introduction

Multicomponent analysis is the process that separates the four different fluorescent dye colors into distinct spectral components. The three fluorescent dyes used in the AmpFℓSTR Profiler Plus PCR Amplification Kit are the NHS-ester dyes 5-FAM, JOE and NED. The fourth dye, ROX, is used for the GeneScan-500 Internal Lane Size Standard.

Each of these fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection on the ABI PRISM® 310 and ABI PRISM® 377 instruments, the fluorescent signals are separated by a diffraction grating according to their wavelengths and projected onto a CCD camera in a predictably spaced pattern. 5-FAM emits at the shortest wavelength and is detected as blue, followed by JOE (green), NED (yellow) and ROX (red), listed in order of increasing wavelength.

Although each of these dyes emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra between the four dyes (Figure 6-1). The goal of multicomponent analysis is to isolate the signal from each dye so that, for example, signals from 5-FAM labeled products are displayed in the electropherogram for blue, but not in those for green, yellow or red.

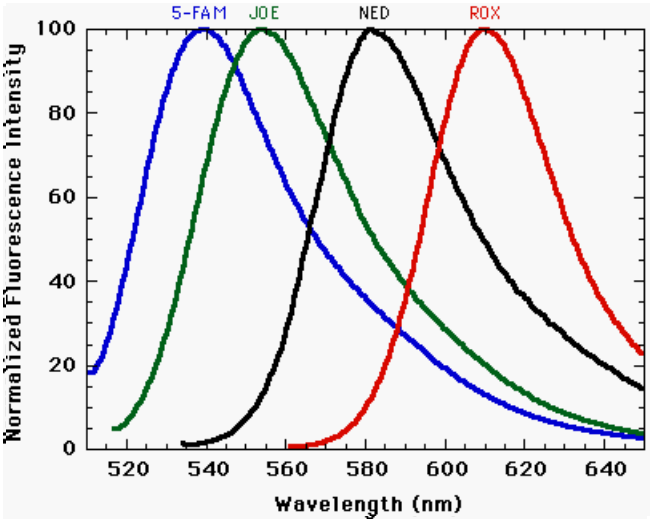


Figure 6-1 Emission spectra of the dyes used in the AmpFℓSTR Profiler Plus kit

Matrix Files

The precise spectral overlap between the four dyes is measured by analyzing DNA fragments labeled with each of the dyes (5-FAM, JOE, NED, or ROX) in separate lanes of a gel or in separate injections on a capillary. These dye-labeled DNA fragments are called matrix standard samples.

The GeneScan® Analysis Software then analyzes the data from each of these four samples and creates a matrix file. The matrix file contains a table of numbers with four columns and four rows. These numbers are normalized fluorescence intensities and represent a mathematical description of the spectral overlap that is observed between the four dyes (Figure 6-2 on page 6-3).

The rows in the matrix file table represent the virtual filters (see page 6-4) and the columns represent the dye-labeled PCR products, indicated as “Reactions.” The top lefthand value, 1.0000, represents the

normalized fluorescence of blue (5-FAM-labeled) PCR products in the blue filter. It follows that all matrix tables should have values of 1.0 on the diagonal from top left to bottom right, as shown in Figure 6-2.

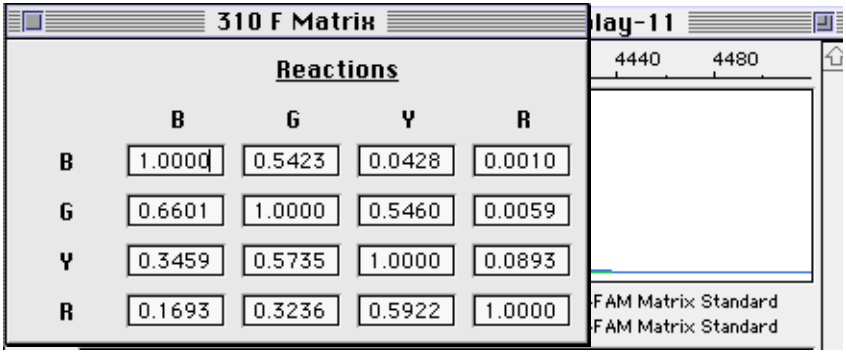


Figure 6-2 Matrix file table, indicating the values obtained on a particular ABI PRISM 310 instrument

The other values in the table should all be less than 1.0. These values represent the amount of spectral overlap observed for each dye in each virtual filter. For example, the values in the first column reflect quantitatively the amount of blue dye detected in each virtual filter. This is shown in Figure 6-1 on page 6-2 by the characteristic tailing emission of 5-FAM with increasing wavelength.

These matrix file values will vary between different instruments, virtual filter sets, and run conditions on a single instrument. A matrix file must be made for each instrument and for a particular set of run conditions.

The appropriate matrix file can be applied to data on subsequent capillary runs or gels on the same instrument, as long as the gel formulation and running conditions are constant from run to run. This is because the spectral overlap between the four dyes is very reproducible under constant run conditions.

Multicomponent analysis is accomplished automatically by the GeneScan Analysis Software, which applies a mathematical matrix calculation (using the values in the matrix file) to all sample data.

Using Filter Set F Module Files

Overview

The ABI PRISM Collection Software collects light intensities from four specific areas on the CCD camera, each area corresponding to the emission wavelength of a particular fluorescent dye. Each of these areas on the CCD camera is referred to as a “virtual” filter, since no physical filtering hardware (like band-pass glass filters) is used.

The information that specifies the appropriate virtual filter settings for a particular set of fluorescent dyes is contained in each appropriate ABI PRISM Collection Software module file. The module files that must be installed and used for the dye set 5-FAM/JOE/NED/ROX on the ABI PRISM 310 Genetic Analyzer, ABI PRISM 377 DNA Sequencer, and ABI PRISM 377 DNA Sequencer with XL Upgrade are the following:

Table 6-1 Filter Set F Module Files

Instrument	Configuration	Module File(s)
ABI PRISM 310	POP-4™ polymer with 1-mL syringe	GS STR POP4 (1 mL) F
ABI PRISM 377 and ABI PRISM 377 with XL Upgrade	36-cm well-to-read 5% Long Ranger gel	Plate Check F GS PR 36F-2400 GS Run 36F-2400

Note AmpFℓSTR Profiler Plus detection is not supported on the ABI™ 373 or ABI 373 with XL Upgrade platforms, as the appropriate band-pass glass filter for the NED dye is not included in the filter wheel.

IMPORTANT Filter Set F module files must be installed on the instrument’s computer before making a matrix file using the 5-FAM, JOE, NED, and ROX matrix standards. Filter Set F module files must also be used on all subsequent runs. Samples that are run on a gel or capillary using Filter Set F must be analyzed using a matrix file that was created using Filter Set F.

Using Filter Set F Module Files

ABI PRISM 310

In the ABI PRISM 310 Collection Software Injection List, choose GS STR POP4 (1 mL) F in the Module pop-up window for each sample.

Note See Chapter 8 for more information.

ABI PRISM 377

Choose the following Filter Set F module files in the ABI PRISM 377 or ABI PRISM 377XL Collection Software Run window when running a gel:

- In the Plate Check Module pop-up menu, choose Plate Check F.
- In the Pre-Run Module pop-up menu, choose GS PR 36F-2400.
- In the Run Module pop-up menu, choose GS Run 36F-2400.

Note See Chapter 7 for more information.

IMPORTANT PCR products from the AmpFℓSTR® Blue and AmpFℓSTR® Green kits (labeled with 5-FAM and JOE, respectively) can be run using Filter Set F or Filter Set A module files. Filter Set F module files must be used when running PCR products that include the NED dye, *i.e.*, those from the AmpFℓSTR® Profiler™ and AmpFℓSTR Profiler Plus PCR Amplification Kits.

Making a Matrix File

Matrix Standards

- Three of the matrix standards used (5-FAM, JOE, and ROX) are found in the Dye Primer Matrix Standards Kit (P/N 401114).

IMPORTANT Do not use the TAMRA matrix standard provided in this kit.

- The fourth is the NED Matrix Standard (P/N 402996).

Making a Matrix File on the ABI PRISM 310

The four matrix standard samples are 5-FAM, JOE, NED, and ROX (see above).

To make the matrix file:

Step	Action
1	Combine 1 µL of each matrix standard with 25 µL of deionized formamide. Prepare one tube for each matrix standard sample. **IMPORTANT** Do not include the GeneScan-500 [ROX] size standard in the samples.
2	Denature the samples at 95 °C for 3 minutes, then quick chill on ice. Place tubes in the 48-well sample tray.
3	Launch the ABI PRISM 310 Collection application.
4	Under the File menu, select New and click the GeneScan Smpl Sheet 48 Tube icon.
5	Complete the sample sheet as described in Chapter 4 of the ABI PRISM 310 *Genetic Analyzer User's Manual*. Enter the sample names/numbers for each row in the Sample Name column. This will identify which sample is in which tube of the sample tray.
6	Under the File menu, select New and click the GeneScan Injection List icon.
7	a. In the Injection List, select the appropriate sample sheet from the Sample Sheet pop-up menu. b. From the Module pop-up menu, choose GS STR POP4 (1 mL) F for every injection. c. Choose None in the Matrix File column for each matrix standard sample.
8	Click Start.

To make the matrix file: *(continued)*

Step	Action
9	When the injections are done, follow these steps in the GeneScan Analysis Software: a. Under the File menu, select New. b. Click the Matrix icon. In the window that appears, indicate the sample files that correspond to each matrix standard dye color. c. Select starting scan numbers of 3300 for each sample. This starting scan number is intended to exclude the primer peaks. d. Enter a value of 2500 Points. e. Click OK. The computer makes the matrix and the matrix file table appears.
10	Save the matrix file in the ABI folder within the System folder.

To verify the accuracy of the matrix file:

Step	Action
1	Apply the new matrix file to the Matrix Standard Sample Files as follows: a. In the Analysis Control window, highlight the Sample File column by clicking in the Sample File title row. b. Under the Sample menu, select Install New Matrix… c. Choose the new matrix file (located in the ABI folder within the System folder) and click Open.
2	Analyze the matrix standard samples as follows: a. Under the Settings menu, select Analysis Parameters, and verify that the settings are as shown in Figure 8-3 on page 8-9. b. In the Analysis Control window, select all four colors in each sample row for all of the matrix standard samples. c. Click the Analyze button.
3	a. In the Results Control window, examine the results for all four colors for each of the matrix standard samples. The 5-FAM matrix standard results should have peaks for Blue. The other colors for this injection should have relatively flat baselines. A pattern of pronounced peaks or dips in any of the other three colors indicates that the color separation is not optimal. b. Examine the results for each matrix standard sample in this way (see “Examining Data” on page 6-12 for examples of accurate and inaccurate spectral separation).

To verify the accuracy of the matrix file: *(continued)*

Step	Action
4	If this verification test fails, then the capillary may not have been aligned properly in the instrument during the run. a. Repeat the experiment, making sure that the capillary is placed carefully in the laser detection window. b. Tape the capillary to the heat plate so that the capillary is immobilized during the run. If the problem persists, laser alignment on the instrument may require optimization. Call technical support.

Once a satisfactory matrix file has been made, this matrix file can be applied to subsequent runs. It is not necessary to run matrix standard samples for each new capillary.

Making a Matrix File on the ABI PRISM 377 and ABI PRISM 377 with XL Upgrade

The four matrix standards used are 5-FAM, JOE, NED, and ROX. (See "Matrix Standards" on page 6-6.)

To make the matrix file:

Step	Action
1	Prepare a 36-cm well-to-read gel and set up the instrument as described on pages 7-6 through 7-13 with the following exceptions: a. In step 6a of "To create a Run file:" on page 7-10, choose None in the Gel's Matrix File pop-up menu. b. In step 6b on page 7-10, in the Sample Sheet area of the Run window, choose None in the Matrix File column for each matrix standard sample.
2	Combine 2.5 µL of each matrix standard with 2.5 µL of deionized formamide. Prepare a separate tube for each matrix standard sample. **IMPORTANT** Do not include the GeneScan-500 [ROX] size standard in the samples.
3	Denature the samples at 95 °C for 2 minutes, then quick chill on ice.

To make the matrix file: *(continued)*

Step	Action
4	Load 1.5 µL of each sample on an ABI PRISM 377 gel or 1.0 µL on an ABI PRISM 377 with XL Upgrade gel: **IMPORTANT** Leave a one-lane space between samples, because there must be no spillover between lanes of matrix standard samples. a. Load a set of four matrix standard samples on the left side of the gel (*e.g.*, lanes 2, 4, 6, and 8). b. Load a set of four matrix standard samples on the right side of the gel (*e.g.*, lanes 27, 29, 31, and 33).
5	Follow the sample electrophoresis instructions on page 7-15.
6	After the gel is run and the gel image is opened, track the lanes of the matrix standard samples.
7	Follow these steps in the GeneScan Analysis Software: a. Under the Gel menu, select Extract Lanes… Fill in the dialog box as shown in Figure 7-4 on page 7-17. b. Under the File menu, select New. c. Click the Matrix icon. In the window that appears, indicate the sample files that correspond to each matrix standard dye color. d. Select starting scan numbers of 1400 for each sample. This starting scan number is intended to exclude the primer peaks. e. Enter a value of 3000 Points. f. Click OK. The computer makes the matrix and the matrix file table appears.
8	Make two matrix files this way, one from the matrix standard samples on the left side of the gel, and one from those on the right side of the gel.
9	Save the matrix files in the ABI folder within the System folder.

To check the matrix files against each other:

Step	Action
1	In the GeneScan Analysis Software, choose Open under the File menu.
2	Click the Matrix icon, and open the left matrix file.
3	Repeat steps 1 and 2 to open the right matrix file.
4	The corresponding values between the two tables should agree to within 0.02 units. If so, proceed with "To verify the accuracy of the matrix file:" If not, see the recommendations on page 6-11.

To verify the accuracy of the matrix file:

Step	Action
1	Apply one of the new matrix files to the Matrix Standard Sample Files from the same gel as follows: a. In the Analysis Control window, highlight the Sample File column by clicking in the Sample File title row. b. Under the Sample menu, select Install New Matrix… c. Choose the new matrix file (located in the ABI folder within the System folder) and click Open.
2	Analyze the matrix standard samples as follows: a. Under the Settings menu, select Analysis Parameters, and verify that the settings are as shown in Figure 7-2 on page 7-7. b. In the Analysis Control window, select all four colors in each sample row for all of the matrix standard samples. c. Click the Analyze button.
3	a. In the Results Control window, examine the results for all four colors for each of the matrix standard samples. The 5-FAM matrix standard results should have peaks for Blue. The other colors for this lane should have relatively flat baselines. A pattern of pronounced peaks or dips in any of the other three colors indicates that the color separation is not optimal. b. Examine the results for each matrix standard sample in this way (see "Examining Data" on page 6-12).

Troubleshooting Matrix Files on the ABI PRISM 377 and ABI PRISM 377 with XL Upgrade

If the left and right matrix files do not match within 0.02 units, or if the verification test fails, then the gel cassette may not have been aligned properly during the experiment. Repeat the experiment, paying close attention to the following:

- When placing the gel plates with the cassette in the instrument, make sure that the cassette is securely in place.
- Make sure that the cassette is aligned flat against the back plate of the instrument.
- Check that the alignment pins are firmly touching the spacers near the bottom of the glass plates. This alignment is very important for making a robust matrix.
- There must be no dried buffer crust on the alignment pins, and the spacers that contact the pins must be clean and free of acrylamide and urea.

If poor spectral resolution persists, laser alignment on the instrument may require optimization. Call technical support.

Once a satisfactory matrix file has been made, it is not necessary to run matrix standard samples on every gel. Choose either the left or right matrix file to be applied to all subsequent gel runs on the same instrument that use the same gel mixture, well-to-read length, and electrophoresis conditions.

We recommend making a unique matrix file for each gel cassette to be used on the instrument (if more than one is used).

Examining Data

Two peaks of each of the four matrix standard samples are shown in Figure 6-3 with no matrix file applied to the analyzed data. All colors are displayed in the electropherogram for each matrix standard. Note that green, yellow, and red peaks are present under the blue peaks in the 5-FAM matrix standard sample (first electropherogram panel). Multiple colors are also present for the other matrix standard samples.

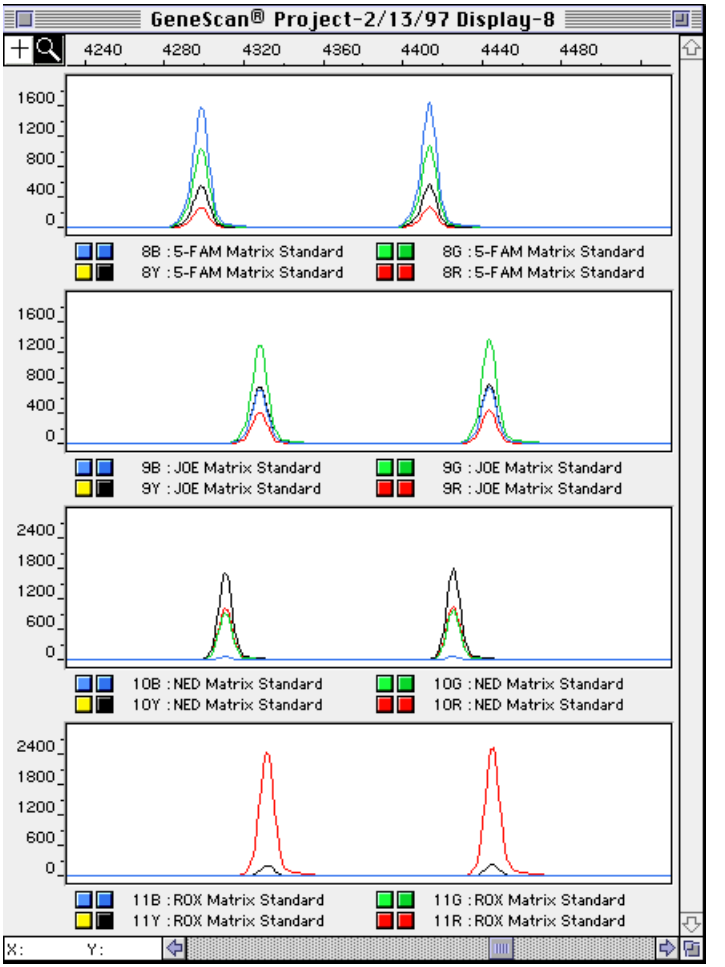


Figure 6-3 No matrix file applied

The matrix standard samples shown in Figure 6-4 on page 6-13 are the same as those in Figure 6-3, but with an accurate matrix file applied to

the analyzed data. Note that each matrix standard sample contains peaks of only the correct color (*e.g.*, the 5-FAM matrix standard sample has only blue peaks).

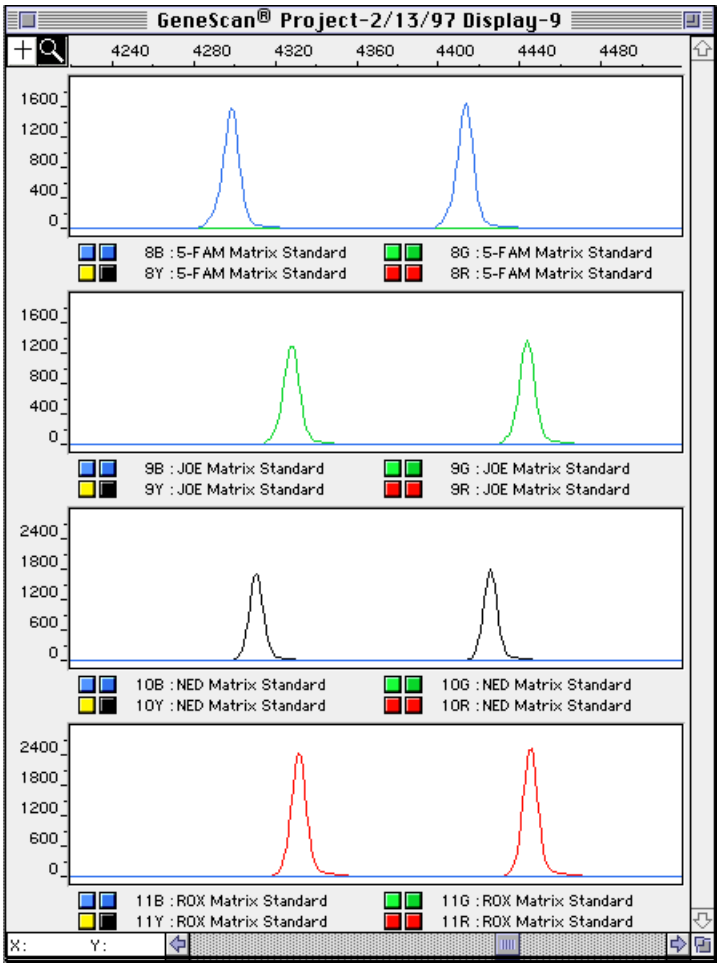


Figure 6-4 Accurate matrix file applied

The matrix standard samples shown in Figure 6-5 on page 6-14 are the same as those in Figures 6-3 and 6-4, but with an inaccurate matrix file applied to the analyzed data. (The matrix file is actually from a different instrument.) The data in Figure 6-5 gives several indications that the matrix file is inaccurate. For example, the 5-FAM matrix standard sample has small green peaks under the blue peaks. This situation is referred to as “pull-up,” because the 5-FAM (blue) signal is “pulling up”

peaks in the green data. Likewise, the second electropherogram panel shows pull-up of blue peaks under the green peaks.

Another indication that the matrix file in Figure 6-5 on page 6-14 is inaccurate is the raised baseline for some colors and dips in the raised baseline that correspond to peaks.

Note Pull-up and raised baselines are also observed under peaks that are off-scale, even when an accurate matrix file is applied (see page 6-15).

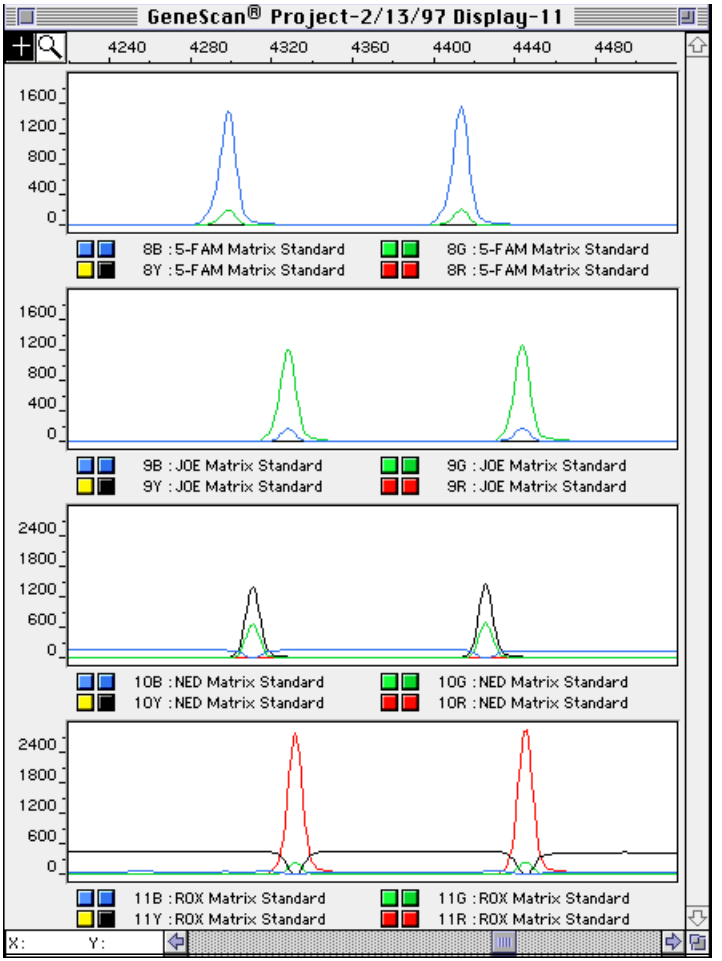


Figure 6-5 Inaccurate matrix file applied

Off-scale Data

Overview

If too much sample DNA is added to the PCR reaction mixtures, the fluorescence intensity from the PCR products may exceed the linear dynamic range for detection by the instrument. This is referred to as “off-scale” data. Multicomponent analysis cannot be performed accurately on data that is off-scale. Samples with off-scale peaks will exhibit raised baselines and/or excessive “pull-up” of one or more colors under the off-scale peaks (see Figure 6-5 on page 6-14).

Analyzed data from off-scale peaks should not be used for quantitative comparisons. For example, the stutter peak that corresponds to an off-scale main peak is likely to be overestimated.

Note Samples with off-scale data should be treated and re-run as described on page 9-23.

The following protocols can be used to determine if any of the peaks in a sample is off-scale.

Off-scale Data on the ABI PRISM 310

To determine if data is off-scale on the ABI PRISM 310:

Step	Action
1	In the GeneScan Analysis Software, highlight the sample file row for the questionable sample in the Analysis Control window.
2	Under the Sample menu, choose Raw Data (or type C R on the keyboard).
3	Examine the fluorescence intensity for the raw data peaks. Any peaks that are greater than 8100 relative fluorescence units (RFU) are off-scale.

Off-scale Data on the ABI PRISM 377 or ABI PRISM 377 with XL Upgrade

The vertical divisions across the read region of a gel where the ABI PRISM 377 or ABI PRISM 377XL Collection Software collects fluorescence signals are referred to as “channels.” One lane of a gel is typically 3–5 channels wide (depending on comb tooth size and scan mode—Full Scan or XL Scan).

When the lanes are tracked in the GeneScan Analysis Software, the tracker line for each lane is placed in the channel showing the strongest fluorescent signal (usually the center portion of the band in the horizontal dimension). The data in the center channel and the adjacent channels on each side (total of three channels) is averaged to determine the raw data for the sample file.

Any peak in the three-channel averaged raw data with a fluorescence intensity greater than approximately 5000 RFU may have off-scale data in one or more of the three channels.

To access the three-channel averaged data:

Step	Action
1	In the GeneScan Analysis Software, highlight the sample file row for the questionable sample in the Analysis Control window.
2	Under the Sample menu, choose Raw Data (or type c R on the keyboard).

ABI PRISM 377 DNA Sequencer Protocols

7

Overview

About This Section

AmpFℓSTR® Profiler Plus™ PCR products are run on 36-cm well-to-read plates on either the ABI PRISM® 377 DNA Sequencer or the ABI PRISM 377 DNA Sequencer with XL Upgrade ("ABI PRISM 377XL"). Protocols for both configurations are included in this chapter.

This chapter was written for use with GeneScan® Analysis 2.1 Software. Refer to the *ABI PRISM 377 DNA Sequencer User's Manual* and *GeneScan Analysis 2.1 User's Manual* for more detailed information on the instrument and software used with these protocols.

GeneScan Analysis 2.0 Software can also be used for analyzing AmpFℓSTR Profiler Plus PCR products. Refer to the *GeneScan Analysis Software User's Manual* for details on using this version of the software.

IMPORTANT Filter Set F must be used for data collection of AmpFℓSTR Profiler Plus PCR products. Filter Set F module files must be installed in the Modules folder within the ABI PRISM 377 or ABI PRISM 377XL folder on the instrument's Macintosh® computer before following the protocols in this chapter (see page 6-4).

Additionally, before running AmpFℓSTR Profiler Plus PCR products on the instrument, a matrix file must be made using the 5-FAM, JOE, NED, and ROX matrix standards and Filter Set F. Refer to Chapter 6, "Multicomponent Analysis," for information on how to do this.

Preparing 36-cm Well-to-Read Gels

Preparing Plates for the ABI PRISM 377

To prepare 36-cm well-to-read plates for the ABI PRISM 377:

Step	Action
1	Clean 36-cm well-to-read gel plates with Alconox detergent using a dedicated wash cloth or Kimwipe tissues. Rinse with deionized water and air dry.
2	Place the larger, unnotched plate with the center etch facing down on a covered benchtop. The narrow width is the bottom portion of the plate.
3	Place 0.2-mm spacers on either side of plate, with notched end of spacer at the top of the plate and the notch facing the center of the plate.
4	Place notched (“rabbit eared”) plate over spacers, making sure that the side that had been previously in contact with the silicone rubber gasket (of the upper buffer chamber) is external. This is the side with the etched writing on it.
5	Clamp plates together using four medium binder clips per side. Place the clamps directly over the spacers.
6	Elevate top of plates approximately 1.0 cm. A pipet tip box top is ideal for this purpose.

Preparing Plates for the ABI PRISM 377 with XL Upgrade

When using the 50-lane comb for the ABI PRISM 377 DNA Sequencer with XL Upgrade, we recommend treating the well-forming region of the notched ("rabbit-eared") glass plate with Bind-Silane to immobilize the wells and facilitate subsequent loading of the gel.

IMPORTANT Apply a fresh coating of Bind-Silane for each gel.

To prepare 36-cm well-to-read plates for the ABI PRISM 377XL:

Step	Action
1	Clean 36-cm well-to-read gel plates with Alconox detergent using a dedicated wash cloth or Kimwipe tissues. Rinse with deionized water and air dry.
2	Place the cleaned, notched ("rabbit-eared") glass plate face up on a covered benchtop.
3	Dip the end of a cotton swab into a bottle of Bind-Silane to wet the tip.
4	Transfer the Bind-Silane to the well-forming region of the notched glass plate (approximately the top 1 cm of the plate) by swiping the swab across the plate three times. Do not treat the side that had been previously in contact with the silicon rubber gasket of the upper buffer chamber.
5	Allow the Bind-Silane to dry on the plate for 1 minute.
6	Dry the treated region with a Kimwipe tissue by wiping three times using moderate pressure.
7	Assemble the plates as described in "Preparing Plates for the ABI PRISM 377" on page 7-2, beginning with step 2.
8	After running the gel, clean the plates as normally done using Alconox detergent. It may be necessary to scrape the gel off the notched plate where it has stuck to the Bind-Silane-treated region.

Preparing 36-cm Well-to-Read Gels

We recommend the use of Long Ranger gel solutions instead of 4% acrylamide for the 36-cm well-to-read plates. Long Ranger gels form better wells around the comb, facilitating loading and potentially enhancing results. This procedure makes enough gel mixture for two 36-cm well-to-read gels.

To prepare 50 mL of 5% Long Ranger/6.0 M urea gel mixture:

Step	Action
1	Combine the following: ♦ Urea 18.0 g ♦ Deionized water 21.5 mL ♦ 5X Maniatis TBE[a] 10.0 mL ♦ 50% Long Ranger stock solution 5.0 mL **! WARNING ! CHEMICAL HAZARD. Urea is a possible mutagen. Do not breathe the dust. Urea can be harmful by inhalation, skin contact, and ingestion. Refer to the MSDS for proper protective equipment.**
2	Warm the mixture in a 37 °C water bath, stirring occasionally, to dissolve the urea. Once the urea is dissolved, allow the gel mixture to equilibrate to room temperature. This gel mixture should be used within 12 hours. Keep the gel mixture covered during this step to prevent evaporative loss.
3	Filter the gel mixture using a 150-mL Nalgene filter apparatus with a 0.2-micron CN filter. Attach to a vacuum source (approximately 20 inches Hg) to pull liquid through filter. This step removes any particulates that may fluoresce or scatter light.
4	Let stand for 5 minutes, swirling occasionally, with the vacuum on to degas the mixture.
5	Turn off vacuum, remove top of filter apparatus and discard. **Note** The filter apparatus can be rinsed thoroughly with deionized water and air-dried to be reused up to five times.
6	Add 250 µL of freshly prepared 10% APS and 35 µL TEMED to the gel mixture. Swirl gently to mix. **IMPORTANT** Proceed to the next step immediately.

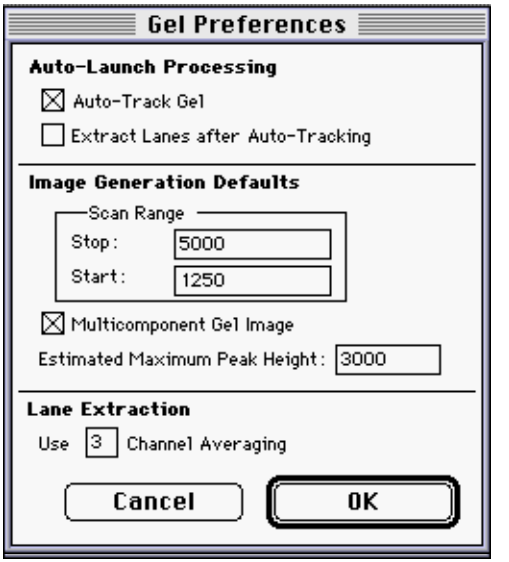
a. See “Preparation of Required Reagents” on page 7-22.

To pour gels:

Step	Action
1	Draw the gel mixture up into a 35-cc syringe. Slowly inject gel mixture into the top center area between the plates. **Note** Tap the gel plates firmly with your palm while pouring to prevent bubbles and move gel mixture down to the bottom of the plates.
2	Stop injecting when the gel mixture is near the bottom of the plates. Immediately lay plates flat on a level surface. Each gel requires approximately 20–25 mL of Long Ranger gel mixture.
3	**If using the ABI PRISM 377:**
	Insert a 34-well 0.2-mm square-tooth comb and clamp into place with three large binder clamps over the comb. **Note** A 24-well square-tooth comb can also be used. Do not use a shark's tooth comb.
	If using the ABI PRISM 377 with XL Upgrade:
	Insert a 50-well 0.2-mm square-tooth comb and clamp into place with three large binder clamps over the comb.
4	Allow gel to polymerize for at least 2.0 hours. **Note** The polymerized gel can be stored for up to 24 hours. After polymerization, place a Kimwipe tissue wetted with deionized water over the top and bottom edges of the gel plates. Do not remove the gel comb. Wrap the top and bottom edges tightly with clear plastic wrap and store at room temperature.
5	Prepare 1.5 L of 1X Maniatis TBE running buffer (89 mM Tris, 89 mM borate, 2 mM EDTA).

Setting up the Gel Preferences and Analysis Parameters

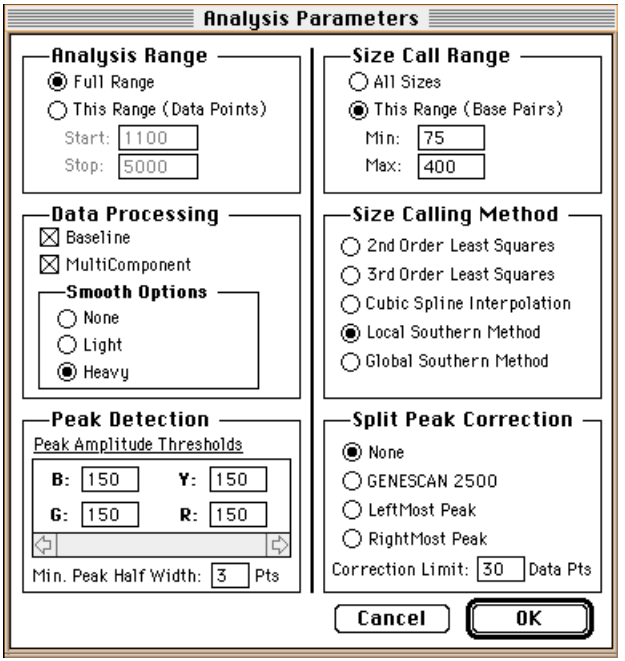
To set up the Gel Preferences:

Step	Action
1	Restart the Power Macintosh® computer to defragment the RAM memory.
2	Launch the GeneScan Analysis 2.1 Software.
3	Under the Settings menu, select Gel Preferences.
4	Fill in the dialog box with the settings shown in Figure 7-1.  **Figure 7-1** Gel Preferences dialog box
5	Click OK when done.

To set up the Analysis Parameters:

Step	Action
1	Under the Settings menu, select Analysis Parameters. A dialog box appears.

To set up the Analysis Parameters: *(continued)*

Step	Action
2	Set the parameters in the dialog box as shown in Figure 7-2. a. You can choose either light or heavy smoothing. Refer to "Smooth Options" in Chapter 4 of the *GeneScan® Analysis 2.1 User's Manual*. b. We generally recommend a Peak Amplitude Threshold of 150 RFU for Blue, Green, and Yellow to avoid typing low amounts of input DNA (see "Effect of DNA Quantity on Results" on page 9-33). However, when GeneScan data will be genotyped automatically using the Genotyper® AmpFℓSTR Profiler Plus template file, lowering the Peak Amplitude Threshold to a value less than 150 RFU for Blue, Green, and Yellow (we recommend as low as 50 RFU) will allow the Genotyper software to identify low signals automatically (see page 10-8). Peaks with heights greater than the Peak Amplitude Threshold but less than 150 RFU will be denoted in the appropriate Genotyper table as "< 150 RFU" and therefore flagged for closer examination.  **Figure 7-2** Analysis Parameters dialog box

To set up the Analysis Parameters: *(continued)*

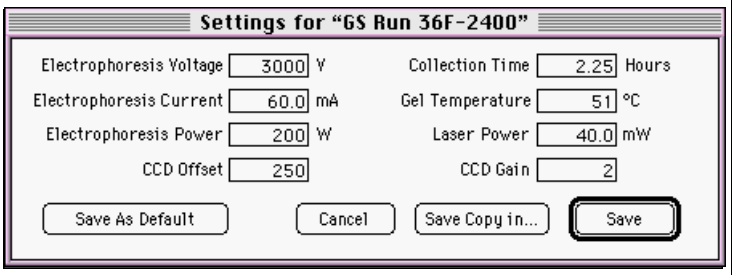
Step	Action
3	Click OK when done.
4	Quit the GeneScan Analysis 2.1 Software.

Setting up the Sample Sheet/ Electrophoresis Parameters

To prepare a sample sheet:

Step	Action
1	Launch the ABI PRISM 377 Collection Software on the Power Macintosh.
2	If a sample sheet needs to be created, then proceed with this step; otherwise skip to step 1 of "To create a Run file:" Under the File menu, select New and click on GeneScan Sample. The sample sheet template appears on the screen.
3	Enter sample names/numbers for each lane in the Sample Name column.
4	Enter the sample description for each row in the Sample Info column (for Blue, Green, and Yellow for each sample). This is necessary for the Genotyper® 2.0 AmpFℓSTR Profiler Plus template to build tables containing the genotypes for each sample. Enter the word "ladder" for the Blue, Green, and Yellow rows for the AmpFℓSTR Profiler Plus Allelic Ladder lane.
5	Be sure that the diamond symbol in the "std" column indicates the red sample as the standard in each lane.
6	Save the sample sheet in the Sample Sheets folder in the ABI PRISM 377 folder.
7	Close the sample sheet. **Note** For more details, see the *ABI PRISM 377 DNA Sequencer User's Manual* for how to create and edit a sample sheet.

To create a Run file:

Step	Action
1	Under the File menu, select New and click on GeneScan Run. The run window appears on the screen.
2	a. In the Plate Check Module pop-up menu, select Plate Check F. b. In the PreRun Module pop-up menu, select GS PR 36F-2400. c. In the Run Module pop-up menu, select GS Run 36F-2400. d. Click the document icon to the right of the Run Module to verify the electrophoresis settings for the particular run. The Settings for "GS Run 36F-2400" window appears. Make sure that the settings are as shown in Figure 7-3.  **Figure 7-3** Settings for GS Run 36F-2400 Run Module
3	Click Save. To save these settings as the default, click Save As Default.
4	Select a sample sheet that has been prepared previously. The run module cannot be started without a sample sheet.
5	**If using the ABI PRISM 377:**
	Choose 34-Well Comb from the Lanes pop-up menu.
	If using the ABI PRISM 377 with XL Upgrade:
	Choose 50-Well Comb from the Lanes pop-up menu.

To create a Run file: *(continued)*

Step	Action
6	Select a matrix file. a. Choose the appropriate matrix file from the Gel's Matrix File pop-up menu. The matrix file must be previously prepared as described in Chapter 6 and saved to the matrix folder in the ABI folder, which is in the System folder. This is necessary to have the matrix file applied automatically to the gel image. **IMPORTANT** The matrix file must be one that was made using the 5-FAM, JOE, NED, and ROX matrix standards and Filter Set F module files. See Chapter 6, "Multicomponent Analysis," for more information. b. In addition, select the appropriate matrix file for each sample in the Sample Sheet area of the run window. This is necessary to have the matrix applied automatically to the sample files. To do so, click the arrow in the Matrix File column for the first sample/lane to view the pop-up menu and choose the appropriate matrix file. After choosing a matrix file for the first sample/lane, select the entire matrix column by clicking on the column heading and choosing Fill Down (under the Edit menu).
7	a. Verify the collection time as 2.25 hours. b. Well-to-read distance should be set at 36 cm for the GS Run 36F-2400 module.
8	**If using the ABI PRISM 377 with XL Upgrade and comb size Š50 lanes:**
	From the Run Mode pop-up menu, choose XL Scan.
9	Under the File menu, choose Save to save all collection settings.

Running the Plate Check and Prerun Modules

To place the gel plates into the instrument:

Step	Action
1	Remove the comb (try removing it slowly while lubricating with 1X TBE for the best results).
2	Remove any acrylamide that remains around the top of the wells.
3	Clean the outside of the gel plates with deionized water and wipe dry. **IMPORTANT** Do not touch the plates in the laser scanning region from this point forward.
4	Place the gel plates into the ABI PRISM 377 cassette. Place the cassette into the instrument, making sure that the lower buffer chamber is already in place.
5	Make sure that the gel plate spacers are pressing against the two positioning pins on the instrument.
6	Close the instrument door.

To run the plate check:

Step	Action	
1	Click on the Plate Check button. Four colored, horizontal lines should appear in the scan window after approximately 30 seconds.	
	IF the lines in the scan window...	**THEN...**
	are relatively flat and level across the screen	♦ The plates are clean. ♦ Click the Cancel button to cancel plate check.
	show peaks	♦ The plates are not clean. ♦ Pause the plate check, remove the cassette, and clean the plates again. ♦ Resume plate check.
	are flat but not level across the screen	♦ The cassette is not positioned properly in the instrument (*i.e.,* the plates are not flush against the back cooling plate and/or the two positioning pins that set the plates the correct distance from the optics). ♦ Pause the plate check and reposition the cassette. ♦ Resume the plate check.
2	After determining that the plates are clean and positioned correctly, clamp the upper buffer chamber onto the gel plates.	
3	Add 1X TBE buffer to the upper buffer chamber and check for leaks.	
4	Clamp on and connect the cooling plate.	
5	Add 1X TBE buffer to the lower buffer chamber. **Optional:** To facilitate the subsequent loading of the gel, drop 15 µL of blue dextran loading buffer (contained in the GeneScan-500 [ROX] Internal Lane Size Standard Kit) over the wells in a sweeping motion using a P10 or P20 pipet tip. The dye outlines the walls and bottom of the wells.	
6	Plug in the electrodes and close the instrument door.	

To perform the prerun:

Note Samples should be prepared while the gel is being prerun.

Step	Action
1	Click the PreRun button in the Run window. Prerun the gel for approximately 15 minutes.
2	Click Pause in the Run window to pause the prerun. Pausing stops the electrophoresis, but continues to heat the gel to 51 °C and then maintains that temperature. **Note** Samples can be loaded onto the gel during this pause. See page 7-15 for sample loading information.
3	Prior to loading the gel, unplug the cathode and check the Status window to verify that the electrophoresis power has been turned off.

Preparing Samples and AmpFℓSTR Profiler Plus Allelic Ladder

To prepare samples and allelic ladder:

Step	Action
1	Prepare formamide loading solution (FLS) by combining: ♦ Blue dextran "Loading Buffer" (contained in GeneScan-500 [ROX] Internal Lane Size Standard Kit), 100 µL ♦ Deionized formamide, 500 µL **! WARNING ! CHEMICAL HAZARD. Formamide is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling.**
2	Vortex to mix. FLS can be stored at 2–6 °C for up to 2 weeks.
3	Prepare the AmpFℓSTR Profiler Plus Allelic Ladder by combining the following in a single microcentrifuge tube: ♦ 10 µL AmpFℓSTR® Blue Allelic Ladder ♦ 10 µL AmpFℓSTR® Green II Allelic Ladder ♦ 10 µL AmpFℓSTR® Yellow Allelic Ladder
4	Mix by pipetting up and down. The AmpFℓSTR Profiler Plus Allelic Ladder can be stored at 2–6 °C for up to 2 weeks.
5	Combine the necessary amount of FLS and GeneScan-500 [ROX] Internal Lane Size Standard in a single microcentrifuge tube as shown: ♦ (Number of samples) × 5.0 µL FLS denaturant ♦ (Number of samples) × 0.55 µL GeneScan-500 [ROX] size standard **Note** The above formulation provides a slight overfill to allow for volume lost in pipetting. Be sure to include two lanes of AmpFℓSTR Profiler Plus Allelic Ladder per gel in the calculations.
6	Vortex the tube to mix, and spin briefly in a microcentrifuge.
7	Aliquot 5.0 µL of FLS/GeneScan-500 [ROX] mixture into 0.5-mL GeneAmp Thin-walled Reaction Tubes with caps (for use in the DNA Thermal Cycler 480) or 0.2-mL MicroAmp tubes with caps (for use in the GeneAmp PCR Instrument System 9600 or 2400). Label one tube per sample.
8	Add 4.0 µL PCR product or AmpFℓSTR Profiler Plus Allelic Ladder per tube and close the caps.

To prepare samples and allelic ladder: *(continued)*

Step	Action
9	Heat samples in a thermal cycler for 2 minutes at 95 °C to denature them.
10	Chill for at least 3 minutes in an ice-water bath. Keep on ice until ready to load.

Loading Samples

Step	Action
1	Rinse the urea out of each sample well with buffer, using a 0.17-mm flat pipet tip attached to a 35-cc syringe. **Note** It may be necessary to cut off the top end of the pipet tip with a razor blade for it to fit on the end of the Luer lock syringe without leaking.
2	**If using the ABI PRISM 377:**
	Load 1.5 µL of denatured sample or allelic ladder per well. The AmpFℓSTR Profiler Plus Allelic Ladder can be loaded into any two wells on the gel.
	If using the ABI PRISM 377 with XL Upgrade:
	Load 1.0 µL of denatured sample or allelic ladder per well. The AmpFℓSTR Profiler Plus Allelic Ladder can be loaded into any two wells on the gel.
3	Attach the upper buffer chamber lid, plug in the cathode, and close the instrument door.

Sample Electrophoresis

To run samples:

Step	Action
1	Select Cancel to stop the pre-run.
2	Select Run to start gel electrophoresis. Name the Gel file and Save.
3	Under the Window menu, select Status to check that the run settings are correct and to monitor the run. **Note** The data collection can be viewed at any time during the run by selecting either Scan or Gel Image under the Window menu. The Gel Image window should not be left open for long periods of time during the run, as it occupies a large block of memory.

To run samples: *(continued)*

Step	Action
4	After electrophoresis is completed, the computer automatically launches the GeneScan Analysis 2.1 Software to generate a gel image on the screen, if chosen as a preference. The gel preferences last saved in this application are used to generate the gel image.

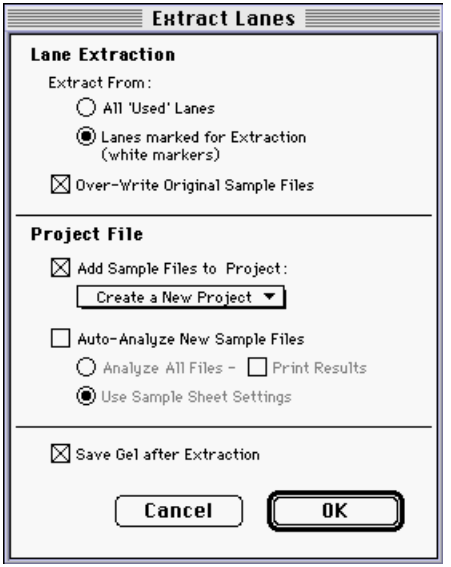
GeneScan Analysis of Data

Note For more information on data analysis, see the *GeneScan Analysis 2.1 User's Manual*.

To analyze data:

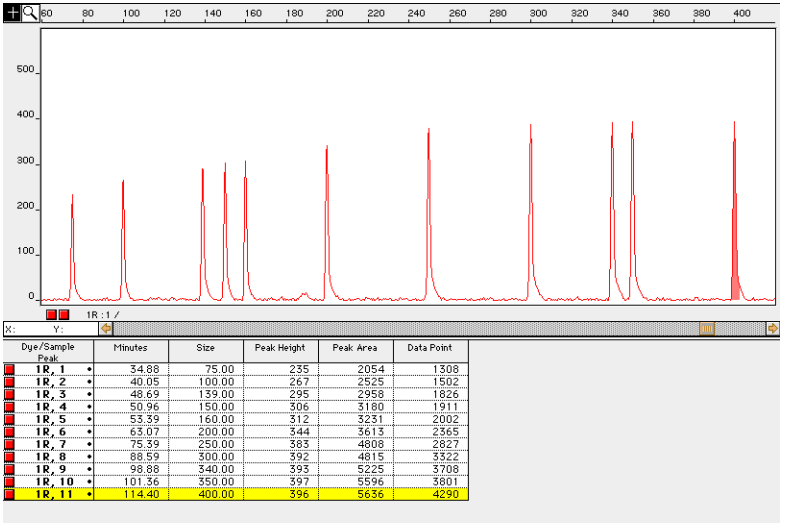
Step	Action
1	Examine the red GeneScan-500 [ROX] size standard bands on the gel picture and check that the 75-bp and 400-bp bands are present. It may be necessary to Adjust Gel Contrast under the Gel menu for red to see all of the red bands clearly. If the 75-bp and/or 400-bp bands are not present, adjust the scan range. Under the Gel menu, choose Regenerate Gel Image…, adjust the values for scan range, and click OK.
2	Verify the automatic lane tracking. It may be necessary to adjust the gel contrast for blue, green, and yellow to see all of the bands clearly. Also, zoom in on the gel image by selecting Zoom In under the View menu. a. Move the lane tracking line over each sample lane, using the arrow keys on the computer keyboard or the mouse. Change the channel and introduce nodes as necessary. When modifications are made to a lane, the associated triangular lane marker turns from blue to white. b. Mark all used lanes as modified (white triangular lane marker) before proceeding to step c. If necessary, option-click the triangular lane marker for used lanes to change them to white. c. Save the changes that have been made by selecting Save under the File menu.
3	Under the Gel menu, create the sample files by choosing Extract Lanes… This opens a dialog box. Fill in the box as shown in Figure 7-4 on page 7-17.

To analyze data: *(continued)*

Step	Action
	 **Figure 7-4** Extract Lanes dialog box
4	Click OK when done. **Note** The computer generates the sample files. After all the sample files have been generated, the Analysis Control window appears.
5	Assign a size standard: a. Command-click the red box column in the Analysis Control window to indicate Red as the standard (a diamond symbol should then appear in the red boxes). b. Click the arrow in the Size Standard box for the first sample to view the pop-up menu and Define New, or choose a correct standard already stored. For more information on defining a size standard, see the *GeneScan Analysis 2.1 User's Manual*. See Figure 7-5 on page 7-18 for the sizes of the peaks in the GeneScan-500 [ROX] size standard.

To analyze data: *(continued)*

Step	Action



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
1R, 1	34.88	75.00	235	2054	1308
1R, 2	40.05	100.00	267	2525	1502
1R, 3	48.69	139.00	295	2958	1826
1R, 4	50.96	150.00	306	3180	1911
1R, 5	53.39	160.00	312	3231	2002
1R, 6	63.07	200.00	344	3613	2365
1R, 7	75.39	250.00	383	4808	2827
1R, 8	88.59	300.00	392	4815	3322
1R, 9	98.88	340.00	393	5225	3708
1R, 10	101.36	350.00	397	5596	3801
1R, 11	114.40	400.00	396	5636	4290

Figure 7-5 GeneScan-500 [ROX] Internal Lane Size Standard, showing the markers from 75–400 bp. Tabular data is shown below the electropherogram. The 400-bp standard is highlighted in both the electropherogram and the table.

	c. To apply one size standard to all lanes, choose that standard in the size standard column header pop-up window (above sample 1).
6	Analyze sample files: a. Highlight the blue, green, yellow, and red columns. b. Click the Analyze button. This calculates the base pair size of each fragment and fills the sample files with the analyzed data from each lane.

To analyze data: *(continued)*

Step	Action
7	After the analysis is complete, confirm that the sizes for the peaks in the GeneScan-500 [ROX] size standard have been correctly assigned. a. Open Results Control (under the Window menu) and examine the red GeneScan-500 peaks in overlapping groups (Quick Tile Off). Command-plus (c +) enlarges the view window for more careful examination; command-minus (c –) reduces it back to full range. Be sure to use the Align By Size option under the View menu. b. Scroll through the tables to verify correct peak assignments (see Figure 7-5 on page 7-18). Check the remaining lanes, taking note of which lanes (if any) have incorrect peak assignments. c. If the size standard peak assignments are incorrect in one lane, define a new size standard for that sample using the peaks in that lane. To do so, select the Define New option in the Size Standard row for that sample (see step 5b). d. Re-analyze any incorrectly sized lanes (select the blue, green, yellow, and red boxes) using the newly defined GeneScan-500 [ROX] size standard file for that lane. This creates a new standard file for each of these lanes, replacing the previous analysis results for those lanes only. e. Confirm that the GeneScan-500 [ROX] size standard peaks are now correctly assigned in the re-analyzed lanes.
8	View the AmpF*ℓ*STR Profiler Plus results (using the Results Control window) and print. Refer to the *GeneScan Analysis 2.1 User's Manual* for printing options. **Note** See Chapter 9, "Results and Interpretation," for more information on interpreting the data.

Dedicated Equipment and Supplies

Equipment Required

Amplified DNA, equipment, and supplies used to handle amplified DNA should not be taken out of the Amplified DNA Work Area. Samples that have not yet been amplified should never come into contact with these supplies and equipment.

- ABI PRISM 377 DNA Sequencer or ABI PRISM 377 DNA Sequencer with XL Upgrade
- ABI PRISM 377 DNA Sequencer Accessories:
  - 36-cm well-to-read plates:
    - 36-cm rear glass plate (P/N 401839)
    - 36-cm front glass plate (P/N 401840)
    - 36-cm gel spacers, 0.2 mm thick (P/N 401836)
  - 34-well squaretooth comb, 0.2 mm thick (P/N 401907)
  - 50-well squaretooth comb, 0.2 mm thick (P/N 402053)
  - optional: 24-well squaretooth comb, 0.2 mm thick (P/N 401904)
  - XL Upgrade for the ABI PRISM 377 DNA Sequencer
- Benchkote absorbent protector sheets
- Binder clips, medium and large
- Freezer, –15 to –25 °C, non-frost-free
- Gloves, disposable, powder-free
- Glassware
- Ice bucket
- Kimwipes
- Lab coat
- Lamp, 27-inch gooseneck with magnetic base (Sunnex, P/N 701-27, or office supply store)
- Microtube racks
- Nalgene filter apparatus, 150-mL, 0.2-µm CN filter
- Permanent ink pen
- Pipet bulb
- Pipets, serological

- Pipet tips, sterile, disposable hydrophobic filter-plugged
- Pipet tips for gel loading, 0.2 mm flat tips (Rainin, P/N GT1514)
- Pipettors, adjustable, 0.5–10 µL, 2–20 µL, 20–200 µL, and 200–1000 µL
- Refrigerator
- Repeat pipettor and Combitips that dispense 1–5 µL (optional)
- Sink
- Syringe, 20 or 35 cc (optional)
- Thermal cycler
- Tube decapper, autoclavable

Reagents Required

- Alconox detergent
- AG501 X8 ion exchange resin (Bio-Rad)
- Ammonium persulfate (APS)
- Bind-Silane (Pharmacia Biotech, P/N 17-1330-01)
- Blue dextran loading buffer (included in GeneScan-500 [ROX] Size Standard Kit)
- Formamide, deionized
- GeneScan-500 [ROX] Internal Lane Size Standard Kit (P/N 401734)
- 50% Long Ranger stock solution (FMC Bioproducts, P/N 50610 or 50611)
- Matrix standards:
  - Dye Primer Matrix Standard Kit (P/N 401114)

IMPORTANT Do not use the TAMRA matrix standard provided in this kit.

  - NED Matrix Standard (P/N 402996)
- 5X TBE (see “Preparation of Required Reagents” on page 7-22)
- N,N,N’,N’-Tetramethylethylenediamine (TEMED)
- Urea

Software and User Documentation Required

- *ABI PRISM 377 DNA Sequencer User's Manual* (P/N 903433)
- ABI PRISM GeneScan Analysis 2.1 Software (P/N 672-18)
- *ABI PRISM GeneScan Analysis 2.1 User's Manual* (P/N 904435)
- Filter Set F module files (located in the Modules folder within the ABI PRISM 377 or ABI PRISM 377XL folder):
  - Plate Check F
  - GS PR 36F-2400
  - GS Run 36F-2400

Preparation of Required Reagents

0.5 M EDTA, pH 8.0

Step	Action
1	Slowly add 186.1 g disodium ethylenediaminetetraacetate dihydrate ($Na_2EDTA•2H_2O$) to 800 mL glass-distilled or deionized water.
2	Stir vigorously on a magnetic stirrer.
3	Adjust to pH 8.0 ± 0.2 by adding NaOH pellets (approximately 20 g). **Note** EDTA will not go into solution without pH adjustment.
4	Adjust final volume to 1 L with glass-distilled or deionized water.
5	Autoclave the solution or filter it through a 0.2 µm Nalgene filter. Store at room temperature.

5X Maniatis TBE (445 mM Tris, 445 mM borate, 10 mM EDTA)

Step	Action
1	To 20 mL of 0.5 M EDTA, pH 8.0, add approximately 900 mL of deionized water.
2	Add 54 g Tris base and 27.5 g boric acid to the diluted EDTA solution. Stir vigorously on a magnetic stir plate.
3	Adjust the volume to 1 L with deionized water and mix thoroughly.
4	Filter the mixture using a 0.2 µm or 0.45 µm Nalgene filter unit to remove particulate matter and prevent formation of a precipitate.
5	Store in a glass container to facilitate visual inspection for precipitates. If a precipitate forms, discard the 5X TBE buffer and remake it.

Deionized formamide

Step	Action
1	Mix 50 mL of formamide and 5 g of AG501 X8 ion-exchange resin.
2	Stir for 30 minutes at room temperature.
3	Check that the pH is greater than 7.0 using pH paper. If the pH is not greater than 7.0, decant the formamide into a beaker containing another 5 g of ion-exchange resin and repeat 30-minute stirring at room temperature.
4	When the pH is greater than 7.0, allow the beads to settle to the bottom of the beaker. Remove the supernatant (formamide), taking care not to disturb the beads.
5	Dispense the deionized formamide into aliquots of 500 µL and store for up to 3 months at –15 to –25 °C.
6	Use one aliquot per set of samples. Discard any unused deionized formamide.

ABI PRISM 310 Genetic Analyzer Protocol

8

Using Performance Optimized Polymer 4 (POP-4) for Analysis of AmpFℓSTR Profiler Plus PCR Products

ABI PRISM 310 Genetic Analyzer

Figure 8-1 shows the ABI PRISM® 310 Genetic Analyzer. The parts mentioned in this section are labeled. Refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* and *GeneScan® Analysis 2.1 User's Manual* for more detailed information on the instrument and software used with this protocol.

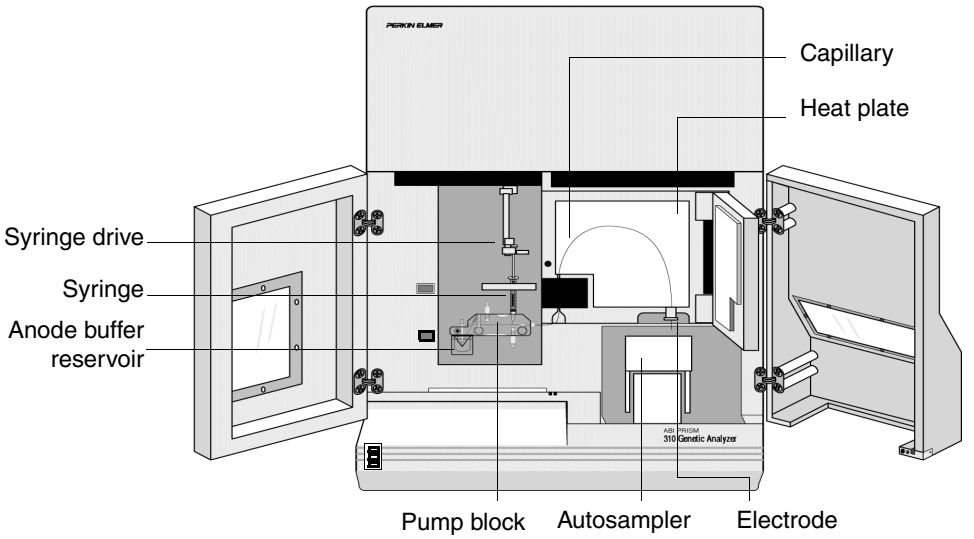


Figure 8-1 ABI PRISM 310 Genetic Analyzer

IMPORTANT Filter Set F must be used for data collection of AmpFℓSTR® Profiler Plus™ PCR products. Filter Set F module files must be installed in the Modules folder within the ABI PRISM 310 folder on the instrument's Macintosh computer before following the protocols in this chapter (see page 6-4).

IMPORTANT Additionally, before running AmpFℓSTR Profiler Plus PCR products on the instrument, a matrix file must be made using the 5-FAM, JOE, NED, and ROX matrix standards and Filter Set F. Refer to Chapter 6, "Multicomponent Analysis," for information on how to do this.

Setting Up the Instrument

Installing a new electrode

Installing and trimming a new electrode is usually necessary only when the instrument is first set up or if the electrode is ever bent severely.

IMPORTANT A new electrode must be trimmed to the correct length. Refer to Figure 8-2 for trimming the electrode.

Step	Action
1	Install the new electrode on the instrument as described in Chapter 2 of the *ABI PRISM 310 Genetic Analyzer User's Manual*.
2	Under the Manual Control menu, select Home Z-Axis.
3	Use the flush-cutting wire cutter (P/N T-6157) provided in the instrument packing kit. Hold the cutters with the flat cutting face toward the top of the instrument.
4	Cut a small amount off the end of the electrode until it is flush with the lower surface of the stripper plate. Be careful not to flex the stripper plate upwards while cutting. Do not cut off more than 1mm beyond the lower surface of the stripper plate (Figure 8-2).

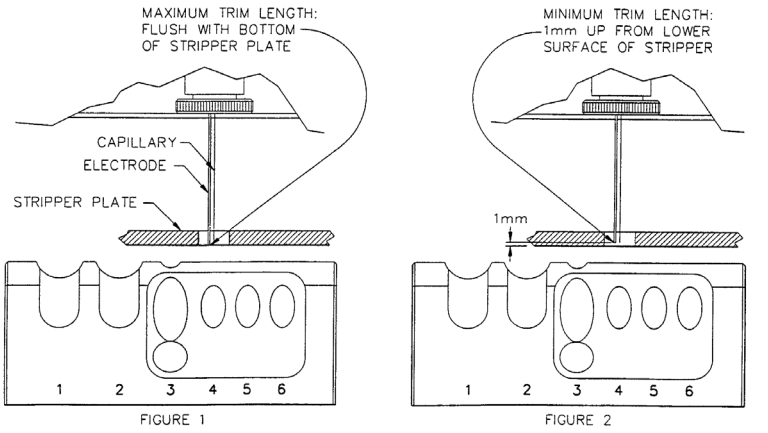


Figure 8-2 Trimming the electrode

Cleaning the electrode (if necessary)

Step	Action
1	Wipe the electrode with a Kimwipe tissue that has been dampened with distilled, deionized water.
2	Dry the electrode with a fresh Kimwipe tissue. **Note** The autosampler should be recalibrated after cleaning the electrode, as described in "Calibrating the Autosampler" in Chapter 2 of the *ABI PRISM 310 Genetic Analyzer User's Manual*.

Checking the syringe

Verify that the 1.0-mL glass syringe (P/N 604418) has a small O-ring (P/N 221102) inside the syringe, and that an O-ring is placed around the ferrule-shaped seal. The ferrule should be firmly seated in the end of the 1.0-mL syringe. If the syringe is dirty, it must be cleaned before use.

Setting Up a Run

Removing the syringe from the instrument

Step	Action
1	Restart the Power Macintosh® computer to defragment the RAM memory.
2	Launch the ABI PRISM 310 Collection Software.
3	Under the Window menu, select Manual Control. Select Syringe Home from the Function pop-up menu. Click Execute. **Note** For all commands in the Manual Control window, the Execute button must be selected to complete the task.
4	Open the instrument doors and move the syringe drive toggle to the left.
5	Unscrew the syringe from the pump block.

Note Before use, the POP-4™ polymer should be allowed to equilibrate to room temperature. If precipitate is present in the bottle when removed from cold storage, it should go back into solution at room temperature. Mix the polymer thoroughly by inversion before using.

Cleaning and priming the syringe

Step	Action
1	Clean and rinse the 1.0-mL syringe with distilled water. Remove all excess water from the inside and outside of the syringe by blowing into it with compressed air. **Note** Place a small droplet of distilled water on the end of the plunger before inserting the plunger into the syringe. Without any water, the PTFE fitting of the plunger will sustain excessive wear.
2	Draw up 0.2 mL of POP-4 solution into the syringe.
3	Pull the plunger up to the 1.0-mL marker. Invert the syringe gently five or six times to coat the walls with polymer.
4	Discard this polymer solution. **Note** This procedure ensures that the running polymer is not diluted with water when added to the syringe. Failure to prime the syringe with polymer will adversely affect your results. See Chapter 11, "Troubleshooting," for more details.

Loading the syringe

Step	Action
1	Fill the 1.0-mL syringe manually with a maximum of 0.8 mL of POP-4 polymer. **Note** The polymer should not stay in the syringe longer than 3 days. Do not return unused polymer to the bottle.
2	Wipe the outside of syringe with a Kimwipe tissue to dry.
3	Remove any air bubbles by inverting the syringe and pushing a small amount of polymer out of the tip.

Removing and cleaning the pump block

Before setting up the instrument for a run, make sure that the pump block is clean of all polymer, especially if the polymer in the syringe has been sitting at room temperature for more than 3 days. Urea decomposition during this interval causes transient current increases (spikes) during electrophoresis.

To remove and clean the pump block, see "Cleaning and Maintaining the Instrument" in Chapter 2 of the ABI PRISM 310 *Genetic Analyzer User's Manual*. Follow the instructions in the sections titled "Removing the Pump Block," "Rinsing the Pump Block," and "Replacing the Pump

Block." We do not recommend following the section titled "Rinsing the Pump Block on the Instrument" for this application.

IMPORTANT Remove all residual water from the pump block and fittings by blowing compressed air through the channels.

Reinstall the pump block on the instrument after cleaning.

Installing the syringe on the pump block

Step	Action
1	Move the syringe drive toggle on the instrument to the left in order to be able to attach the syringe to the pump block.
2	Place the 1.0-mL syringe through the right-hand port of the plastic syringe guide plate and screw the syringe into the pump block. The syringe should be finger-tight in the block.
3	Hand-tighten the valves on the pump block to the left of and below the syringe.

Installing the capillary (if necessary)

Step	Action
1	Clean capillary window with 95% ethanol on a Kimwipe tissue. Do not touch capillary window after cleaning.
2	Install the 47-cm, 50-µm i.d. capillary (P/N 402839, green mark) as described in Chapter 2 of the *ABI PRISM 310 Genetic Analyzer User's Manual*. Follow the instructions in the section titled "Installing the Capillary."
3	After installing the capillary, secure it into place by pressing a piece of tape over it onto the heat plate just above the electrode. **Note** The capillary should be approximately flush with the end of the electrode.
4	Calibrate the autosampler if the electrode was touched during installation of a fresh capillary. Make sure that it is calibrated in the X, Y, and Z directions. The capillary should almost touch the metal calibration points. Refer to "Calibrating the Autosampler" in Chapter 2 of the *ABI PRISM 310 Genetic Analyzer User's Manual*. **IMPORTANT** The sample tray must be removed before calibrating the autosampler. If the sample tray is not removed, the electrode will be bent.

Filling the buffer reservoirs

Step	Action
1	Dilute 5 mL of 10X Genetic Analyzer Buffer with EDTA (P/N 402824) to 1X concentration (50 mL) with distilled, deionized water.
2	Fill the anode buffer reservoir to the red line with 1X Genetic Analyzer Buffer and place the reservoir on the pump block.
3	Fill a 4-mL glass buffer vial (P/N 401955) to the fill line with 1X Genetic Analyzer Buffer. Insert the plastic vial lid with attached septum into the glass vial and place the buffer vial into position 1 on the autosampler. This will serve as the cathode buffer.
4	Fill a second 4-mL glass buffer vial to the fill line with distilled water. Insert the plastic vial lid with attached septum into the glass vial and place the vial into position 2 on the autosampler.
5	Fill a 1.5-mL Eppendorf tube full with distilled water and place it into position 3 on the autosampler. **Note** Do not use a screw-cap tube. The lids on screw-cap tubes are too high to clear the electrode and capillary. Use a 1.5-mL Eppendorf tube with the lid clipped off.

Priming the pump block

Step	Action
1	Under the Window menu, select Manual Control. Select Buffer Valve Close from the pop-up menu. Click Execute.
2	Partly unscrew the waste valve on the pump block (below the syringe).
3	Manually press down on the 1.0-mL syringe plunger until the valve space is filled with polymer. This will remove the air bubbles at this valve site, and should use about 0.1 mL of polymer.
4	Tighten the waste valve to close.
5	Open the pin valve at the anode buffer reservoir on the pump block by selecting Buffer Valve Open from the pop-up menu in the Manual Control window. Click Execute.

Priming the pump block *(continued)*

Step	Action
6	Manually press down on the 1.0-mL syringe plunger to push enough gel through the block so that all of the air bubbles are removed from the polymer channel in the block. (This process should use about 0.2 mL of polymer). **IMPORTANT** There should be no air bubbles in the pump block channels.
7	Close the pin valve by selecting Buffer Valve Close from the pop-up menu in the Manual Control window. Click Execute.
8	Move the syringe drive toggle to the right so that it is positioned over the syringe plunger.
9	a. Select Syringe Down from the pop-up menu in the Manual Control window. Click Execute. b. Select 50-step intervals. Execute until the toggle almost makes contact with the syringe plunger. c. Select smaller step intervals until the toggle makes contact with the syringe plunger.

Setting the Analysis Parameters

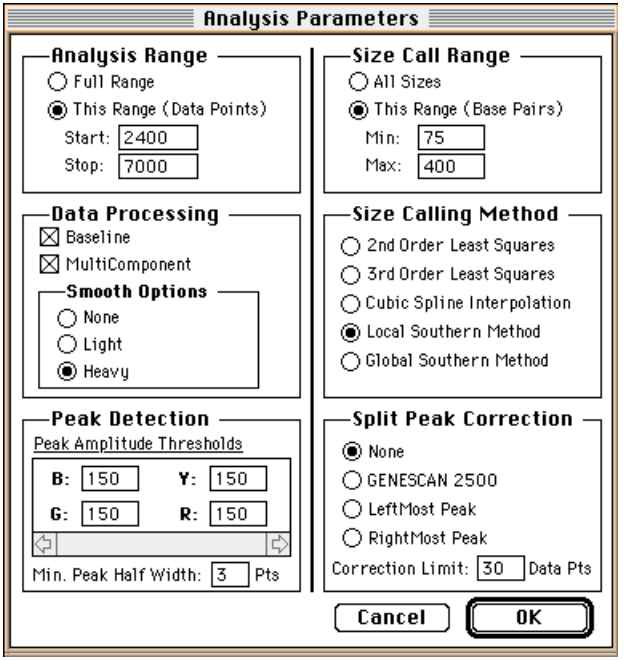
To set the analysis parameters:

Step	Action
1	Launch the GeneScan Analysis 2.1 Software.
2	Under the Settings menu, select Analysis Parameters.

To set the analysis parameters: *(continued)*

Step	Action
3	Fill in the dialog box with the settings shown in Figure 8-3 on page 8-9. a. You can choose either light or heavy smoothing. Refer to "Smooth Options" in Chapter 4 of the *GeneScan® Analysis 2.1 User's Manual*. b. We generally recommend a Peak Amplitude Threshold of 150 RFU for Blue, Green, and Yellow to avoid typing low amounts of input DNA (see "Effect of DNA Quantity on Results" on page 9-33). However, when GeneScan data will be genotyped automatically using the Genotyper® AmpFℓSTR Profiler Plus template file, lowering the Peak Amplitude Threshold to a value less than 150 RFU for Blue, Green, and Yellow (we recommend as low as 50 RFU) will allow the Genotyper software to identify low signals automatically (see page 10-8). Peaks with heights greater than the Peak Amplitude Threshold but less than 150 RFU will be denoted in the appropriate Genotyper table as "< 150 RFU" and therefore flagged for closer examination. c. The primer peak is detected in the 2600–3000 scan number range. The analysis range can be set to start immediately after the primer peak in order to see only the allelic data. Examine the raw data to determine the exact position of the primer peak.

To set the analysis parameters: *(continued)*

Step	Action
	 **Figure 8-3** Analysis Parameters dialog box
4	Click OK when done.
5	Quit the GeneScan Analysis 2.1 Software.

Setting the Run Temperature

This procedure is optional, but saves time. This heating step occurs automatically at the beginning of the GS STR POP4 (1 mL) F run module.

Step	Action
1	Close the instrument doors.
2	Return to the ABI PRISM 310 Collection Software.
3	Under the Window menu, select Manual Control. Select Temperature Set from the pop-up menu.
4	Set the temperature to 60 °C and click Execute. It takes up to 30 minutes for the instrument to reach the 60 °C run temperature. Samples can be prepared while the instrument is heating.

Preparing Samples and AmpFℓSTR Profiler Plus Allelic Ladder

To prepare the samples:

Step	Action
1	Combine the necessary amount of deionized formamide and GeneScan-500 [ROX] Internal Lane Size Standard (P/N 401734) in a single microcentrifuge tube as shown: ♦ (number of samples + 2) × 24 µL deionized formamide ♦ (number of samples + 2) × 1 µL GeneScan-500 [ROX] size standard **! WARNING ! CHEMICAL HAZARD. Formamide is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling.** **IMPORTANT** Use only deionized formamide (see "Preparation of Required Reagents" on page 8-20). Over time, formamide decomposes to formate. Formate ions are injected preferentially into the capillary, causing a loss of signal intensity. **Note** We recommend that enough volume for an additional couple of samples be included in the calculation to account for volume lost in pipetting. Be sure to include at least two injections of AmpFℓSTR Profiler Plus Allelic Ladder per run in the calculations. The formamide/GeneScan-500 [ROX] solution can be prepared as a master mix (1.20 mL deionized formamide + 50 µL GeneScan-500 [ROX] size standard) and stored at 2–6 °C for up to 2 weeks.
2	Vortex the tube to mix, then spin briefly in a microcentrifuge.
3	Prepare the AmpFℓSTR Profiler Plus Allelic Ladder by combining the following in a single microcentrifuge tube: ♦ 10 µL AmpFℓSTR® Blue Allelic Ladder ♦ 10 µL AmpFℓSTR® Green II Allelic Ladder ♦ 10 µL AmpFℓSTR® Yellow Allelic Ladder
4	Mix by pipetting up and down. The AmpFℓSTR Profiler Plus Allelic Ladder can be stored at 2–6 °C for up to 2 weeks.

To prepare the samples: *(continued)*

Step	Action
5	Aliquot 25 µL of formamide/GeneScan-500 [ROX] solution into 0.5-mL Genetic Analyzer sample tubes. Label one tube for each sample. **Note** To pipet the deionized formamide/size standard solution, we recommend using an Eppendorf repeating pipettor with a 25-µL Combitip.
6	Add 1.5 µL of PCR product or AmpFℓSTR Profiler Plus Allelic Ladder per tube. Mix by pipetting up and down and by stirring with pipet tip.
7	Seal each tube with a septum.
8	Denature each sample by placing in a thermal cycler for 3 minutes at 95 °C.
9	Chill tubes for at least 3 minutes in an ice-water bath.

Loading Samples

Step	Action
1	Open the instrument door and press the Tray button to present the autosampler.
2	Place the tubes in the 48-well sample tray. Tube #1 will go into sample tray position A1, tube #2 into sample tray position A3, etc. Place allelic ladder tubes in positions A1, D12, and H11 (the first, 24th, and 48th samples) if running a full 48-well set of samples (*i.e.*, 45 samples).
3	Place the sample tray on the autosampler.
4	Press the Tray button on the instrument to retract the autosampler.
5	Close the instrument door.
6	If a new capillary has been installed, select Change Capillary under the Instrument menu. Select OK in the Reset window to set the injection counter to zero.

Sample Electrophoresis

To run the samples:

Step	Action
1	If not already open, launch the ABI PRISM 310 Collection Software.
2	Under the File menu, select New and click the GeneScan Smpl Sheet icon.
3	Complete the sample sheet as described in Chapter 4 of the *ABI PRISM 310 Genetic Analyzer User's Manual*. The sample sheet can be prepared at any time before the preparation of samples and saved in the Sample Sheet folder. a. Enter sample names/numbers for each injection in the Sample Name column. This will indicate later which sample is in which tube of the sample tray. b. Enter the sample description for each row in the Sample Info column (for Blue, Green, and Yellow for each sample). This is necessary for the Genotyper® 2.0 AmpFℓSTR Profiler Plus template to build tables containing the genotypes for each sample. Enter the word "ladder" for the Blue, Green, and Yellow rows for the AmpFℓSTR Profiler Plus Allelic Ladder injection.
4	Under the File menu, select New and click on the GeneScan Injection List icon.
5	Choose the appropriate sample sheet from the Sample Sheet pop-up menu (at the top left of the Injection List window).
6	From the Module pop-up menu, choose Module GS STR POP4 (1 mL) F for every injection. To do so, click the arrow in the Module column for the first sample/injection to view the pop-up menu and choose the GS STR POP4 (1 mL) F module file. Next, select the entire Module column by clicking the Module column heading and choosing Fill Down (under the Edit menu).

To run the samples: *(continued)*

Step	Action
7	From the Matrix file pop-up menu, choose the appropriate matrix file for every injection. To do so, click the arrow in the Matrix column for the first sample/injection to view the pop-up menu and choose the appropriate matrix file. Next, select the entire Matrix column by clicking the Matrix column heading and choosing Fill Down (under the Edit menu). **IMPORTANT** The matrix file must be one that was made using the 5-FAM, JOE, NED, and ROX matrix standards and Filter Set F module files. See Chapter 6, “Multicomponent Analysis,” for more information.
8	Click the Start button. **Note** If you have not preheated the heat plate, the module has an initial step in which the plate is heated to 60 °C before running the first sample. This step takes up to 30 minutes. Once the plate reaches 60 °C, the run begins.

GeneScan Analysis of Data

Perform the following steps in the GeneScan Analysis 2.1 application.

To analyze the sample files:

Step	Action
1	Assign a size standard: a. Command-click the red box column in the Analysis Control window to indicate Red as the standard (a diamond symbol should then appear in the red boxes). b. Click the arrow in the standard column for the first sample to view the pop-up menu and select Define New. For more information on defining a size standard, refer to the *GeneScan Analysis 2.1 User's Manual*. See Figure 7-5 on page 7-18 for the sizes of the peaks in the GeneScan-500 [ROX] size standard. **IMPORTANT** Do **not** assign a size for the 250-bp peak (*i.e.*, assign a size of zero). This peak is used as an indicator of run-to-run precision. c. To apply one standard to all injections, assign the standard to one sample and save the standard file in the GS Standards folder within the ABI PRISM GeneScan 2.1 folder. Then choose that standard in the size standard column header (above sample 1) in the Analysis Control window.
2	Analyze sample files: a. Highlight the blue, green, yellow, and red columns. b. Click the Analyze button. This fills the sample files with the analyzed data from each injection.

To analyze the sample files: *(continued)*

Step	Action
3	After the analysis is complete, confirm that the sizes for the peaks in the GeneScan-500 [ROX] size standard have been correctly assigned. a. Open Results Control (under the Window menu) and examine the red GeneScan-500 [ROX] peaks in overlapping groups of 16 samples (Quick Tile Off). Command-plus (c +) enlarges the view window for more careful examination; command-minus (c –) reduces it back to full range. Be sure to use the Align By Size option under the View menu. b. While the samples are tiled, check the 250-bp peaks (sized as approximately 246 bp) in the enlarged view window. Remember that this peak was not defined in the size standard. The tiled 250-bp peaks should size precisely, *i.e.*, should all overlap. In a typical run, the 250-bp peaks all fall within a size window of approximately 1 bp. c. Scroll through the tables to verify correct GeneScan-500 [ROX] peak assignments (see Figure 7-5 on page 7-18). Check the GeneScan-500 [ROX] peaks in the remaining samples, taking note of which samples (if any) have incorrect peak assignments.
4	If the size standard peak assignments are incorrect for one injection, define a new size standard for that sample using the peaks in that injection. To do so, select the Define New option in the Size Standard pop-up menu for that sample (see step 1b).
5	Re-analyze any incorrectly sized samples (select the blue, green, yellow, and red boxes) using the newly defined GeneScan-500 [ROX] size standard file. This creates a new standard file for each of these samples, replacing the previous analysis results for those samples only.
6	Confirm that the GeneScan-500 [ROX] size standard peaks are now correctly assigned in the re-analyzed samples.
7	View AmpF*ℓ*STR Profiler Plus results (using the Results Control window) and print. Refer to the *GeneScan Analysis 2.1 User's Manual* for printing options. **Note** See Chapter 9, "Results and Interpretation," for more information on interpreting the data.

Ending the Run

To shut down the instrument:

Step	Action
1	Remove and store the capillary if the capillary has been used for fewer than 100 injections.
2	Store the capillary ends in two sample tubes filled with water and closed with septa. Do not allow these tubes to dry out over time. **Note** Do not run the GS-Wash Capillary and Block module to wash the capillary. This module is intended for a different polymer and application. After re-installing a capillary that has been stored, run a known standard to verify the condition of the capillary.
3	Remove and clean the syringe and block.
4	Discard unused polymer in the proper waste container. **Note** Do not put unused polymer back into the bottle. Polymer in the syringe decomposes over time at room temperature.
5	In the Manual Control window, select Autosampler Home X, Y Axis and click Execute. Select Autosampler Home Z Axis and click Execute.
6	Turn off the instrument.

Dedicated Equipment and Supplies

Equipment Required

Amplified DNA, equipment, and supplies used to handle amplified DNA should not be taken out of the Amplified DNA Work Area. Samples that have not yet been amplified should never come into contact with these supplies and equipment.

- ABI PRISM 310 Genetic Analyzer
- ABI PRISM 310 Genetic Analyzer Accessories:
  - ABI PRISM 310 Genetic Analyzer Capillary, L_t = 47 cm, L_d = 36 cm, i.d. = 50 µm (P/N 402839), labeled with a green mark
  - ABI PRISM 310 Genetic Analyzer Vials, 4.0 mL, (P/N 401955)
  - ABI PRISM 310 Genetic Analyzer 0.5-mL Sample Tubes (P/N 401957)
  - ABI PRISM 310 Genetic Analyzer Septa for 0.5-mL Sample Tubes (P/N 401956)
  - Syringe, Hamilton 1.0-mL (P/N 604418)
- Benchkote absorbent protector sheets
- Flush-cutting wire cutter (P/N T-6157)
- Freezer, –15 to –25 °C, non-frost-free
- Gloves, disposable, powder-free
- Glassware
- Ice bucket
- Kimwipes
- Lab coat
- Microcentrifuge tubes, 1.5-mL
- Microtube racks
- Nalgene filter apparatus, 150-mL, 0.2-µm CN filter
- Permanent ink pen
- Pipet bulb
- Pipets, serological
- Pipet tips, sterile, disposable hydrophobic filter-plugged

- Pipettors, adjustable, 1–10 µL, 2–20 µL, 20–200 µL, and 200–1000 µL
- Refrigerator
- Repeat pipettor and Combitips that dispense 25–125 µL (optional)
- Sink
- Syringe, 35 cc (optional)
- Tape, labeling
- Thermal cycler
- Tube, 50 mL Falcon
- Tube decapper, autoclavable

Reagents Required

- ABI PRISM 310 10X Genetic Analyzer Buffer with EDTA (P/N 402824)
- AG501 X8 ion exchange resin (Bio-Rad)
- Deionized water
- Formamide, deionized
- GeneScan-500 [ROX] Internal Lane Size Standard Kit (P/N 401734)
- Matrix standards:
  - Dye Primer Matrix Standard Kit (P/N 401114)

IMPORTANT Do not use the TAMRA matrix standard provided in this kit.

  - NED Matrix Standard (P/N 402996)
- Performance Optimized Polymer 4 (POP-4), (P/N 402838)

Software and User Documentation Required

- ABI PRISM 310 Genetic Analyzer Firmware, version 1.0.2 or higher
- ABI PRISM 310 Collection Software, version 1.0.2 or higher
- *ABI PRISM 310 Genetic Analyzer User's Manual* (P/N 903565)
- ABI PRISM 310 Module GS STR POP4 (1 mL) F
- GeneScan Analysis Software, version 2.1
- *GeneScan Analysis 2.1 User's Manual* (P/N 904435)

Preparation of Required Reagents

Deionized formamide

Step	Action
1	Mix 50 mL of formamide and 5 g of AG501 X8 ion-exchange resin.
2	Stir for 30 minutes at room temperature.
3	Check that the pH is greater than 7.0 using pH paper. If the pH is not greater than 7.0, decant the formamide into a beaker containing another 5 g of ion-exchange resin and repeat 30-minute stirring at room temperature.
4	When the pH is greater than 7.0, allow the beads to settle to the bottom of the beaker. Remove the supernatant (formamide), taking care not to disturb the beads.
5	Dispense the deionized formamide into aliquots of 500 µL and store for up to 3 months at –15 to –25 °C.
6	Use one aliquot per set of samples. Discard any unused deionized formamide.

Results and Interpretation

9

Overview

In This Section

This chapter describes various aspects of AmpFℓSTR® Profiler Plus™ results interpretation.

Topic	See page
GeneScan Results	9-2
Determining Genotypes	9-5
Precision Data	9-11
Extra Peaks in the Electropherogram	9-17
Stutter Products	9-17
Addition of 3´ A Nucleotide	9-21
Mixed Samples	9-24
Lack of Amplification of Some Loci	9-28
Effect of Inhibitors	9-28
Degraded DNA	9-30
Effect of DNA Quantity on Results	9-33

GeneScan Results

GeneScan Analysis Software

After the sample files have been analyzed, the Results Control window is used to display the results from each lane of a gel or each injection into a capillary. This window displays the newly analyzed sample files and allows the user to specify the format of the results. Selecting both the Electropherogram and Tabular Data icons is recommended for reviewing the results. For more information on displaying the results, refer to Chapter 6 of the *GeneScan® Analysis 2.1 User's Manual.*

Information Provided in the Electropherogram and Table

Both the electropherogram and the tabular data can be displayed as in Figure 9-1 on page 9-3.

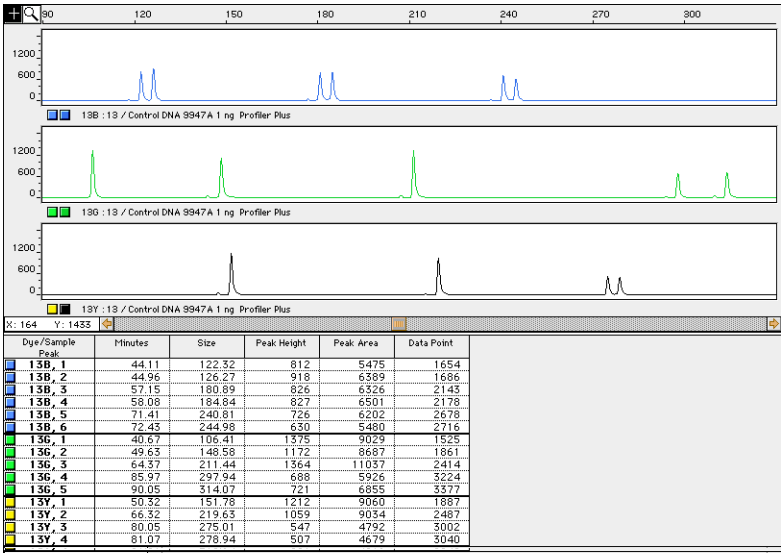
The electropherogram is a chromatographic display with fluorescence intensity indicated as relative fluorescence units (RFU) on the y-axis. After the internal lane size standard has been defined and applied, the electropherogram can be displayed with the base pair size on the x-axis.

Peaks of all heights within the Analysis Range specified in the Analysis Parameters are displayed on the electropherogram, but those peaks below the Peak Amplitude Threshold (minimum peak height) defined in the Analysis Parameters will not be listed in the tabular data.

The columns of the table list the following:

- Column 1 lists the Dye/Sample and Peak (*e.g.*, "13B, 1" indicates the first blue peak in project sample 13).
- Column 2 lists the time it took the dye-labeled molecules to reach the detector (in this case it took the first blue molecule 44.11 minutes to reach the laser detection region on the ABI PRISM® 377 instrument).
- Column 3 lists the base pair size of the peak, as calculated from the GeneScan-500 [ROX] internal size standard curve.
- Column 4 lists the height (relative fluorescence intensity) of the peak.
- Column 5 lists the relative peak area, which is the integral of the fluorescence intensity times the data point (scan number). This value depends on the velocity of the band as it passes the detector.
- Column 6 lists the data point (scan number) of the fragment at its maximum peak height; the data point correlates with the number of

laser scans (or data points collected) from the beginning of the run until the time that the peak maximum is detected.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
13B, 1	44.11	122.32	812	5475	1654
13B, 2	44.96	126.27	918	6389	1686
13B, 3	57.15	180.89	826	6326	2143
13B, 4	58.08	184.84	827	6501	2178
13B, 5	71.41	240.81	726	6202	2678
13B, 6	72.43	244.98	630	5480	2716
13G, 1	40.67	106.41	1375	9029	1525
13G, 2	49.63	148.58	1172	8687	1861
13G, 3	64.37	211.44	1364	11037	2414
13G, 4	85.97	297.94	688	5926	3224
13G, 5	90.05	314.07	721	6855	3377
13Y, 1	50.32	151.78	1212	9060	1887
13Y, 2	66.32	219.63	1059	9034	2487
13Y, 3	80.05	275.01	547	4792	3002
13Y, 4	81.07	278.94	507	4679	3040

Figure 9-1 GeneScan electropherogram of AmpFℓSTR Profiler Plus alleles in AmpFℓSTR® Control DNA 9947A

The GeneScan Analysis Software offers two main options in the Results Control window for viewing formats for electropherograms: Quick Tile Off and Quick Tile On.

- The “Quick Tile Off” format provides the option of displaying results either for multiple colors within a single lane or injection or from multiple lanes or injections in the same panel, *i.e.*, the results are overlaid. This is demonstrated in panel 1 of Figure 9-2.
- The “Quick Tile On” format displays each color of each lane or injection separately, as shown in panels 2–5 of Figure 9-2.

The Quick Tile Off and On feature offers the user versatility in customizing the display of results. Up to eight electropherograms can be tiled at a single time and up to 16 electropherograms may be overlaid at the same time.

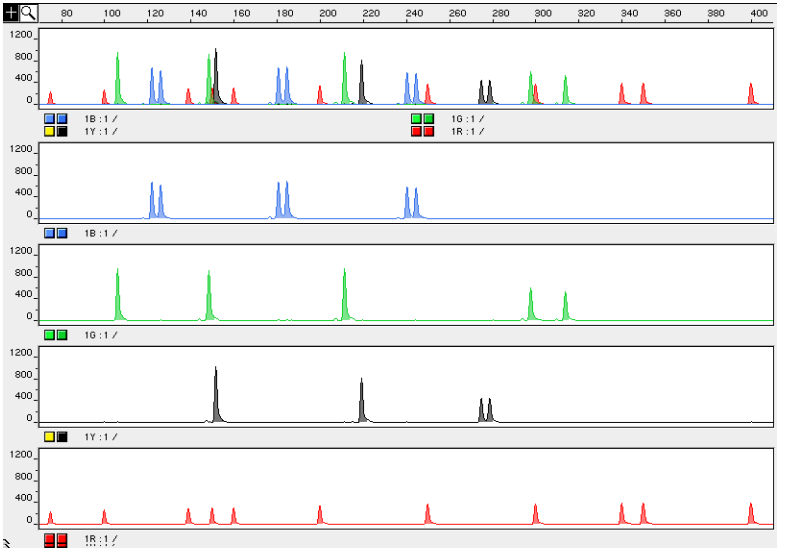


Figure 9-2 Quick Tile Off and Quick Tile On options

Note For a more detailed description see Chapter 6 of the *GeneScan Analysis 2.1 User's Manual*.

Determining Genotypes

AmpFℓSTR Allelic Ladders

The AmpFℓSTR® Blue, AmpFℓSTR® Green II, and AmpFℓSTR® Yellow Allelic Ladders are provided separately in the kit and contain the most common alleles for each locus. Genotypes are assigned by comparing the sizes obtained for the unknown samples with the sizes obtained for the alleles in the allelic ladders.

Alleles contained in the AmpFℓSTR® Allelic Ladders and from population samples have been sequenced to verify both length and repeat structure. The size ranges shown in Table 1-1 on page 1-6 indicate the actual number of nucleotides contained in the smallest and largest allelic ladder alleles for each locus. The size range is the actual base pair size of the sequenced alleles including the 3´ A nucleotide addition. The AmpFℓSTR Profiler Plus PCR Amplification Kit is designed so that a majority of the PCR products contain the non-templated 3´ A nucleotide. For more detail see “Addition of 3´ A Nucleotide” on page 9-21.

The alleles have been named in accordance with the recommendations of the DNA Commission of the ISFH (DNA Recommendations, 1994; Bär *et al.*, 1997). The number of complete 4-bp repeat units observed is designated by an integer. Variant alleles that contain a partial repeat are designated by a decimal followed by the number of bases in the partial repeat. For example, an FGA 26.2 allele contains 26 complete repeat units and a partial repeat unit of 2 bp.

Additional variation has been seen at some loci where alleles exist that differ from integer allele lengths by one or three base pairs (see Table 1-3 on page 1-8). For example, D21S11 allele 33.1 contains 33 complete repeat units and one nonconsensus base pair. Likewise, a D21S11 29.3 allele contains 29 complete repeat units and a partial 3-bp unit (Möller *et al.*, 1994; Gill *et al.*, 1997). Electropherograms of the AmpFℓSTR Blue, AmpFℓSTR Green II, and AmpFℓSTR Yellow Allelic Ladders listing the designation for each allele are shown in Figure 9-3 on page 9-6.

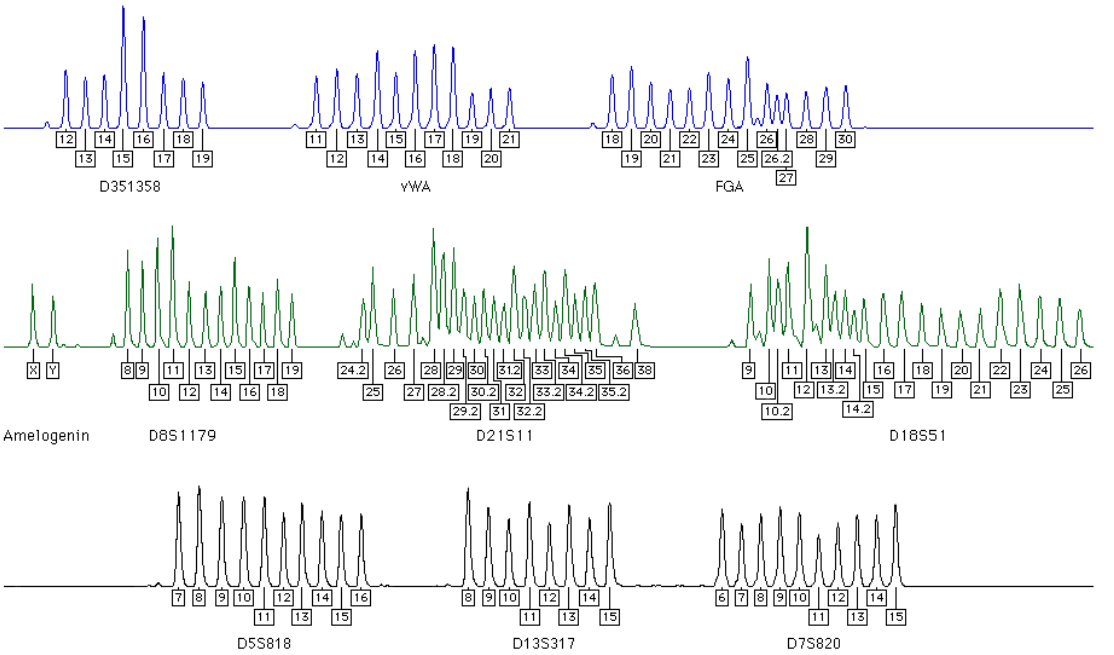


Figure 9-3 Genotyper® plot of the AmpF*ℓ*STR Blue, AmpF*ℓ*STR Green II, and AmpF*ℓ*STR Yellow Allelic Ladders, indicating the designation for each allele

Genotyping Using the AmpF*ℓ*STR Profiler Plus Allelic Ladder

When interpreting AmpF*ℓ*STR Profiler Plus results, genotypes are assigned to sample alleles by comparison of their sizes to those obtained for the known alleles in the AmpF*ℓ*STR Allelic Ladders. Genotypes, not sizes, are used for comparison of data between runs, instruments, and laboratories. We strongly recommend that laboratories use the AmpF*ℓ*STR Profiler Plus Allelic Ladder on each gel or set of capillary injections to convert the allele sizes to genotypes. The main reasons for this approach are outlined below:

- The size values obtained for the same sample can differ between instrument platforms (ABI PRISM® 310 Genetic Analyzer versus ABI PRISM 377 DNA Sequencer) because of differences in the type and concentration of the gel/polymer matrices and in electrophoresis conditions. (See Table 9-2 on page 9-12.)
- Sizes may differ between protocols for the same instrument platform because of differences in gel or polymer concentration, run temperature, gel or capillary thickness, and well-to-read length.

- Slight procedural and reagent variations between gels or between capillaries result in greater size variation than that found between samples on the same gel or between samples injected in the same capillary.

The GeneScan-500 [ROX] Internal Lane Size Standard must be used with the AmpFℓSTR Profiler Plus PCR Amplification Kit. This is because some of the D18S51 alleles are greater than 340 bp. The recommended sizing method, Local Southern, works optimally when there are two internal lane size standard peaks above and two below each possible allele to be sized. Thus, it is important that the 400-bp peak in the GeneScan-500 [ROX] size standard be defined and used in sizing.

The GeneScan-500 [ROX] Internal Lane Size Standard is the same as the GeneScan-350 [ROX] Internal Lane Size Standard, except that additional fragments of 400, 450, 490 and 500 bp are present. The 400-bp peak is the only additional peak in the GeneScan-500 [ROX] size standard that needs to be used for Local Southern Sizing.

The internal lane size standard is run with every sample (AmpFℓSTR Profiler Plus PCR products and AmpFℓSTR Profiler Plus Allelic Ladder) and is used to normalize lane-to-lane or injection-to-injection migration differences, thereby providing excellent sizing precision within a gel or within a set of capillary injections. Size windows based on the allelic ladder are used to assign allele designations and genotypes to the samples. The procedure for running the allelic ladder and determining genotypes is described in detail below.

Note The genotyping of samples by comparison to the AmpFℓSTR Profiler Plus Allelic Ladder can be automated using the AmpFℓSTR Profiler Plus template and Genotyper 2.0 software, as described in Chapter 10.

Loading Allelic Ladder onto the ABI PRISM 310 Genetic Analyzer

Aliquots of the AmpFℓSTR Blue, AmpFℓSTR Green II, and AmpFℓSTR Yellow Allelic Ladders are combined in a single tube to prepare the AmpFℓSTR Profiler Plus Allelic Ladder (see Chapter 8). Inject the AmpFℓSTR Profiler Plus Allelic Ladder at least twice for every set of samples run on the ABI PRISM 310 instrument. For example, when using the 48-well autosampler tray, 45 samples can be analyzed and the allelic ladder analyzed for injections 1, 24, and 48. Include GeneScan-500 [ROX] Internal Lane Size Standard with the AmpFℓSTR Profiler Plus Allelic Ladder.

Loading Allelic Ladder onto the ABI PRISM 377 DNA Sequencer and ABI PRISM 377 DNA Sequencer with XL Upgrade

Aliquots of the AmpFℓSTR Blue, AmpFℓSTR Green II, and AmpFℓSTR Yellow Allelic Ladders are combined in a single tube to prepare the AmpFℓSTR Profiler Plus Allelic Ladder (see Chapter 7). Load two lanes of allelic ladder on each gel (regardless of comb size). For example, load one lane of allelic ladder in lane 1 of the gel and one lane of allelic ladder in the middle of the gel. Include GeneScan-500 [ROX] Internal Lane Size Standard with the AmpFℓSTR Profiler Plus Allelic Ladder.

Note For the loading volume of the AmpFℓSTR Profiler Plus Allelic Ladder refer to the specific instrument protocol section.

To size the AmpFℓSTR Profiler Plus Allelic Ladder alleles:

Step	Action
1	Refer to the “GeneScan Analysis of Data” sections in Chapters 7 and 8 for information on obtaining size values for the alleles.
2	Analyze the lanes/injections containing allelic ladder using the same parameters used for samples.

To compare lanes of AmpFℓSTR Profiler Plus Allelic Ladder:

Step	Action
1	Compare the base pair sizes of one lane or injection of allelic ladder to those obtained for the other lanes or injections of allelic ladder. All corresponding peaks (peaks at the same position in the allelic ladder) should be within ±0.5 bp of each other.

To compare lanes of AmpFℓSTR Profiler Plus Allelic Ladder: *(continued)*

Step	Action
2	If one or more corresponding peaks are not within ±0.5 bp of each other, check the GeneScan-500 [ROX] Internal Lane Size Standard peaks in all allelic ladder lanes or injections to confirm that all GeneScan-500 [ROX] peaks have been assigned the correct size and/or that all peaks are clearly resolved.

To genotype samples:

Note Genotypes can be assigned automatically using the AmpFℓSTR Profiler Plus template file and Genotyper 2.0 software (see Chapter 10).

Step	Action
1	Select one lane or injection of allelic ladder to use for genotyping (our studies have shown that it does not matter which lane or injection of allelic ladder is selected if the alleles in the allelic ladder samples are within ±0.5 bp of each other).
2	Compare the base pair size obtained for each sample allele peak to the sizes obtained for the allelic ladder peaks. An example is shown in Table 9-1 on page 9-9.
3	Assign genotypes to those sample allele peaks falling within ±0.5 bp of the corresponding allelic ladder peak. The allele designation for each allelic ladder peak is given in Figure 9-3 on page 9-6.

Table 9-1 Comparison of sample and allelic ladder allele sizes from an ABI PRISM 377 gel for the AmpFℓSTR Blue STR loci

Locus	Allele	Allelic ladder size (lane 34)	Sample allele size (lane 2)	Difference
D3S1358	14	122.34	122.32	–0.02
	15	126.36	126.29	–0.07
vWA	14	168.96	169.02	+0.06
	17	180.87	180.85	–0.02
FGA	19	224.34	224.37	+0.03
	20	228.48	228.53	+0.05

Figure 9-4 on page 9-10 illustrates the size differences that are typically observed between sample alleles and allelic ladder alleles on the ABI PRISM 310 Genetic Analyzer with POP-4 polymer. The x-axis in Figure 9-4 represents the nominal base pair sizes for a single injection

of AmpFℓSTR Profiler Plus Allelic Ladder, and the dashed lines parallel to the x-axis represent the ±0.5-bp windows. The y-axis is the deviation of each sample allele size from the corresponding allelic ladder allele size. The data includes a total of 542 alleles from 31 population database samples. In this representative example, all sample alleles are within 0.5 bp of a corresponding allelic ladder allele.

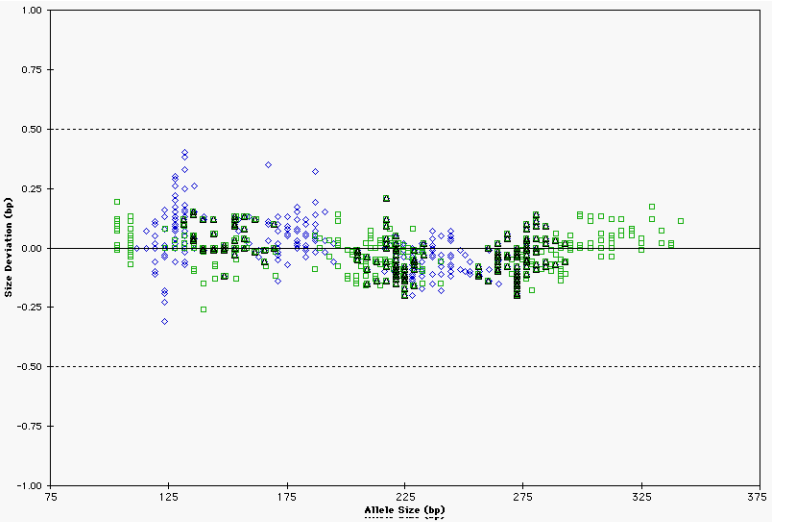


Figure 9-4 Size deviation of 31 samples and two allelic ladders from one injection of allelic ladder on a single ABI PRISM 310 run

The AmpFℓSTR Allelic Ladders contain the common alleles for the D3S1358, vWA, FGA, amelogenin, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820 loci. However, alleles not found in the AmpFℓSTR Allelic Ladders do exist. These “off-ladder” alleles may contain full and/or partial repeat units. An “off-ladder” allele should flag itself by not falling inside the ±0.5 bp window of any known allelic ladder allele.

Note If a sample allele peak is found to be Š0.5 bp from the corresponding allelic ladder peak, then the sample must be rerun to verify the result.

See Table 1-3 on page 1-8 for examples of known “off-ladder” alleles.

Precision Data

Precision and Size Windows

As indicated in the previous section, the recommended method for genotyping is to employ a ±0.5-bp "window" around the size obtained for each allele in the AmpFℓSTR Profiler Plus Allelic Ladder. A ±0.5-bp window allows for the detection and correct assignment of potential off-ladder sample alleles whose true size is only one base different from an allelic ladder allele. Alleles of all possible sizes (within the range of 75–400 bp) should be readily identifiable. Any sample allele that sizes outside a window could be either of the following:

- an "off-ladder" allele, *i.e.*, an allele of a size that is not represented in the AmpFℓSTR Profiler Plus Allelic Ladder (see Table 1-3 on page 1-8 for examples of known off-ladder alleles)
- an allele that does correspond to an allelic ladder allele, but whose size is just outside a window because of measurement error

The measurement error inherent in any sizing method can be defined by the degree of precision in sizing an allele multiple times. Precision is measured by calculating the standard deviation in the size values obtained for an allele that is run in several lanes of one gel or in several injections in one capillary run.

Table 9-2 on page 9-12 indicates typical precision results obtained from 31 database samples and three AmpFℓSTR Profiler Plus Allelic Ladder samples analyzed on two Applied Biosystems instrument platforms: the ABI PRISM 310 Genetic Analyzer (47-cm capillary and POP-4 polymer) and ABI PRISM 377 DNA Sequencer (36-cm wtr plates and 5% Long Ranger). The internal lane size standard used was GeneScan-500 [ROX]. These results were obtained within a set of injections on a single capillary or within a single gel.

As indicated above, sample alleles may occasionally size outside of the ±0.5-bp window for a respective allelic ladder allele because of measurement error. The frequency of such an occurrence is lowest in detection systems having the smallest standard deviations in sizing. Figure 9-4 on page 9-10 illustrates the tight clustering of allele sizes obtained on the ABI PRISM 310 Genetic Analyzer, where the standard deviation in sizing is typically less than 0.15 bp. The instance of a sample allele sizing outside of the ±0.5-bp window because of measurement error is relatively rare when the standard deviation in sizing is approximately 0.15 bp or less (Smith, 1995).

For sample alleles that do not size within a ±0.5-bp window, the PCR product must be rerun to distinguish between a true off-ladder allele *vs.* measurement error of a sample allele that corresponds with an allele in the allelic ladder. Repeat analysis, when necessary, provides an added level of confidence to the final allele assignment. Genotyper software automatically flags sample alleles that do not size within the prescribed window around an allelic ladder allele.

It is important to note that while the precision within a gel or set of capillary injections is very good, the determined allele sizes vary between platforms. Cross-platform sizing differences arise from a number of parameters, including type and concentration of gel/polymer mixture, well-to-read distance, gel thickness, run temperature, and electrophoresis conditions. Variations in sizing can also be found between instrument runs because of these parameters. We strongly recommend that the allele sizes obtained be compared to the sizes obtained for known alleles in the AmpFℓSTR Profiler Plus Allelic Ladder and then converted to genotypes (as described in the previous section on page 9-6).

Table 9-2 Example of precision results

		ABI PRISM 310		ABI PRISM 377	
Allele	**n**	**Mean**	**S.D.**	**Mean**	**S.D.**
D3S1358					
12	3	111.34	0.01	114.10	0.08
13	3	115.51	0.04	118.24	0.07
14	11	119.43	0.07	122.27	0.06
15	14	123.42	0.14	126.34	0.06
15	22	127.48	0.10	130.45	0.08
17	16	131.62	0.15	134.59	0.07
18	6	135.75	0.11	138.75	0.05
19	4	139.96	0.08	143.29	0.04
vWA					
11	4	154.59	0.05	157.20	0.01
12	3	158.84	0.07	160.97	0.01
13	3	163.01	0.02	164.91	0.03
14	6	167.18	0.12	168.88	0.03
15	13	171.09	0.07	172.80	0.04
16	13	175.09	0.05	176.76	0.04

Table 9-2 Example of precision results *(continued)*

		ABI PRISM 310		ABI PRISM 377	
Allele	**n**	**Mean**	**S.D.**	**Mean**	**S.D.**
17	11	179.11	0.06	180.77	0.06
18	12	183.04	0.05	184.79	0.07
19	13	187.00	0.09	188.80	0.07
20	4	190.93	0.08	192.81	0.05
21	3	194.86	0.04	196.90	0.04
FGA					
18	3	216.23	0.05	220.00	0.07
19	5	220.27	0.07	224.15	0.05
20	6	224.28	0.02	228.25	0.04
21	16	228.30	0.05	232.38	0.05
22	12	232.34	0.06	236.48	0.05
23	14	236.39	0.06	240.60	0.04
24	9	240.41	0.07	244.75	0.06
25	12	244.50	0.06	248.82	0.06
26	3	248.53	0.05	252.82	0.04
26.2	3	250.56	0.05	254.84	0.02
27	5	252.61	0.04	256.68	0.06
28	3	256.67	0.06	260.69	0.05
29	4	260.74	0.07	264.60	0.07
30	3	264.85	0.08	268.62	0.08
Amelogenin					
X	34	103.44	0.05	106.31	0.06
Y	22	109.12	0.05	112.02	0.05
D8S1179					
8	3	123.68	0.05	127.06	0.05
9	4	127.69	0.02	131.17	0.02
10	6	131.82	0.04	135.33	0.04
11	7	135.95	0.06	139.55	0.06
12	11	140.22	0.11	144.08	0.04
13	17	144.77	0.05	148.58	0.06
14	16	149.24	0.04	152.71	0.04

Table 9-2 Example of precision results *(continued)*

		ABI PRISM 310		ABI PRISM 377	
Allele	**n**	**Mean**	**S.D.**	**Mean**	**S.D.**
15	14	153.64	0.07	156.65	0.03
16	4	157.98	0.06	160.46	0.05
17	3	162.13	0.08	164.37	0.03
18	3	166.25	0.04	168.34	0.02
19	3	170.33	0.06	172.32	0.06
D21S11					
24.2	3	187.10	0.07	189.08	0.03
25	3	189.08	0.02	191.08	0.07
26	3	192.97	0.04	195.11	0.02
27	8	196.93	0.07	199.21	0.06
28	14	200.77	0.06	203.22	0.02
28.2	3	202.76	0.02	205.22	0.02
29	10	204.73	0.06	207.30	0.04
29.2	3	206.73	0.04	209.38	0.03
30	12	208.68	0.05	211.41	0.04
30.2	5	210.63	0.03	213.46	0.05
31	7	212.67	0.06	215.53	0.03
31.2	7	214.57	0.07	217.54	0.03
32	4	216.61	0.05	219.58	0.04
32.2	6	218.54	0.06	221.62	0.04
33	3	220.55	0.05	223.67	0.03
33.2	5	222.48	0.06	225.66	0.03
34	3	224.52	0.02	227.73	0.03
34.2	3	226.49	0.02	229.73	0.02
35	6	228.60	0.08	231.84	0.03
35.2	3	230.45	0.04	233.81	0.04
36	6	232.46	0.06	235.74	0.07
38	4	240.42	0.06	243.85	0.06

Table 9-2 Example of precision results *(continued)*

		ABI PRISM 310		ABI PRISM 377	
Allele	**n**	**Mean**	**S.D.**	**Mean**	**S.D.**
D18S51					
9	3	270.49	0.06	274.25	0.05
10	4	274.62	0.02	278.17	0.04
10.2	5	276.61	0.06	280.06	0.03
11	4	278.70	0.07	282.10	0.03
12	5	282.83	0.05	286.01	0.03
13	5	286.93	0.05	289.95	0.02
13.2	4	288.97	0.04	291.87	0.04
14	9	291.03	0.06	293.90	0.05
14.2	3	293.01	0.06	295.78	0.04
15	10	295.06	0.02	297.84	0.02
16	16	299.16	0.06	301.81	0.04
17	10	303.54	0.05	305.80	0.04
18	9	307.94	0.05	309.80	0.04
19	10	312.31	0.04	313.82	0.04
20	5	316.61	0.04	317.89	0.05
21	4	320.93	0.04	321.96	0.02
22	4	325.11	0.02	326.12	0.06
23	3	329.28	0.09	330.19	0.07
24	3	333.45	0.04	334.33	0.05
25	3	337.55	0.01	338.50	0.05
26	3	341.56	0.06	342.71	0.06
D5S818					
7	3	131.32	0.06	134.49	0.05
8	8	135.40	0.06	138.65	0.00
9	5	139.61	0.06	143.14	0.05
10	10	144.04	0.05	147.60	0.06
11	14	148.47	0.05	151.80	0.05
12	21	152.85	0.06	155.72	0.04
13	12	157.13	0.04	159.52	0.05
14	4	161.39	0.08	163.45	0.02

Table 9-2 Example of precision results *(continued)*

		ABI PRISM 310		ABI PRISM 377	
Allele	**n**	**Mean**	**S.D.**	**Mean**	**S.D.**
15	3	165.41	0.03	167.27	0.02
16	3	169.53	0.06	171.32	0.04
D13S317					
8	8	205.04	0.02	207.15	0.06
9	4	209.05	0.06	211.26	0.08
10	3	213.03	0.07	215.38	0.05
11	13	217.06	0.09	219.54	0.05
12	25	221.02	0.05	223.56	0.05
13	10	225.01	0.05	227.63	0.05
14	7	229.00	0.05	231.71	0.05
15	3	233.02	0.03	235.76	0.02
D7S820					
6	4	256.05	0.05	259.42	0.03
7	3	260.09	0.08	263.30	0.04
8	12	264.18	0.04	267.18	0.05
9	8	268.15	0.06	271.13	0.04
10	22	272.24	0.06	274.99	0.04
11	14	276.29	0.06	278.87	0.04
12	12	280.31	0.07	282.72	0.04
13	6	284.38	0.06	286.62	0.02
14	3	288.49	0.05	290.51	0.04
15	3	292.43	0.04	294.37	0.06

Extra Peaks in the Electropherogram

Overview

Peaks other than the target alleles may be detected on the electropherogram displays. Described below are several causes for the appearance of the extra peaks, including the stutter product (found at the n–4 position), incomplete 3´ A nucleotide addition (found at the n–1 position), and mixed DNA samples.

Stutter Products

The PCR amplification of tetranucleotide STR loci typically produces a minor product peak four bases shorter (n–4) than the corresponding main allele peak. This is referred to as the stutter peak or product. Sequence analysis of stutter products at tetranucleotide STR loci has revealed that the stutter product is missing a single tetranucleotide core repeat unit relative to the main allele (Walsh *et al.*, 1996).

The proportion of the stutter product relative to the main allele (percent stutter) is measured by dividing the height of the stutter peak by the height of the main allele peak. Such measurements have been made for hundreds of samples at the loci used in the AmpFℓSTR Profiler Plus PCR Amplification Kit.

Some of the general conclusions from these measurements and observations are as follows:

- Within each AmpFℓSTR Profiler Plus locus, the percent stutter generally increases with allele length, as shown in Figures 9-5 through 9-7 on pages 9-18 through 9-20. Smaller AmpFℓSTR Profiler Plus alleles display a lower level of stutter relative to the longer alleles within each locus. This is reflected in Figures 9-5 through 9-7, where minimal data points are plotted for some smaller alleles, as stutter could not be detected for many of these samples.
- For the alleles within a particular locus, the percent stutter is generally greater for the longer allele in a heterozygous sample (this is related to the first point above).
- The highest percent stutter observed for any D5S818, D13S317, or D7S820 allele was less than 8%, for any D8S1179 allele less than 9%, for any D3S1358, vWA, FGA, or D21S11 allele less than 10%, and for any D18S51 allele less than 13%.

- Each allele within a locus displays a percent stutter that is quite reproducible. The average standard deviation is 0.8% for the Green II loci and 0.6% for the Blue and Yellow loci. This means that most of the time the percent stutter will be less than ±2.5 percentage points from the mean. For example, if the percent stutter for a particular allele averages 5% for multiple replicates, then it is rare that the percent stutter for this allele will ever be less than 2.5% or greater than 7.5%.
- Given the above two observations, stutter percentages greater than approximately 12% (D8S1179, D5S818, D13S317, D7S820), 15% (D3S1358, vWA, FGA, D21S11), or 18% (D18S51) are not expected to be observed in single-source samples. (The maximum percent stutter expected for a particular allele can be derived from its specific mean percent stutter and standard deviation.) See page 9-24 for evaluation of mixed samples.
- The percent stutter does not change significantly with the quantity of input DNA, based on a minimum peak height of 150 relative fluorescence units (RFU) and a maximum of approximately 4500 RFU. The measurement of percent stutter may be unnaturally high for main peaks that are off-scale. See Chapter 6 for identification of off-scale data. Loading or injecting less of the PCR product will yield accurate quantitation (as described in the protocol on page 9-23).

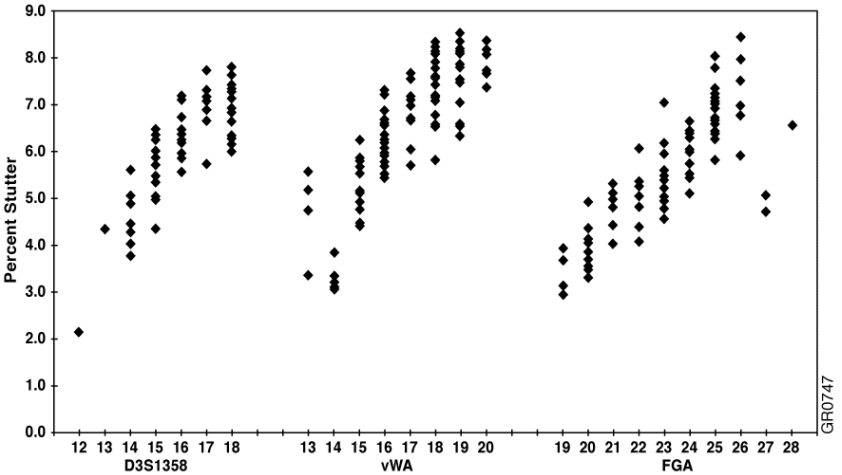


Figure 9-5 Stutter percentages for the D3S1358, vWA, and FGA loci. Off-scale and "shoulder" peaks (see Figure 9-8 on page 9-21) are not included in the data.

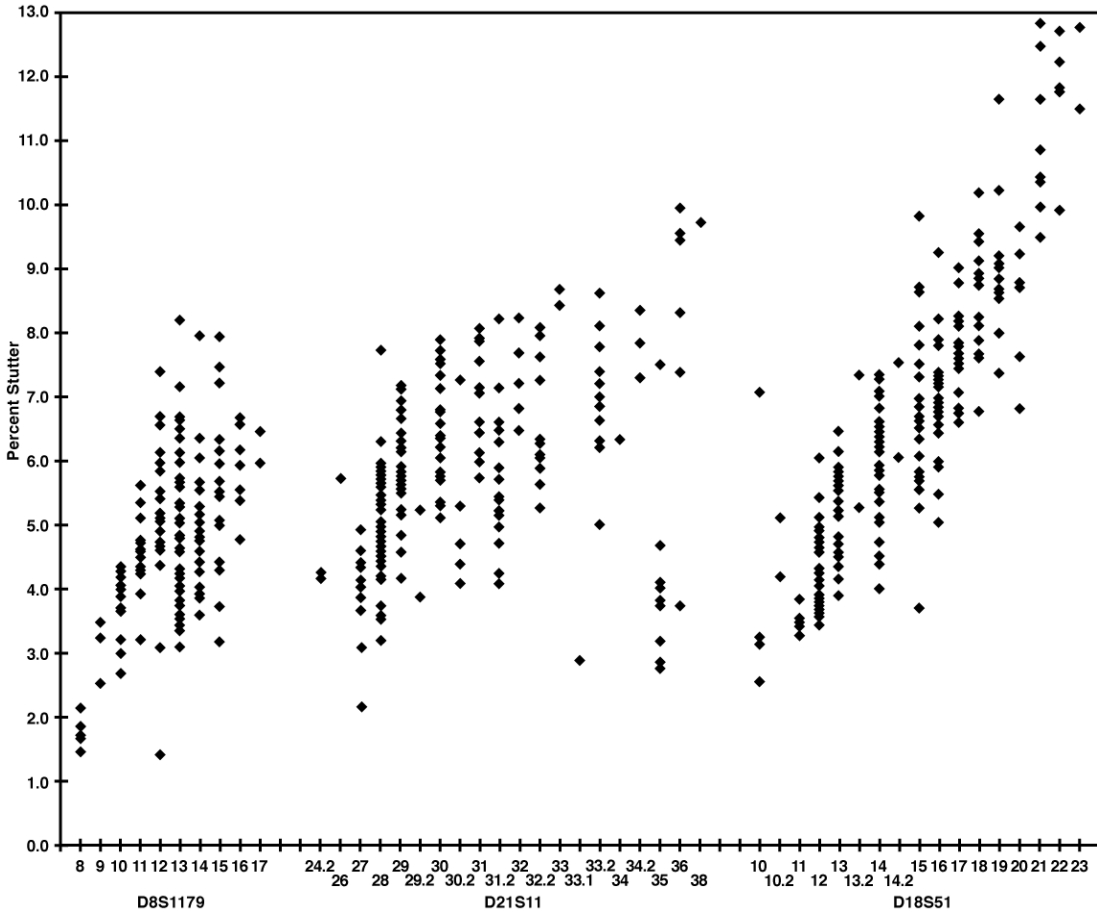


Figure 9-6 Stutter percentages for the D8S1179, D21S11, and D18S51 loci. Off-scale and “shoulder” peaks (see Figure 9-8 on page 9-21) are not included in the data.

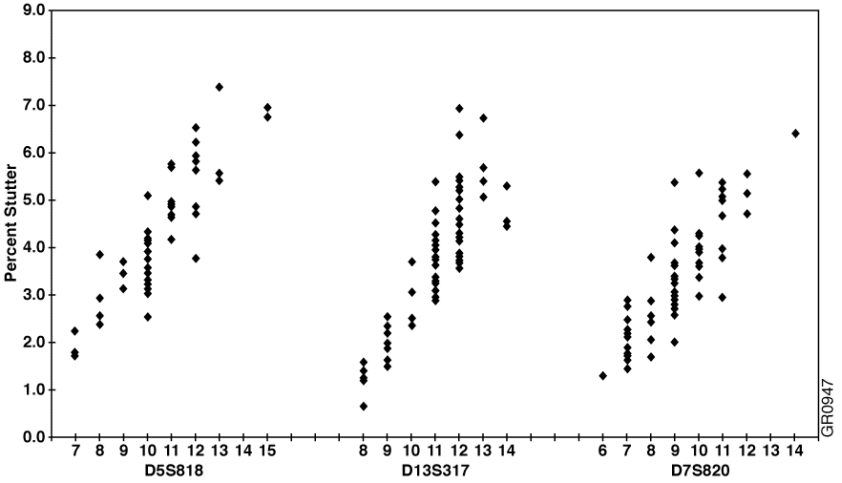
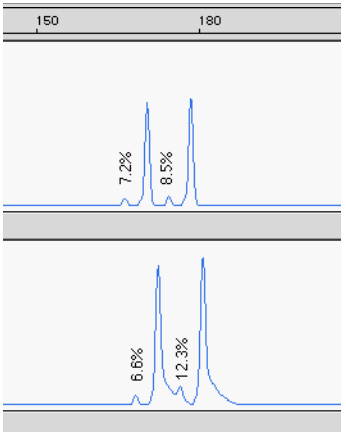


Figure 9-7 Stutter percentages for the D5S818, D13S317, and D7S820 loci. Off-scale and "shoulder" peaks (see Figure 9-8 on page 9-21) are not included in the data.

- When two alleles differ in size by eight base pairs, the stutter peak for the long allele can reside on the shoulder of the peak for the shorter allele. In this instance, the height of the stutter peak is additive with the height of the shoulder.

  Because of this "shoulder" effect, the percent stutter for stutter peaks in this position depends on the resolution of the gel or capillary system (Figure 9-8) and may exhibit more variability between samples and between detection platforms than stutter peaks not on a shoulder. If the electropherogram shows all peaks resolved to baseline, then this "shoulder" effect should not apply.



High resolution of peaks

Low resolution of peaks

Figure 9-8 Shoulder effect (lower panel) observed for two vWA alleles differing in size by 8 bp

Addition of 3´ A Nucleotide

AmpliTaq Gold®, like many other DNA polymerases, can catalyze the addition of a single nucleotide (predominately adenosine) to the 3´ ends of double-stranded PCR products (Clark, 1988). This non-template addition results in a PCR product that is one base pair longer than the actual target sequence, and the PCR product with the extra nucleotide is referred to as the "+A" form.

The efficiency of "A addition" is related to the particular sequence of the DNA at the 3´ end of the PCR product. The AmpFℓSTR Profiler Plus PCR Amplification Kit includes two main design features that promote maximum A addition:

- The primer sequences have been optimized to encourage A addition.
- The last thermal cycling step is 60 °C for 45 minutes.

This final extension step gives the AmpliTaq Gold DNA polymerase extra time to complete A addition to all double-stranded PCR product. STR systems that have not been optimized for maximum A addition may have "split peaks", where each allele is represented by two peaks one base pair apart (Figure 9-9 on page 9-22).

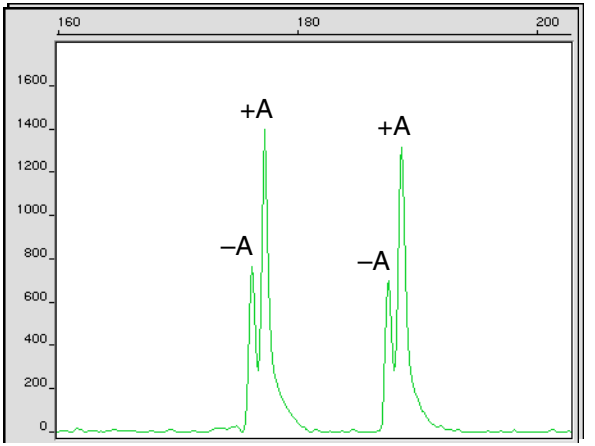


Figure 9-9 Split peaks resulting from incomplete A nucleotide addition due to omission of the 45-minute extension step

Note that the final extension step for the AmpFℓSTR Profiler Plus kit is 45 minutes, 15 minutes longer than for the AmpFℓSTR Blue or AmpFℓSTR Green kits. For a given amount of input DNA, more overall PCR product is generated with the AmpFℓSTR Profiler Plus kit because ten loci are co-amplified as opposed to three or four loci for the other AmpFℓSTR kits. Thus, in the case of the AmpFℓSTR Profiler Plus kit, the AmpliTaq Gold DNA Polymerase generally requires this extra time to complete the A nucleotide addition at the 3´ ends.

Lack of full A nucleotide addition may be observed in AmpFℓSTR Profiler Plus results when the amount of input DNA is greater than approximately 2.5 ng. The reason for this is that more time is needed for AmpliTaq Gold DNA Polymerase to add the A nucleotide to all molecules as more PCR product is generated. Amplification of too much input DNA will also result in off-scale data (see Chapter 6 for more information on off-scale data).

Sample tubes that contain too much PCR product should be incubated again at 60 °C (to complete A addition), diluted (to bring the data on-scale) and then rerun, as described below:

Step	Action
1	Remove the tube of PCR product from the refrigerator or freezer.
2	Place the tube in the appropriate thermal cycler and incubate at 60 °C for 45 minutes. Residual AmpliTaq Gold DNA Polymerase activity will complete the A nucleotide addition.
3	Dilute the PCR product in 1X TE buffer. For example, combine: ♦ 2 µL of PCR product ♦ 6 µL of 1X TE buffer
4	**If using the ABI PRISM 310:**
	a. Combine 1.5 µL of diluted PCR product and 25 µL of deionized formamide/GeneScan-500 [ROX] solution (see page 8-11). b. Proceed to step 8 of "Preparing Samples and AmpFℓSTR Profiler Plus Allelic Ladder" on page 8-12.
	If using the ABI PRISM 377 or ABI PRISM 377 with XL Upgrade:
	a. Combine 4 µL of diluted PCR product and 5 µL of FLS/GeneScan-500 [ROX] solution (see page 7-14). b. Proceed to step 9 of "Preparing Samples and AmpFℓSTR Profiler Plus Allelic Ladder" on page 7-15.

The dilution factor necessary to achieve on-scale data for any specific sample will depend on the extent to which the sample was initially overamplified. It may be wise to prepare more than one dilution (each at a different dilution factor) for a sample. Also, in some extreme cases—for example, if more than approximately 10 ng of input DNA was used in the initial PCR—optimal results may be achieved by reamplifying the sample using less input DNA.

Mixed Samples

Evidence samples may contain DNA from more than one individual. The possibility of multiple contributors should be considered when interpreting the results. In the discussion below, a peak is defined as any peak that is greater than 150 RFU. We recommend a minimum peak height threshold to avoid typing less than 35 pg of input DNA (see page 9-33).

Detection of mixed samples

Each of the following can aid in determining whether a sample is a mixture:

- the presence of greater than two alleles at a locus
- the presence of a peak at a stutter position that is significantly greater in percentage than what is typically observed in a single-source sample

  See the section on “Stutter Products” beginning on page 9-17 and Figures 9-5 through 9-7.

- significantly imbalanced alleles for a heterozygous genotype

  The peak height ratio is defined as the height of the lower peak (in RFU) divided by the height of the higher peak (in RFU), expressed as a percentage. Mean peak height ratios and standard deviations observed for alleles in the AmpFℓSTR Profiler Plus loci in unmixed population database samples are as follows:

– D3S1358	93 ± 4%	(n = 68 observations)
– vWA	93 ± 5%	(n = 74 observations)
– FGA	93 ± 5%	(n = 80 observations)
– Amelogenin	90 ± 6%	(n = 46 observations)
– D8S1179	92 ± 6%	(n = 93 observations)
– D21S11	91 ± 7%	(n = 95 observations)
– D18S51	91 ± 6%	(n = 100 observations)
– D5S818	92 ± 5%	(n = 65 observations)
– D13S317	93 ± 5%	(n = 73 observations)
– D7S820	93 ± 6%	(n = 79 observations)

  For all ten loci, the mean peak height ratios indicate that the two alleles of a heterozygous individual are generally very well

balanced. Ratios less than 70% are rare in normal, unmixed samples.

If the peak height ratio is less than 70% for one locus, and there are no other indications that the sample is a mixture, the sample should be reamplified and reanalyzed to determine if the imbalance is reproducible. Reproducible imbalance at only one locus may indicate a mixture. Other possible causes of imbalance at a locus are degraded DNA, presence of inhibitors, extremely low amounts of input DNA, or the presence of an allele containing a rare sequence that does not amplify as efficiently as the other allele. Amplification and analysis of additional loci may assist in the interpretation of the sample.

Resolution of genotypes in mixed samples

A sample containing DNA from two sources can be comprised (at a single locus) of any of the seven genotype combinations listed below.

- heterozygote + heterozygote, no overlapping alleles (four peaks)
- heterozygote + heterozygote, one overlapping allele (three peaks)
- heterozygote + heterozygote, two overlapping alleles (two peaks)
- heterozygote + homozygote, no overlapping alleles (three peaks)
- heterozygote + homozygote, overlapping allele (two peaks)
- homozygote + homozygote, no overlapping alleles (two peaks)
- homozygote + homozygote, overlapping allele (one peak)

Specific genotype combinations and input DNA ratios of the samples contained in a mixture determine whether it is possible to resolve the genotypes of the major and minor component(s) at a single locus.

The ability to obtain and compare quantitative values for the different allele peak heights on Applied Biosystems instruments provides additional valuable data to aid in resolving mixed genotypes. This quantitative value is much less subjective than comparing relative intensities of bands on a stained gel.

Ultimately, the likelihood that any sample is a mixture must be determined by the analyst in the context of each particular case, including the information provided from known reference samples.

Limit of detection of the minor component

Mixtures of the two DNA samples shown in Figure 9-10 were examined at ratios from 1:1–20:1, where sample A was the major component and sample B was the minor component. The total amount of genomic input DNA mixed at each ratio was 1 ng.

The samples were amplified in a GeneAmp® PCR System 9600 and were electrophoresed and detected using an ABI PRISM 377 DNA Sequencer. The results are shown in Figure 9-11 on page 9-27.

The genotypes of the samples in Figure 9-10 are the following:

	Genotype	
Allele	**Sample A (Male)**	**Sample B (Female)**
Amelogenin	X, Y	X, X
D3S1358	15, 16	15, 18
vWA	14, 16	17, 19
FGA	24, 26	23, 24
D8S1179	12, 13	13, 13
D21S11	28, 31	30, 33
D18S51	12, 15	17, 19
D5S818	11, 11	11, 13
D13S317	11, 11	11, 11
D7S820	7, 12	9, 10

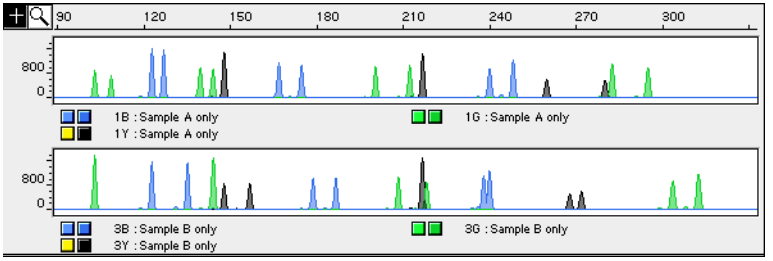


Figure 9-10 Reference samples for mixture study shown in Figure 9-11 on page 9-27

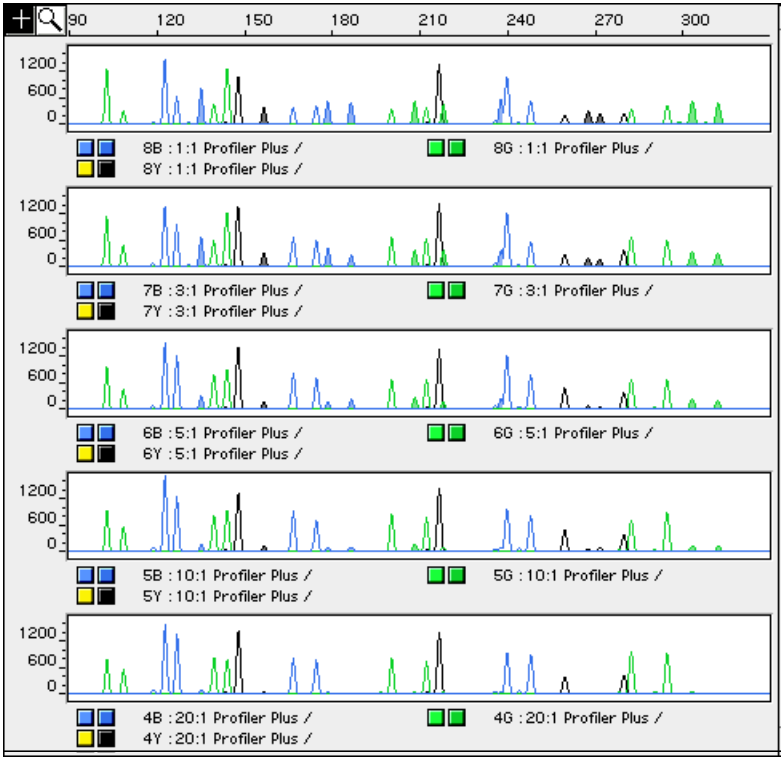


Figure 9-11 Results of the two DNA samples from Figure 9-10 mixed together at defined ratios and amplified with the AmpF*ℓ*STR Profiler Plus PCR Amplification Kit. The A:B ratios shown are 1:1, 3:1, 5:1, 10:1, and 20:1 (top to bottom). Alleles attributable only to the minor component are highlighted.

For these 1 ng total DNA mixtures, the limit of detection is when the minor component is present at approximately one tenth of the concentration of the major component. The limit of detection for the minor component is influenced by the combination of genotypes in the mixture.

Lack of Amplification of Some Loci

Overview

As with any multi-locus system, the possibility exists that not every locus will amplify. This is most often observed when the DNA substrate has been severely degraded or when the DNA sample contains PCR inhibitors. Since each locus is an independent marker, whose results are not based upon information provided by the other markers, results generally can still be obtained from the loci that do amplify.

Effect of Inhibitors

Heme compounds have been identified as PCR inhibitors in DNA samples extracted from bloodstains (Akane *et al.*, 1994; DeFranchis *et al.*, 1988). It is believed that the inhibitor is co-extracted and co-purified with the DNA and subsequently interferes with PCR by inhibiting polymerase activity.

Bovine serum albumin (BSA) can prevent or minimize the inhibition of PCR, most likely by binding to the inhibitor (Comey *et al.*, 1994). Since the presence of BSA can improve the amplification of DNA from blood-containing samples, BSA has been included in the AmpF*ℓ*STR® PCR Reaction Mix at a concentration of 8 µg per 50-µL amplification. BSA has also been identified as an aid in overcoming inhibition from samples containing dyes, such as in denim (Comey *et al.*, 1994).

To examine the effects of hematin on the AmpF*ℓ*STR Profiler Plus amplification results, DNA samples were amplified using the AmpF*ℓ*STR Profiler Plus PCR Amplification Kit reagents (including the BSA-containing PCR reaction mix) in the presence of varying concentrations of purified hematin. The concentrations of hematin used were 0 µM, 20 µM, 22 µM, 28 µM, and 30 µM. When the amount of hematin was increased to a concentration that started to inhibit the PCR, D18S51 and D7S820 were the first loci to drop out in each experiment, followed by FGA (Figure 9-12 on page 9-29).

When the JOE (green) dye-labeled STR loci (D8S1179, D21S11, and D18S51) were amplified in singleplex reactions, inhibition occurred at slightly lower concentrations of hematin than when the loci were amplified in the AmpF*ℓ*STR Profiler Plus multiplex reaction.

These results suggest that the increased overall concentration of oligonucleotide primers in the AmpF*ℓ*STR Profiler Plus multiplex reaction helps to overcome hematin inhibition, *i.e.*, multiplex reactions may be more resistant to inhibition than singleplex reactions.

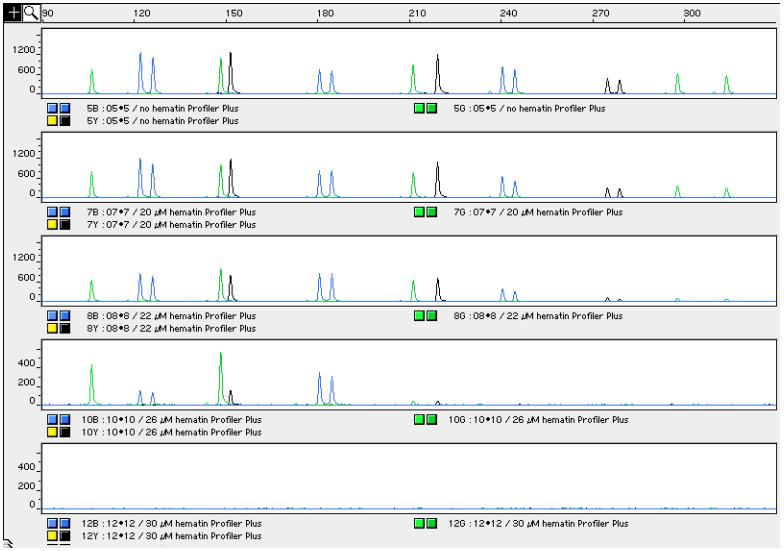


Figure 9-12 DNA amplified with the AmpF*ℓ*STR Profiler Plus PCR Amplification Kit in the presence of varying concentrations of hematin: 0, 20 µM, 22 µM, 26 µM, and 30 µM

Degraded DNA

As the average size of the DNA approaches the size of the target sequence, the amount of PCR product generated is reduced. This is due to the reduced number of intact templates in the size range necessary for amplification.

Degraded DNA was prepared to examine the potential for differential amplification of loci. High molecular weight DNA was incubated with the enzyme DNase I for varying amounts of time. The DNA was examined by agarose gel analysis to determine the average size of the DNA fragments at each timepoint. The results of the gel analysis of the degraded DNA are shown in Figure 9-13.

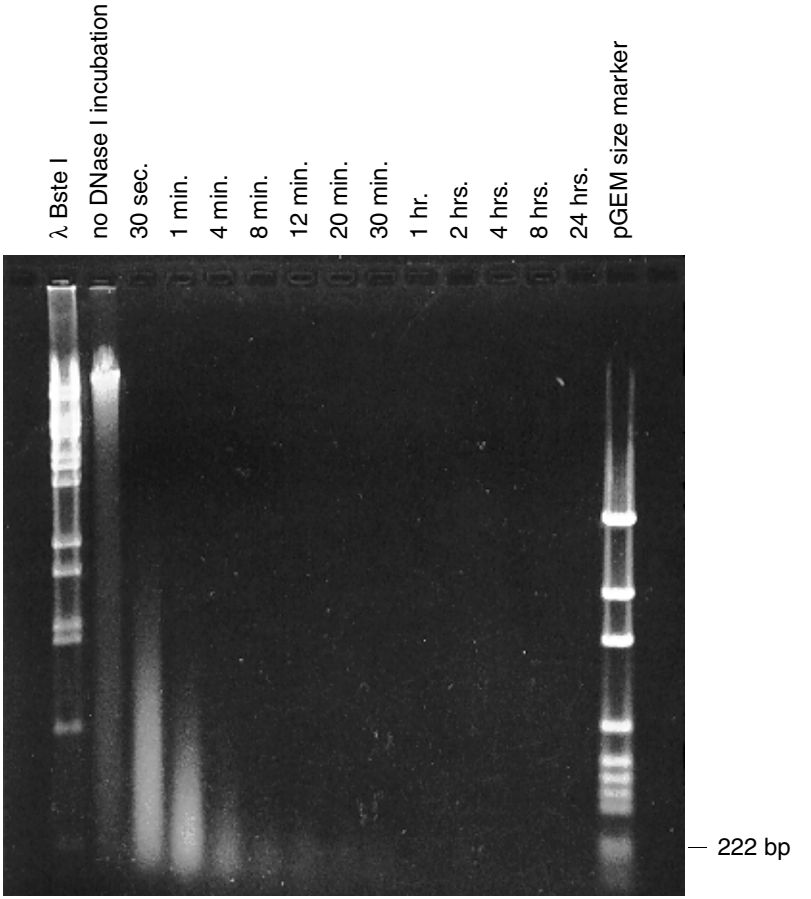


Figure 9-13 Agarose gel of degraded genomic DNA

Four nanograms of degraded DNA (or 1.5 ng undegraded DNA) was amplified using the AmpF*ℓ*STR Profiler Plus PCR Amplification Kit (all ten primer pairs together) and also in reactions containing each locus-specific primer pair individually.

The electropherograms in Figure 9-14 show the AmpF*ℓ*STR Profiler Plus amplification results of a DNA sample with no DNase I treatment (1.5 ng amplified) and those of the 30-second, 1-, 4-, and 8-minute incubations (approximately 4 ng amplified).

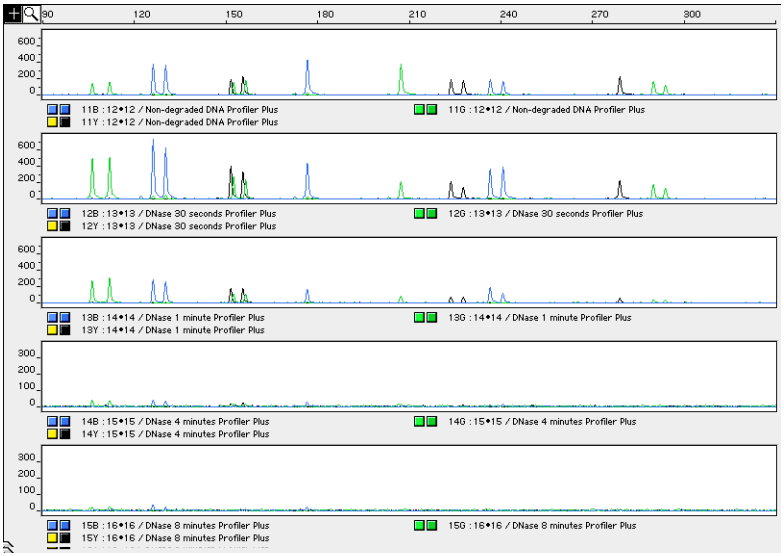


Figure 9-14 Amplifications of DNA incubated for various times with DNase I

The loci failed to amplify in the order of decreasing size as the extent of degradation progressed: D18S51 was the first locus to drop out, followed by FGA and so forth. The same result at each timepoint was obtained whether the DNA samples were amplified for each locus alone or co-amplified with the AmpF*ℓ*STR Profiler Plus PCR Amplification Kit (Figure 9-15 on page 9-32). No advantage in amplifying each locus alone was observed.

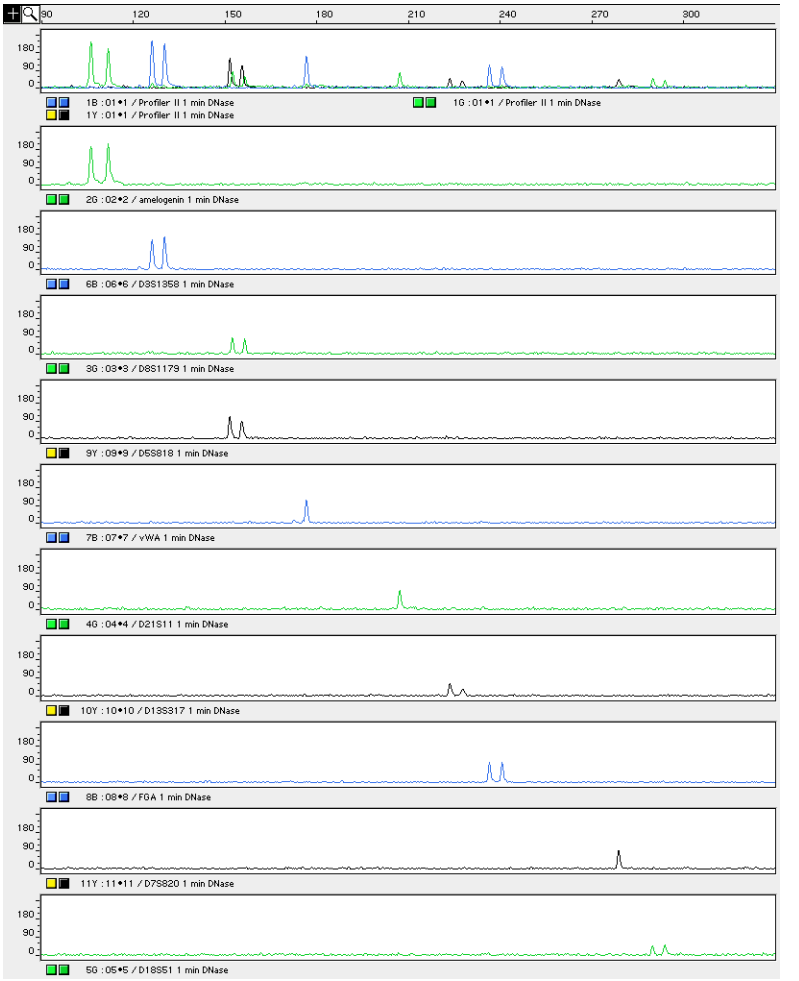


Figure 9-15 Multiplex and single-locus amplifications of the DNA sample incubated for 1 minute with DNase I

When degraded DNA is suspected to have compromised amplification of one or more loci, the molecular weight of the DNA can be assessed by agarose gel analysis. If the DNA is degraded to an average of 400 bp in size or less, adding more DNA template to the AmpFℓSTR Profiler Plus amplification reaction can help produce a typeable signal for all loci. Adding more DNA to the amplification provides more of the necessary size template for amplification.

Effect of DNA Quantity on Results

Importance of Quantitation

The amount of input DNA added to the PCR reaction should be between 1.0 and 2.5 ng. The DNA sample should be quantitated prior to amplification using a system such as the QuantiBlot® Human DNA Quantitation Kit (P/N N808-0114). The final DNA concentration should be in the range of 0.05–0.125 ng/µL so that 1.0–2.5 ng of DNA will be added to the PCR reaction in a volume of 20 µL. If the sample contains degraded DNA, amplification of additional DNA may be beneficial.

If too much DNA is added to the PCR reaction, then the increased amount of PCR product that is generated can result in the following:

- fluorescence intensity that exceeds the linear dynamic range for detection by the instrument ("off-scale" data)

  Off-scale data is a problem for two reasons:

  – Quantitation (peak height and area) for off-scale peaks is not accurate. For example, an allele peak that is off-scale can cause the corresponding stutter peak to appear higher in relative intensity, thus increasing the calculated percent stutter.

  – Multicomponent analysis of off-scale data is not accurate, which results in poor spectral separation ("pull-up").

  Identification of off-scale peaks and multicomponent analysis are discussed in Chapter 6.

- incomplete A nucleotide addition

Follow the protocol on page 9-23 for treatment of samples that have incomplete A nucleotide addition and/or off-scale peaks. Alternatively, the sample can be re-amplified using less DNA.

When the total number of allele copies added to the PCR is extremely low, unbalanced amplification of the two alleles of a heterozygous individual may occur. This is due to stochastic fluctuation in the ratio of the two different alleles.The PCR cycle number and amplification conditions have been specified to produce peak heights of <150 RFU for a sample containing 35 pg human genomic DNA (corresponding to ten total allele copies). Peak heights <150 RFU should be interpreted with caution.

Individual laboratories may find it useful to determine an appropriate minimum peak height threshold based on their own results using low amounts of input DNA. Typically, peak heights >150 RFU are

consistently obtained when approximately 250–500 pg of DNA is added to the PCR amplification.

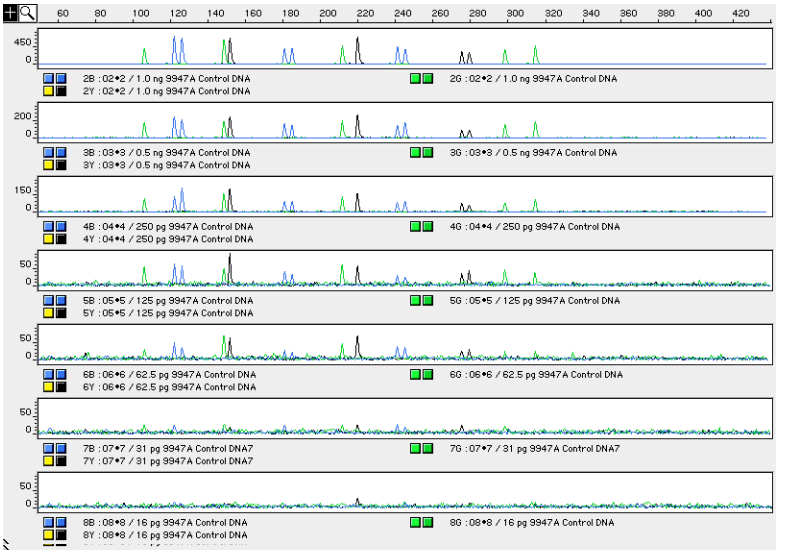


Figure 9-16 Effect of amplifying various amounts of AmpF*ℓ*STR Control DNA 9947A ranging from 16 pg to 1 ng. Note that the y-axis scale differs in many of these panels.

Amplification Using Bloodstained FTA Cards

FTA™-treated DNA collection cards can be useful for the collection, storage, and processing of biological samples. For example, a small punch of the bloodstained card can be placed directly into an amplification tube, purified, and amplified without transferring the evidence. Our studies have indicated that a 1.2-mm bloodstained punch contains approximately 15–20 ng DNA. Accordingly, an appropriate cycle number for this high quantity of DNA is 25 cycles.

In the example shown in Figure 9-17, a 1.2-mm punch of a bloodstained FTA card was purified according to the “DNA Purification Using FTA Cards” procedure (provided with the FTA Purification Reagent). The punch was then amplified directly in the MicroAmp® tube for only 25 cycles.

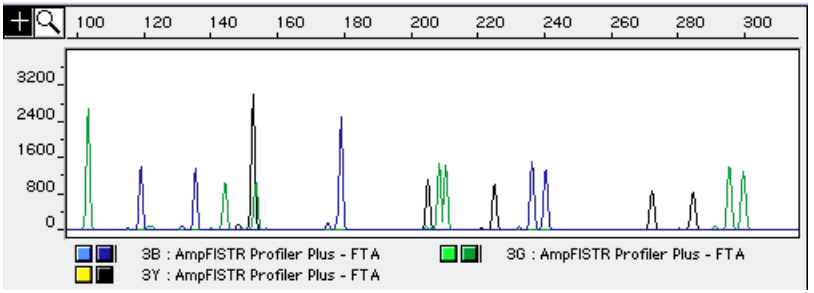


Figure 9-17 AmpF*ℓ*STR Profiler Plus results from a 1.2-mm FTA bloodstain punch (25 cycle amplification)

Automated Genotyping

10

Using Genotyper Software for Automated Genotyping

Overview

Genotyper® 2.0 Software is used to convert allele sizes obtained from GeneScan® Analysis 2.1 Software into allele designations automatically and to build tables containing the genotype information. Genotypes are assigned by comparing the sizes obtained for the unknown sample alleles with the sizes obtained for the alleles in the allelic ladder.

A Genotyper template file that contains macros specifically written for use with the AmpFℓSTR® Profiler Plus™ PCR Amplification Kit is provided with this manual and should be used with AmpFℓSTR Profiler Plus data. Install the template onto your computer following the instructions in the ReadMe file.

Note You must have Genotyper software version 2.0 or higher to run the AmpFℓSTR Profiler Plus template. This version of Genotyper software requires a Macintosh® Quadra or Power Macintosh® computer. Refer to the *Genotyper User's Manual* (P/N 904648) and *Genotyper Applications Tutorials* (P/N 904649) for more detailed information about the Genotyper software. The Human Identification Tutorial and HID template file included with the Genotyper 2.0 software package are for tutorial purposes only.

Before Running Genotyper

GeneScan Analysis Software sample data (particularly the allelic ladder) must meet a few specific requirements before the macros in the AmpFℓSTR Profiler Plus template file can be used. These requirements are described in this section.

Sample Info

All samples must have a unique sample description in the Sample Info column of the GeneScan sample sheet so that the macros in the AmpFℓSTR Profiler Plus template file can build a table. Samples with an empty Sample Info field will not be incorporated into the table of genotypes. In particular, lanes or injections that contain the AmpFℓSTR

Profiler Plus Allelic Ladder (*i.e.*, the combined AmpFℓSTR® Blue, AmpFℓSTR® Green II, and AmpFℓSTR® Yellow Allelic Ladders) must have the word “ladder” in the Sample Info. The first lane or injection of “ladder” that is found is the one that is used by the Kazam macro in the AmpFℓSTR Profiler Plus template file to determine the sizes in the allele categories that will be used for genotyping.

It is possible to skip the first lane or injection of allelic ladder and use the second lane or injection of allelic ladder for genotyping instead. After importing the sample files, but before running the Kazam macro, remove the word “ladder” from the Sample Info in all three dye colors for the first lane or injection of allelic ladder in the Dye/lanes Window. Make sure that the word “ladder” is entered for Sample Info in the second lane or injection of allelic ladder. See step 3 on page 10-3 for a description of how to access the Sample Info field in the Dye/lanes Window.

GeneScan Analysis Peak Recognition

All allele peaks in the allelic ladder for each locus must be “recognized” in GeneScan, meaning that each allele peak must have an entry in the GeneScan table. Thus, all allele peaks in each allelic ladder must have a peak height value in relative fluorescence units (RFU) that is greater than the Peak Amplitude Threshold that was specified in the GeneScan Analysis Parameters. Also, all allele peaks in each allelic ladder must be resolved. For example, the FGA 26, 26.2 and 27 alleles must be resolved such that each peak has an entry in the GeneScan table.

AmpFℓSTR Profiler Plus Template

The AmpFℓSTR Profiler Plus template file contains macros that perform the following steps automatically:

- find the lane or injection containing the allelic ladder
- create allele size categories that are centered on the sizes obtained for the allelic ladder alleles
- assign the appropriate allele label to sample alleles that size within the allele size categories
- remove labels from stutter peaks by applying a filter
- build a table containing genotypes for all samples

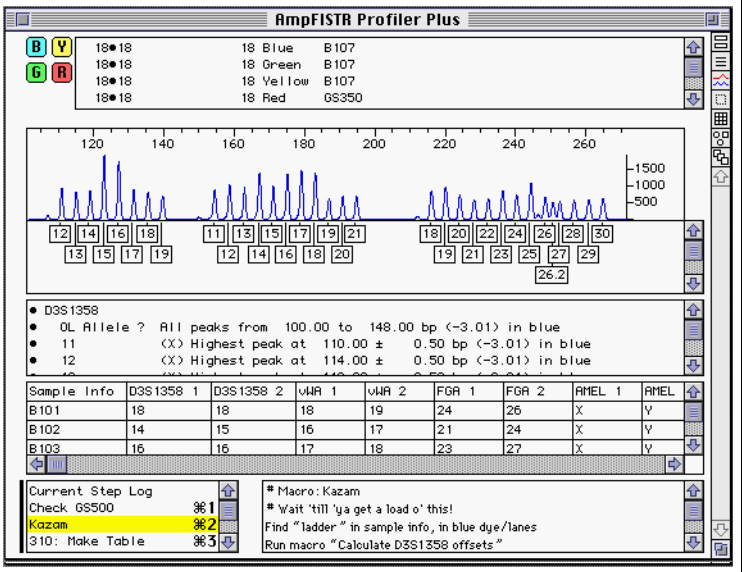
Using the AmpFℓSTR Profiler Plus Template

Use the following procedure to assign genotypes to AmpFℓSTR Profiler Plus alleles automatically.

To use the AmpFℓSTR Profiler Plus template:

Step	Action
1	Double-click the AmpFℓSTR Profiler Plus template file to launch the Genotyper 2.0 application and open the template file simultaneously. **Note** The AmpFℓSTR Profiler Plus template is a Stationery pad, which means that a new document is created when the template file is opened. The original template file is not overwritten.
2	To import the GeneScan sample files: a. Under the File menu, choose Import GeneScan File(s). b. Select all checkboxes (Import raw data, Blue, Green, Yellow, and Red). c. Select the project file and click Import.
3	If each sample does not already have Sample Info completed in the sample sheet, this can be accomplished in Genotyper as follows: a. Under the Views menu, choose Show Dye/lanes Window. b. Select the first sample row by clicking on the row. c. Click the mouse cursor in the Sample Info box at the top of the window, and type the sample designation or description. d. Repeat steps b and c to enter a sample description for every dye/lane in the list. Enter the same sample description for all dye colors of a single sample.
4	From the Macro list at the bottom left of the Main Window, select Check GS500.
5	Under the Macro menu, choose Run Macro. In the plot window that appears, scroll through each sample to verify that each GeneScan-500 peak (from 75–400 bp) was assigned the correct size in the GeneScan Analysis Software.

To use the AmpFℓSTR Profiler Plus template: *(continued)*

Step	Action
6	From the Macro list at the bottom left of the Main Window, select Kazam (Figure 10-1).  **Figure 10-1** Genotyper Main Window with Kazam macro highlighted
7	Under the Macro menu, choose Run Macro. This macro may take a few minutes to run. When it is finished, a plot window opens with the AmpFℓSTR Blue Allelic Ladder and sample allele peaks labeled.
8	Examine data and edit peaks (see “Examining Data” on page 10-5).
9	Print the electropherograms in the plot window by choosing Print under the File menu.
10	In the Main Window, click the green G button at the top left. Under the Views menu, select Show Plot Window. Repeat steps 8 and 9.
11	In the Main Window, click the yellow Y button at the top left. Under the Views menu, select Show Plot Window. Repeat steps 8 and 9.

Examining Data

Check that the peaks in the allelic ladder are labeled correctly, as shown in Figure 9-3 on page 9-6. If the peaks are not labeled correctly, see “Troubleshooting Automated Genotyping” on page 10-10. Scroll through the samples below the allelic ladder to examine the peak labels in each electropherogram.

Peak labeling

- Allele categories (which appear as dark gray bars in the plot window) are defined to be ±0.5 bp wide. Peaks that size within ±0.5 bp of an allele category will have a label indicating the allele designation (Figure 10-2 on page 10-6).
- Peaks that do not size within an allele category will have a label indicating “OL Allele?” (off-ladder allele).
- The Kazam macro includes a step that removes labels from stutter peaks by applying a percentage filter. Labels are removed from peaks that are within 5 bp shorter than a main peak and that have peak heights less than a specified percentage of the main peak. The percentage filters for these loci are 12% for D8S1179, D5S818, D13S317, and D7S820, and 15% for D3S1358, vWA, FGA, and D21S11, and 18% for D18S51.
- A sample allele peak must have been “recognized” in GeneScan before it can be recognized by Genotyper. Thus, sample allele peaks that are below the Peak Amplitude Threshold that was specified in the GeneScan Analysis Parameters cannot be labeled in Genotyper.

  Also, because no information is imported for peaks that are not recognized in GeneScan, they will not align exactly by size relative to the x-axis size scale in the Genotyper Plot Window.

Peak Editing

- Clicking on a labeled peak removes the label. Clicking again on the same peak labels the peak by size (in bp).
- To change the label back to allele designation, select Change Labels... under the Analysis menu, then select the checkbox next to “the category’s name” and click OK.

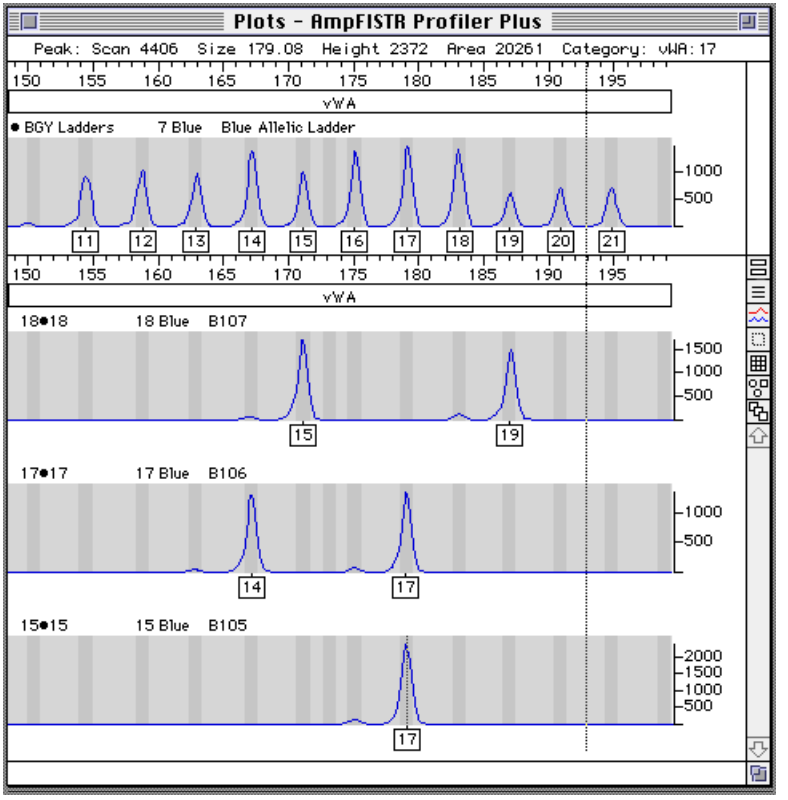


Figure 10-2 Genotyper plot of allelic ladder and sample results for the vWA locus

Plot Window Viewing Options

♦ Zoom in and out on regions of the plot window as follows:

Step	Action
1	Click and drag in a region of an electropherogram in the plot window to draw a box around the desired size range (the vertical size of the box is not important).
2	Type c R (hold down the command key and type the letter R) to zoom in.
3	Type c H to zoom out completely.

♦ To view electropherograms from more than one dye color in the Plot Window:

Step	Action
1	Under the Views menu, choose Show Dye/lanes Window.
2	Click on the desired Dye/lane rows. **Note** Hold down the shift key on the keyboard to select multiple adjacent Dye/lane rows. Hold down the command (c) key to select Dye/lane rows that are not adjacent.
3	Under the Views menu, choose Show Plot Window.

Making Tables

Four macros for making tables are included in the AmpFℓSTR Profiler Plus template file. Two of these tables, Make Allele Table and Make Codis Table, are very simple and contain only Sample Info and genotype data. The other two tables, 310: Make Table and 377: Make Table, contain additional information.

The contents and unique features of each table are described below. All four of the tables have two features in common:

♦ A locus that has no labeled peaks will have zeros in the cells of the table for that locus.

♦ Loci that have homozygous alleles will have the allele designation indicated twice in the table.

Make Allele Table

This table has Sample Info in the first column, and allele designations for each locus in columns 2–21. The first two labeled peaks within each locus appear in the table.

Make Codis Table

This table has Sample Info in the first column, locus name in the second column, and allele designations in columns 3–4. This alternative table is for laboratories that will be importing the table into DataBanker software, which creates a CODIS compatible file.

310: Make Table

This table can be used if the data was generated on the ABI PRISM 310 instrument. This table has Sample Info in the first column, Sample Comment in the second column, locus name in the third column, and

allele designations in columns 4–7. Four columns are provided for allele designations to accommodate mixed samples. The first four labeled peaks within each locus appear in the table. The remaining five table columns are as follows:

- Overflow: If more than two peaks are labeled at one locus, the text "> two labels" will appear in this column.
- Low Signal: If the height of any peak at a locus is greater than the Peak Amplitude Threshold specified in the GeneScan Analysis Parameters (see page 7-6 or page 8-7) but less than 150 RFU, the text "< 150 RFU" will appear in this column.
- Saturation: If the raw data signal for any peak at a locus is greater than 8100 RFU, the text "310: off-scale" will appear in this column.
- Edited Label: The text "Edited" will appear in this column for any loci where the peak labels were edited manually. For example, clicking on an unlabeled peak in the Plot Window to add a label constitutes an edit.
- Edited Row: The text "Edited" will appear in this column for any rows in the table that contain cells that have been edited after initial creation of the table.

377: Make Table

This table can be used if the data was generated on the ABI PRISM 377 instrument. This table is the same as 310: Make Table, except the Saturation warning, "377: off-scale likely," will appear for raw data signals greater than 5000 RFU. See page 6-15 for more information on off-scale data.

IMPORTANT All electropherograms should be examined, and their peaks edited as described in the previous section, before making a table.

Creating and Using Tables

Step	Action
1	From the Macro list at the bottom of the Genotyper Main Window, click on one of the four table macros.
2	Under the Macro menu, choose Run Macro.
3	Under the Views menu, select Show Table Window to view the table in full screen mode.

Creating and Using Tables *(continued)*

Step	Action
4	**For all tables except the Make Allele Table:**
	Clicking in a cell of the table causes the corresponding sample electropherogram to appear in the plot window, as follows: a. Click on any cell in the table. This causes this locus region of the corresponding electropherogram to appear in the plot window for that sample. b. Zoom out (c H) to view all loci for a particular dye color for the corresponding sample.
5	To edit the cells of the table: a. Click in a cell of the table that contains an allele designation. b. Under the Edit menu, select Edit Cell… c. Type the desired information in the box and click OK.
6	Print the table by selecting Print under the File menu.
7	**Optional**Under the Table menu, select Export to File… to save the table as a Microsoft Excel-readable document.
8	Under the File menu, select Save to save the Genotyper template with data.

Troubleshooting Automated Genotyping

Observation	Probable Cause	Recommended Action
Warning message: "Could not complete 'Run Macro' command because no dye/lanes are selected"	The word "ladder" is not in Sample Info for the lane or injection of allelic ladder.	Type the word "ladder" in Sample Info, as described in step 3 of "Using the AmpFℓSTR Profiler Plus Template" on page 10-3. The word "ladder" must be entered for each dye color (Blue, Green, Yellow) in Sample Info for the AmpFℓSTR Profiler Plus Allelic Ladder sample.
Warning message: "Could not complete 'Run Macro' command because the labeled peak could not be found"	One or more peaks in the allelic ladder are below the Peak Amplitude Threshold that was specified in the GeneScan Analysis Parameters.	a. In the GeneScan Analysis 2.1 Software, lower the Peak Amplitude Threshold values for each dye color in the Analysis Parameters. b. Reanalyze the sample file(s) containing the allelic ladder. c. Import all sample files into a new Genotyper project, and run the Kazam macro again.

Understanding the AmpFℓSTR Profiler Plus Template File

Overview

This section describes the organization and functionality of the AmpFℓSTR Profiler Plus template file. Read this section for a greater understanding of the macros and steps that are used in the AmpFℓSTR Profiler Plus template file.

Categories

In the Genotyper software, each allele is defined by a category. Each category contains information about the allele size, size range, and dye color. To view the list of categories in the AmpFℓSTR Profiler Plus template file, choose Show Categories Window, under the Views menu. The categories for each locus are listed together under the locus name. The locus is called a group.

In the Categories Window, each locus actually has two sets of categories. For example, the D3S1358 locus has one list of categories under the group “D3S1358” and another list of categories under the group “D3S1358.os.” The categories in the D3S1358 group are allele categories and are used for allele assignment. Categories with the “.os” suffix are called offset categories and are described below.

The base pair size indicated in each category is the actual nucleotide length of the allele (including the 3’ A nucleotide) as determined by sequencing. This is the “reference size.” One main function of the macros in the AmpFℓSTR Profiler Plus template is to offset the reference sizes relative to the sizes obtained for the alleles in the lane or injection of allelic ladder. These offset steps are performed by the Calculate [locus] Offsets macros, located in the Macro list of the Genotyper Main Window. After the macros are run, the calculated offset values are indicated in parentheses near the end of each category line in the Categories Window.

An example of how to interpret the offset values is given here for D3S1358 allele 14. The reference size for this allele is 122 bp. On a particular ABI PRISM 310 injection, the size obtained for D3S1358 allele 14 was 119.06 bp. The offset value is calculated as 119.06 – 122 = –2.94. In this example, the actual category size used for allele assignment is 119.06 (equals 122–2.94), which is the size of the D3S1358 allele 14 in this particular injection of the allelic ladder. In other words, the category sizes used for genotyping are equivalent to the allele sizes obtained in the lane or injection of allelic ladder.

Offset Categories

As noted above, the offset values are determined automatically by the Calculate [locus] Offsets macros. These macros use the offset categories (categories with an ".os" suffix) to find the allele peaks in the allelic ladder and to determine the correct offset values for each allele category.

Find and recognize the leftmost (first) allele peak in each allelic ladder

- Identification of the leftmost peak is accomplished through the specifications of the first ".os" category listed within each group of offset categories. This first ".os" category (12.os in the case of D3S1358) is specified to find all peaks in a range of ±7 bp around the reference size for the indicated allele.
- Each Calculate [locus] Offsets macro applies a 20% filter to all peaks in the ±7-bp range in the allelic ladder. This avoids the first stutter peak in each allelic ladder and thus identifies the first allele peak as the leftmost peak.

Apply the appropriate offset value to each allele in succession

Once the leftmost allele peak in each allelic ladder is identified, the offset value determined for this allele is applied to the relevant allele(s) in the allele categories.

For example, assume that the offset value determined by the 12.os category in the D3S1358.os group is (–3.01) for a particular lane or injection of allelic ladder. This offset value is then applied to the allele 12 category in the D3S1358 group, thus setting the correct offset value for the 12 allele.

In order for the software to find the next allele peak in the D3S1358 allelic ladder (allele 13), the offset value for the 12.os allele is also applied to the 13.os category. The result of this operation is that the 13.os category size will be 4 bp longer than the 12.os category. In other words, allele 13 is expected to be found at a size that is 4 bp longer than allele 12.

To maximize the ease of peak recognition, the size width for all offset categories is ±1 bp, as compared to the allele categories, which have a width of ±0.5 bp. Once allele 13 is recognized in the D3S1358 allelic ladder, the correct offset value is calculated and assigned to the appropriate categories.

This process of peak recognition, offset calculation, and offset assignment is carried out for each of the alleles in each of the allelic ladders.

Off-ladder Alleles and Virtual Alleles

In the example above, the 12.os offset value (-3.01) is also applied to two other categories in the D3S1358 group: “OL Allele?” and allele 11.

The OL Allele? category is specified to span the range of known D3S1358 alleles and is intended to catch off-ladder alleles that do not size within one of the allele categories.

Allele 11 in this case is a “virtual” allele category, meaning that this allele is not present in the allelic ladder. The virtual category exists to assign an allele designation to allele 11, which is a known allele not included in the allelic ladder.

Because allele 11 is specified to have the same offset value as allele 12, the allele category sizes for these two alleles will differ by exactly 4 bp, which is the same as the difference in their reference sizes. Specifying a size for allele 11 that is 4 bp shorter than allele 12 is generally expected to be a reasonable estimate since alleles 11 and 12 differ by a single repeat unit (4 bp).

The D3S1358 group also contains virtual allele categories for two other alleles, 15.2 and 20. The offset value for the 15.2 allele is specified to be the same as for the 15 allele. In this case, since reference sizes for these two alleles differ by 2 bp, the category size used for allele 15.2 will be 2 bp longer than for allele 15. Likewise, the offset for allele 20 is specified to be the same as for allele 19, so the allele category size for allele 20 will be 4 bp longer than for allele 19.

Many of the loci in the Categories Window contain virtual allele categories. For example, the FGA locus contains a virtual category for most of the possible 2-bp length variants, except for FGA allele 26.2, which is included in the allelic ladder.

Kazam Macro

The Kazam macro is the top level macro that contains all of the instructions and steps necessary for determination of genotypes relative to the allelic ladder. Kazam references the Calculate [locus] Offsets macros for each locus, and contains further instructions to label peaks at each locus and to filter (remove labels from) the stutter peaks. The various steps in Kazam can be viewed in Genotyper by clicking on the Kazam line in the Macro list, and then choosing Show Step Window, under the Views menu.

To illustrate the steps involved in filtering the stutter peaks, consider again the example of the D3S1358 locus:

- In the Step Window for the Kazam macro, scroll down to the line that reads “Select category: D3S1358”.
- Click on the line four rows below that reads “Remove labels from peaks followed by a 666% higher, labeled peak within 0.00 to 5.00 bp”.
- Under the Macro menu, choose Edit Step.

  In the Filter Labels window that appears, there are four options (checkboxes) for filtering. In this example, the filtering option for D3S1358 is denoted in the last checkbox. This filtering option includes another checkbox that reads “(higher by at least 666%).”

  The percentage value in this filtering option refers to the higher (in peak height) peak relative to a peak that is within 5 bp shorter in size. For example, a peak with a peak height of 1000 RFU is 666% higher than a peak with a height of 150 RFU (1000 /150 × 100% = 666%). Likewise, the 150 RFU peak is 15% of the 1000 RFU peak (150/1000 × 100% = 15%). The value of 666% is the inverse of 15%, which is the recommended way to express percent stutter.
- To use a filter value different than 666% for D3S1358, simply input another value (no more than three digits) and click Replace. For example, to use a filter threshold of 12% (remove labels from peaks that are ð12% of a higher peak), enter a value of 833% (100/12 × 100% = 833%).

Because the filtering option in the Filter Labels window described above will accept no more than three digits for filter percentage (up to 999%), filtering of peaks ð10% of a higher peak cannot be specified by this filtering option.

When the desired filtering level for a particular locus is below 10%, a different filtering option in the Filter Labels window is used. In this case, the second filtering option (checkbox) in the Filter Labels window is used. This option reads, “Remove labels from peaks whose height is less than ____% of the highest peak in a category’s range.”

Note that this particular option does not include any condition regarding the bp size of the filtered peak relative to a higher peak. Indeed, this second filtering option will remove labels from all peaks that are less than a specified percentage of the highest peak observed anywhere in the locus range. Also, note that this second filtering option does not require the ‘inverse’ percentage to be used.

Kazam (20% Filter)

The standard Kazam macro is written so that a different filter threshold can be used for each locus (the steps for each locus are written separately in the macro—see “Examining Data” on page 10-5). The Kazam macro thus provides maximum flexibility and the opportunity to customize the filter that is used for each locus.

A different version of the Kazam macro called “Kazam (20% filter)” is also provided in the Macro list. This macro is simpler in that the same filtering step is specified for all loci. To view the various steps in the Kazam (20% filter) macro, click on the Kazam (20% filter) line in the Macro list, and then choose Show Step Window from the Views menu. The first filter step for this macro (which applies to the sample alleles) reads, “Remove labels from peaks whose height is less than 20% of the highest peak in a category’s range.”

To edit this filter value, proceed as described earlier: click on this step in the Step Window, then choose Edit Step from the Macro menu. Note that this macro uses the second filter option in the Filter Labels window. If desired, change the value from 20% to some other value, and click Replace.

The Kazam (20% filter) macro is provided as an option for laboratories that would like to use one general filter value for all loci. This macro can also be used when a high level of filtering specificity is not required, as in the typing of single source samples, like database samples or paternity samples.

Modifying the Template

The original AmpFℓSTR Profiler Plus template file can be modified so that the changes made to the macros or settings are used as the default:

Step	Action
1	Close all Genotyper windows, but do not quit the application.
2	Find the AmpFℓSTR Profiler Plus template icon. Click on the icon to select it.
3	Under the File menu, select Get Info (or type c I).
4	Deselect the checkbox for Stationery pad at the bottom of the window. Close the Get Info window.
5	Open the original AmpFℓSTR Profiler Plus template by double-clicking on its icon.
6	Make any desired changes.
7	Save the template file by choosing Save under the File menu.
8	Repeat steps 2 and 3.
9	Select the checkbox for Stationery pad at the bottom of the window. Close the Get Info window.

11 Troubleshooting

Table 11-1 Troubleshooting for the AmpFℓSTR Profiler Plus PCR Amplification Kit

Observation	Possible Causes	Recommended Actions
Faint or no signal from both the AmpFℓSTR® Control DNA 9947A and the DNA test samples at all loci	Incorrect volume or absence of either AmpFℓSTR® PCR Reaction Mix, AmpFℓSTR® Profiler Plus™ Primer Set, or AmpliTaq Gold® DNA Polymerase	Repeat amplification, carefully following the protocol in Chapter 5.
	No activation of AmpliTaq Gold DNA Polymerase	Repeat amplification, making sure to hold reactions initially at 95 °C for 11 minutes.
	PCR Master Mix not vortexed thoroughly before aliquoting	Vortex PCR Master Mix thoroughly.
	AmpFℓSTR Profiler Plus Primer Set exposed to too much light	Store Primer Set protected from light.
	GeneAmp® PCR Instrument System malfunction	Refer to the thermal cycler user's manual and check instrument calibration.
	Incorrect thermal cycler parameters	Check the protocol in Chapter 5 for correct thermal cycler parameters.
	Tubes not seated tightly in the thermal cycler during amplification (DNA Thermal Cycler 480)	Push reaction tubes firmly into contact with block after first cycle. Repeat test.
	GeneAmp PCR System 9600 or 2400 heated cover misaligned	Align GeneAmp 9600 or 2400 heated cover properly so that white stripes align after twisting the top portion clockwise.

Table 11-1 Troubleshooting for the AmpFℓSTR Profiler Plus PCR Amplification Kit *(continued)*

Observation	Possible Causes	Recommended Actions
Faint or no signal from both the AmpFℓSTR Control DNA 9947A and the DNA test samples at all loci	Wrong PCR reaction tube	Use Applied Biosystems GeneAmp Thin-Walled Reaction Tubes for the DNA Thermal Cycler 480 and MicroAmp Reaction Tubes with Caps for the GeneAmp 9600 and 2400.
	MicroAmp® Base used with tray/retainer set and tubes in GeneAmp 9600 or 2400	Remove MicroAmp Base from tray/retainer set and repeat test.
	Insufficient PCR product loaded onto gel or injected into capillary	**For ABI PRISM® 377 gels:** Mix 4 µL of PCR product and 5 µL of FLS/GeneScan-500 [ROX] solution (see page 7-14).
		For ABI PRISM® 310 runs: Mix 1.5 µL of PCR product and 25 µL of deionized formamide/GeneScan-500 [ROX] solution (see page 8-11).
Positive signal from AmpFℓSTR Control DNA 9947A but no signal from DNA test samples	Quantity of test DNA sample is below assay sensitivity	Quantitate DNA and add 1.0–2.5 ng of DNA. Repeat test.
	Test sample contains PCR inhibitor (*e.g.*, heme compounds, certain dyes)	Quantitate DNA and add minimum necessary volume. Repeat test.
		Wash the sample in a Centricon-100 (see page 3-12). Repeat test.
	Test sample DNA is degraded	If possible, evaluate the quality of DNA sample by running an agarose gel. If DNA is degraded, re-amplify with an increased amount of DNA.
	Dilution of test sample DNA in H_2O or wrong buffer (*e.g.*, wrong EDTA concentration)	Re-dilute DNA, making sure to use TE Buffer (with 0.1 mM EDTA).

Table 11-1 Troubleshooting for the AmpFℓSTR Profiler Plus PCR Amplification Kit *(continued)*

Observation	Possible Causes	Recommended Actions
More than two alleles present at a locus	Presence of exogenous DNA	Use appropriate techniques to avoid introducing foreign DNA during laboratory handling.
	Too much DNA in reaction	Use recommended amount of template DNA (1.0–2.5 ng).
	Mixed sample	See Chapter 9.
	Amplification of stutter product (n-4 bp position)	See Chapter 9.
	Lack of 100% 3´ A base addition (n-1 bp position)	See Chapter 9. Be sure to include the final extension step of 60 °C for 45 minutes in the PCR.
	Signal exceeds dynamic range of instrument (off-scale data). See Chapters 6 and 9.	Treat and dilute the PCR product as described on page 9-23.
		Quantitate DNA and re-amplify sample, adding 1.0–2.5 ng of DNA.
	Poor spectral separation (bad matrix)	Follow the steps in Chapter 6 for creating a matrix file.
		Confirm that Filter Set F modules are installed and used for analysis.
	Spillover between gel comb lanes	Follow the recommended sample loading protocol. Do not use a shark's tooth comb.
Some but not all loci visible on electropherogram	Test sample DNA is degraded	If possible, evaluate the quality of DNA sample by running an agarose gel. If DNA is degraded, re-amplify with an increased amount of DNA.
	Test sample contains PCR inhibitor (*e.g.*, heme compounds, certain dyes)	Quantitate DNA and add minimum necessary volume. Repeat test.
		Wash the sample in a Centricon-100 (see page 3-12). Repeat test.

Table 11-1 Troubleshooting for the AmpFℓSTR Profiler Plus PCR Amplification Kit *(continued)*

Observation	Possible Causes	Recommended Actions
ABI PRISM 377 DNA Sequencer and ABI PRISM 377 DNA Sequencer with XL Upgrade		
Misshapen wells	Suction when removing comb	Lay gel flat, pour 1X TBE over comb, and remove comb slowly.
Severely bowed gel image	Clamping bottom of gel plates	Clamp plates as recommended in protocol.
	Gel extruded between plates into upper buffer reservoir	After cleaning plates, wash briefly in 3M HCl, then rinse with water. Remake gel.
Fuzzy or smeared bands in electropherogram	Dirty gel plates	Clean plates with Alconox cleaner and a soft cloth. Rinse thoroughly with deionized water.
	Excess urea in wells before loading	Flush wells immediately before loading.
	Wrong TBE buffer formulation	Remake buffer, carefully following protocol.
Signal gets weaker over time	Outdated or mishandled reagents	Check expiration dates on reagents and store and use according to manufacturers instructions. Compare with fresh reagents.
Red or green smearing on gel	Gel dried out before running	Wrap gel ends with damp Kimwipes and plastic wrap before storing. Use gel within 24 hours.
Inconsistent mobilities from gel to gel	Total polymer percentage wrong	Remake gel, carefully following protocol.
	Wrong TBE buffer concentration	Remake buffer, carefully following protocol.
	Poor quality reagents	Remake 5X TBE and gel solution stock using fresh reagents from a reliable source. Urea must also be ultrapure.
	Dissolved O_2 concentration	When degassing the acrylamide solution, keep vacuum strength at ~20 in. Hg and leave under vacuum for 5 minutes. Stir and pour gel solutions gently. Filter and pour gels at 20–23 °C.
	Variations in spacers	Use spacers and combs that are of equal thickness.

Table 11-1 Troubleshooting for the AmpFℓSTR Profiler Plus PCR Amplification Kit *(continued)*

Observation	Possible Causes	Recommended Actions
Poor resolution	Poor quality or old reagents	Use fresh reagents from a reliable source.
	Small bubble between load and read region	Cast gel as described in protocol.
	Well shape not flat	Ensure that no air is trapped by comb. Remove comb carefully, and only load in flat wells.
	Wrong TBE buffer formulation	Remake buffer, carefully following protocol.
Gel image not showing 75 bp and/or 400 bp bands	Gel ran slower or faster than expected	Under Gel Menu, select Regenerate Gel Image… Adjust the scan range. Click OK.
Size standard peaks not recognized when defining size standard	Height of a size standard peak <150 RFU	Rerun sample, adding recommended volume of size standard.
		Lower Peak Amplitude Threshold for Red in GeneScan Analysis parameters and reanalyze samples.
	Gel image does not display all analysis peaks	Under Gel Menu, select Regenerate Gel Image… Adjust the scan range. Click OK.
Extraneous peaks in GeneScan-500 [ROX] electropherogram display	Samples not fully denatured	Heat samples to 95 °C for 2 minutes in deionized formamide prior to loading. Snap-cool on ice.
	Renaturation of denatured samples	Load sample immediately following denaturation, or store on ice until you are ready to load.
	Too much fluorescence signal. Saturating the CCD camera with one color can cause signals in other colors	Treat and dilute the PCR product as described on page 9-23 or amplify again using less input DNA.
Lower than usual peak heights	Cassette not flush with back heat transfer plate and alignment pins	Place cassette flush against back heat transfer plate. The spacers must touch the alignment pins.
	Optics/detector misalignment	Call technical support.
	Insufficient PCR product loaded onto the gel	Mix 4 µL of PCR product and 5 µL of FLS/GeneScan-500 [ROX] solution (see page 7-14).

Table 11-1 Troubleshooting for the AmpFℓSTR Profiler Plus PCR Amplification Kit *(continued)*

Observation	Possible Causes	Recommended Actions
ABI PRISM 310 Genetic Analyzer		
Data was not automatically analyzed	Sample sheet not completed	Complete sample sheet as described.
	Injection list not completed	Complete injection list as described.
	Preferences not set correctly in ABI PRISM 310 Collection software	In Preferences under the Window menu, select Injection List Defaults and the Autoanalyze checkbox.
Extra peaks visible when sample is known to contain DNA from a single source	Incomplete denaturation before loading onto detection instrument	Heat samples to 95 °C for 3 minutes in deionized formamide solution. Snap cool on ice. Use Genetic Analyzer 0.5-mL Sample Tubes and a thermal cycler.
Current too high	Decomposition of urea in the POP-4™ polymer solution	Add fresh POP-4 polymer solution to the syringe. See Chapter 8.
	Incorrect buffer concentration	Replace buffer with 1X Genetic Analyzer Buffer.
No current	No 1X Genetic Analyzer buffer	Refill buffer vials with 1X Genetic Analyzer buffer.
	Pump block channel blockage	Remove and clean block. Refer to the ABI PRISM 310 *Genetic Analyzer User's Manual.*
	Loose valve fittings or syringe	Tighten valve fittings and syringe.
	Capillary not flush with electrode	Tape capillary securely to heat plate. Refer to the *ABI PRISM 310 Genetic Analyzer User's Manual*.
	Electrode bent	Check calibration of autosampler. See Chapter 8.

Table 11-1 Troubleshooting for the AmpFℓSTR Profiler Plus PCR Amplification Kit *(continued)*

Observation	Possible Causes	Recommended Actions
No signal	Capillary misaligned with electrode	Align capillary and electrode. See Chapter 8.
	No PCR product added	Add 1.5 µL PCR product to formamide/GeneScan-500 [ROX] mixture.
	Capillary bent out of sample tube	Align capillary and electrode. Recalibrate autosampler.
	Autosampler not calibrated correctly	Calibrate autosampler in X, Y, and Z directions.
	PCR product not at bottom of tube	Spin sample tube in microcentrifuge.
	Air bubble at bottom of sample tube	Spin tube in microcentrifuge to remove air bubbles.
	Sealed sample tube septum	Replace septum.
Low signal	PCR product added to non-deionized formamide	Always use deionized formamide for sample preparation.
	PCR product not mixed well with formamide/GeneScan-500 [ROX] mixture	Mix PCR product with formamide/GeneScan-500 [ROX] mixture by pipetting up and down several times.
Loss of resolution after 100 bp	Excess salt in sample	Do not concentrate PCR product by evaporation. Use Centricon-100 if necessary (see page 3-12).
	Too much DNA in sample	Treat and dilute the PCR product as described on page 9-23.
	Oil in sample (from DNA Thermal Cycler 480)	Carefully pipet PCR product without oil carryover.
		Remove oil by organic extraction.
	Bad water	Use autoclaved or freshly prepared deionized water.
	Incorrectly prepared and/or old solutions	Replace buffer and polymer with fresh solutions.
Runs get progressively slower, *i.e.*, size standard peaks come off at higher and higher scan numbers	Leaking syringe: polymer is not filling capillary before every injection	Clean syringe thoroughly.
		Replace syringe.

Table 11-1 Troubleshooting for the AmpFℓSTR Profiler Plus PCR Amplification Kit *(continued)*

Observation	Possible Causes	Recommended Actions
Runs get progressively faster, *i.e.*, size standard peaks come off at lower and lower scan numbers	Water in syringe	Prime syringe with small volume of polymer and discard. Fill syringe with polymer.
High baseline	Dirty capillary window	Clean capillary window with 95% ethanol.
	Capillary moved out of position in laser window.	Position capillary in front of laser window.

TWGDAM Validation 12

Overview

About This Section

In this section, the validation studies performed to meet each of the recommended Technical Working Group on DNA Analysis Methods (TWGDAM) guidelines are summarized (Technical Working Group on DNA Analysis Methods, 1995).

4.1 General Considerations for Developmental Validation of the DNA Analysis Procedure

Overview

The following are subsections of the TWGDAM Guideline 4.1.5, a subtitle of 4.1, *General Considerations for Developmental Validation of the DNA Analysis Procedure*. The 4.1.5 guideline states, "The validation process should include the following studies *(Report of a Symposium on the Practice of Forensic Serology*, 1987; Budowle *et al.,* 1988; *Guidelines for a Quality Assurance Program for DNA Analysis*, 1991 and 1995)."

4.1.5.1 Standard Specimens

"The typing procedure should have been evaluated using fresh body tissues, and fluids obtained and stored in a controlled manner. DNA isolated from different tissues from the same individual should yield the same type."

In the following study, body fluids and tissues were collected and DNA extracts were prepared by the Santa Clara County Crime Laboratory DNA Unit, San Jose, CA. Blood, saliva, hair, and either a semen sample or a vaginal swab were collected from four individuals and DNA was extracted following the phenol/chloroform procedure (see page 3-5) and stored at the crime laboratory for approximately 1 year at –15 to –25 °C.

DNA was also extracted from the brain, kidney, liver, muscle, and skin of a human cadaver using the phenol/chloroform procedure.

Additionally, four individuals contributed blood and saliva and two individuals contributed blood, saliva, and hair that were processed using the Chelex DNA extraction protocol (see page 3-20) and stored at the crime laboratory for approximately 1 week at 4 °C. These thirty samples were amplified using AmpF*ℓ*STR® Profiler Plus™ PCR Amplification Kit reagents and PCR products were analyzed using an ABI PRISM® 310 Genetic Analyzer and GeneScan® Analysis 2.1 Software. DNA isolated from each of the different tissues/fluids from each individual yielded the same genotype.

4.1.5.2 Consistency

"Using specimens obtained from donors of known type, evaluate the reproducibility of the technique both within the laboratory and among different laboratories."

Two collaborators were paired and exchanged ten genomic DNA samples with one another. The instrumentation used included both the DNA Thermal Cycler 480 and GeneAmp® PCR System 9600 thermal cyclers along with the ABI PRISM® 377 DNA Sequencer. The genotype results and keys of all 20 samples were forwarded to Applied Biosystems where they were evaluated. Both laboratories obtained the correct results. Reproducibility within the Applied Biosystems laboratories has been tested with several samples many times. Each time, the correct results have been obtained.

4.1.5.3 Population Studies

"Establish population distribution data in different racial and/or ethnic groups."

The ten AmpFℓSTR Profiler Plus loci were amplified and typed from 200 U.S. Caucasian and 195 African-American individuals. For more information regarding these samples, see Chapter 13, "Population Genetics."

4.1.5.4 Reproducibility

"Prepare dried stains using body fluids from donors of known types and analyze to ensure that the stain specimens exhibit accurate, interpretable and reproducible DNA types or profiles that match those obtained on liquid specimens."

Liquid specimens and dried bloodstains from five individuals were prepared and extracted by the Santa Clara County Crime Laboratory DNA Unit following both phenol/chloroform and Chelex methods (see pages 3-5 and 3-20). Extracted samples were amplified using the AmpFℓSTR Profiler Plus PCR Amplification Kit reagents and analyzed using an ABI PRISM 310 Genetic Analyzer and GeneScan Analysis 2.1 Software. The results obtained from the liquid and dried samples were the same for each individual.

4.1.5.5 Mixed Specimen Studies

"Investigate the ability of the system to detect the components of mixed specimens and define the limitations of the system."

Evidence samples that contain body fluids and/or tissues originating from more than one individual are an integral component of forensic casework. Therefore it is essential to ensure that the DNA typing system is able to detect DNA mixtures. In the case of STRs, stutter peaks may be considered in the interpretation of mixed samples. The AmpF*ℓ*STR Profiler Plus alleles give similar peak height values for a heterozygous genotype within a locus. This balance can be used as an aid in detecting and interpreting mixtures.

Several studies were carried out to assist in the identification and interpretation of mixtures. All samples were amplified using the AmpF*ℓ*STR Profiler Plus PCR Amplification Kit and analyzed using ABI PRISM 377 DNA Sequencers or ABI PRISM 310 Genetic Analyzers and GeneScan Analysis 2.1 Software.

Studies performed included the following:

- examination of peak height ratios of heterozygous alleles within a locus
- determination of the range of stutter percentage for each allele of each locus
- analysis of purified DNA samples mixed in defined ratios

The results of these studies are summarized in Chapter 9, "Results and Interpretation." Other studies performed included the following:

- analysis of purified DNA from various body fluids mixed in defined ratios

  Simulation of forensic casework scenarios was achieved by combining various body fluids (blood:blood, semen:blood, saliva:blood, and semen:saliva) from two donors in defined ratios, by volume, from 50:1–1:50.

  Mixed stains were prepared and DNA extracted at the Santa Clara County Crime Laboratory following the phenol/chloroform procedure (differential lysis of stains containing semen). At Applied Biosystems, a total of 3 ng of DNA was amplified from each dried fluid mixture in a GeneAmp PCR System 9600 and detected using

an ABI PRISM 310 Genetic Analyzer and GeneScan Analysis 2.1 Software.

As in mixtures of purified DNA, the limit of detection occurred in blood:blood mixtures when the minor component was present at one tenth the volume of the major genotype in 3 ng total DNA amplifications. Likewise, the limit of detection of the AmpFℓSTR Profiler Plus genotype of saliva mixed with blood is when saliva was present at one tenth the blood volume. The blood genotype was detected even at the saliva:blood ratio of 50:1.

In epithelial cell fractions of differential extractions of semen:blood or semen:saliva stains, the blood or saliva was detectable until present at one tenth the volume of semen. Sperm DNA carryover into the epithelial cell fraction of these stains became apparent when semen was mixed at a 10:1 ratio with either blood or saliva. In sperm fractions, the male genotype was detectable from every semen:blood or semen:saliva mixture with no trace of the female DNA.

Again, the limit of detection for the minor component is influenced by the specific combination of genotypes present in mixtures.

- analysis of sexual assault evidence

  The results of AmpFℓSTR Profiler Plus amplifications of DNA extracted from adjudicated and nonprobative sexual assault evidence were also examined to assess the performance of the AmpFℓSTR Profiler Plus PCR Amplification Kit on typical casework samples comprised of mixed body fluids. These samples were extracted, amplified, and analyzed in collaboration with the Santa Clara County Crime Laboratory, San Jose, CA. See Part 4.1.5.8, “Nonprobative Evidence,” on page 12-8 for discussion of results.

4.1.5.6 Environmental Studies

"Evaluate the method using known or previously characterized samples exposed to a variety of environmental conditions. The samples should be selected to represent the types of specimens to be routinely analyzed by the method. They should resemble actual evidence materials as closely as possible so that the effects of factors such as matrix, age and degradative environment (temperature, humidity, UV) of a sample are considered."

Environmentally stressed samples, previously tested using the AmpliType® HLA DQα PCR Amplification and Typing Kit and the AmpliFLP™ D1S80 Amplification Kit, were provided by the Santa Clara County Crime Laboratory. These samples were comprised of dried blood stains (two donors) exposed to sunlight or shade as well as dried blood stains and semen stains (two donors) exposed to variations in temperature (room temperature and 37 °C). Samples were collected at zero, 1-month, 4-month, and 12-month timepoints.

Stains were stored frozen at the crime laboratory for approximately 4 years. DNA was then extracted from these samples using the phenol/chloroform method (see page 3-5), examined for degradation on an agarose gel, and quantitated using the QuantiBlot® Human DNA Quantitation Kit. One and a half nanograms of DNA was amplified using the AmpFℓSTR Profiler Plus PCR Amplification Kit reagents and the resulting PCR products were analyzed using the ABI PRISM 310 Genetic Analyzer and GeneScan Analysis 2.1 Software.

The results showed a reduction in PCR product yield for a locus as the level of DNA degradation increased. The first locus to show reduced yield was the longest, D18S51, followed by D7S820, and then FGA and so forth. Reduced peak heights seen in amplified products reflected the level of genomic DNA degradation observed by agarose gel analysis. These results are consistent with those described in Chapter 9 where purified DNA was degraded using DNase I. Additionally, while exhibiting the peak height pattern typical of partially degraded DNA, amplification of all ten loci was accomplished from a semen stain stored at room temperature for 40 months.

Furthermore, results found with the AmpFℓSTR Profiler Plus PCR Amplification Kit reflected those obtained with the AmpliType DQα Amplification Kit: FGA and HLA DQA1 (242 bp), of similar base pair size, failed to amplify at the same time point.

4.1.5.7 Matrix Studies

*"Examine prepared body fluids mixed with a variety of commonly encountered substances (*e.g.*, dyes, soil) and deposited on commonly encountered substrates (*e.g.*, leather, denim)."*

Analysts at the Santa Clara Crime Laboratory, San Jose, CA, prepared a panel of blood and semen specimens deposited on a variety of commonly encountered substrates. Blood samples from two donors were deposited on wool, cotton, nylon, glass, metal, leather, and blue denim. Semen samples from two donors were deposited on wool, cotton, nylon, leather, blue denim, acetate, vinyl upholstery, facial tissue, a condom with spermicide (5% nonoxynol-9), a condom with water soluble lubricant, and a latex glove. Specimens were stored at room temperature and at specified timepoints a sampling of each stain was removed for extraction. The blood and semen stains were extracted using the organic extraction procedure. Additionally, a portion of each blood specimen was extracted using the Chelex method followed by Centricon-100 ultrafiltration (Chapter 3). Extracted samples were then stored at –15 to –25 °C for 3–4 years.

The 1-week and 1-year timepoints were analyzed by Applied Biosystems scientists. The samples were amplified using AmpFℓSTR Profiler Plus PCR Amplification Kit reagents and analyzed using an ABI PRISM 310 Genetic Analyzer and GeneScan Analysis 2.1 Software. A complete ten-locus genotype was obtained for all 36 blood and semen samples exposed to the tested matrices for 1 week and for 1 year. No non-specific artifacts were generated and the samples all yielded expected genotypes.

4.1.5.8 Nonprobative Evidence

"Examine DNA profiles in nonprobative evidentiary stain materials. Compare the DNA profiles obtained for the known liquid blood versus questioned blood deposited on typical crime scene evidence."

DNA extracts from four adjudicated sexual assault cases were prepared by DNA analysts at the Santa Clara County Crime Laboratory. Sexual assault evidence materials were processed using the differential lysis and organic extraction procedure, while victim/suspect reference blood samples were processed using the Chelex extraction procedure (Chapter 3). Following amplification with the AmpFℓSTR Profiler Plus PCR Amplification Kit reagents, the PCR products were analyzed using the ABI PRISM 377 DNA Sequencer and GeneScan Analysis 2.1 Software.

- Case 1 and Case 2 contained a victim reference blood sample, a suspect reference blood sample, and a victim vaginal swab. The AmpFℓSTR Profiler Plus genotype of the epithelial cell fraction was the same as that of the victim reference and did not contain alleles foreign to the victim. The AmpFℓSTR Profiler Plus genotype of the sperm cell fraction did not contain detectable epithelial cell fraction DNA and included the suspect as a possible semen donor.
- Case 3 contained a victim reference blood sample and a victim vaginal swab. The AmpFℓSTR Profiler Plus genotype of the epithelial cell fraction was the same as that of the victim reference and did not contain alleles foreign to the victim. In accordance with the victim's account, AmpFℓSTR Profiler Plus typing of the sperm fraction revealed DNA from multiple semen donors. No suspect(s) were developed in this case.
- Case 4 contained victim and suspect reference samples and a victim vaginal swab. The AmpFℓSTR Profiler Plus genotype of the epithelial cell fraction was the same as that of the victim reference and did not contain alleles foreign to the victim. The major sperm fraction genotype included the suspect. A minor genotype, attributable to carryover from the epithelial cell fraction, was present in the sperm fraction.

4.1.5.9 Nonhuman Studies

"Determine if DNA typing methods designed for use with human specimens detect DNA profiles in nonhuman source stains."

DNA samples extracted from primates (2.5 ng each from gorilla, chimpanzee, and orangutan) and non-primates (50 ng each from bacteria, yeast, mouse, cat, dog, pig, chicken, cow, and horse) were amplified in AmpFℓSTR Profiler Plus reactions and analyzed using the ABI PRISM 377 DNA Sequencer with GeneScan Analysis 2.1 Software. The primate DNA samples all amplified, producing fragments within the 75–350 base pair region. The primate samples were subsequently sequenced by Applied Biosystems scientists. The data revealed significant sequence homology between the primate and human DNA for the AmpFℓSTR Profiler Plus loci.

The bacteria, yeast, cat, chicken, and mouse samples did not yield any detectable product. The dog, pig, cow, and horse samples produced a 103-bp fragment. This 103-bp fragment was also amplified using the amelogenin primers alone. This confirms amplification of the product obtained by Buel *et al.* (1995). The 103-bp fragment is 4 bp shorter than the primate 107-bp X-specific product (including +A addition).

4.1.5.10 Minimum Sample

"Establish quantity of DNA needed to obtain a reliable result."

The AmpFℓSTR Profiler Plus PCR Amplification Kit has been optimized to amplify and type approximately 1.0–2.5 ng of sample DNA reliably. The suggested minimum peak height threshold for detection and assignment of genotypes is 150 relative fluorescence units (RFU) (see pages 9-33 to 9-34). In the Human Identification Group's laboratory at Applied Biosystems this amount is obtained from between 250 and 500 pg of AmpFℓSTR® Control DNA 9947A. These results have been obtained on both the DNA Thermal Cycler 480 and GeneAmp PCR System 9600. Analysis was performed using the ABI PRISM 377 DNA Sequencer and GeneScan Analysis 2.1 Software.

4.1.5.12

"It is essential that the results of the developmental validation studies be shared as soon as possible with the scientific community through presentations at scientific/professional meetings. It is imperative that details of these studies be available for peer review through timely publications in scientific journals."

There have been several AmpFℓSTR Profiler Plus presentations at scientific/professional meetings since Spring 1997. These include presentations at the 1997 International Society for Forensic Haemogenetics (ISFH) meeting, Eighth International Symposium on Human Identification, and other international, national and local meetings. Also, the AmpFℓSTR Profiler Plus PCR Amplification Kit development and optimization studies, along with the results of the validation studies, will be submitted for publication in peer-reviewed scientific journals.

4.2 Characterization of Loci

Overview

The following are in response to 4.2, *Characterization of Loci*, which states, "During the development of a DNA analysis system, basic characteristics of the loci must be determined and documented (Baird, 1989; AABB Standards Committee, 1990)."

4.2.1 Inheritance

"DNA loci used in forensic testing shall have been validated by family studies to demonstrate the mode of inheritance. Those DNA loci used in parentage testing should have a low frequency of mutation and / or recombination."

The Centre d'Etude du Polymorphisme Humain (CEPH) has collected DNA from 39 families of Utah Mormon, French Venezuelan, and Amish descent. These DNA sets have been extensively studied all over the world and are routinely used to characterize the mode of inheritance of various DNA loci. Each family set contains three generations, generally including four grandparents, two parents, and several offspring. Consequently, the CEPH family DNA sets are ideal for studying inheritance patterns.[1]

Four CEPH family DNA sets were examined. One and a half nanograms of DNA from each sample was amplified using the AmpF*ℓ*STR Profiler Plus kit, followed by analysis using an ABI PRISM 310 Genetic Analyzer and GeneScan Analysis 2.1 Software.The families examined included #884 (14 offspring), #1340 (eight offspring), #1341 (ten offspring), and #1345 (eight offspring), representing eighty meiotic divisions. The results confirmed that the loci are inherited according to Mendelian rules, as has also been reported in the literature.[2, 3, 4, 5, 6, 7]

1. Begovich *et al.*, 1992.
2. Kimpton *et al.*, 1992.
3. Li *et al.*, 1993.
4. Mills *et al.*, 1992.
5. Nakahori *et al.*, 1991.
6. Sharma and Litt, 1992.
7. Straub *et al.*, 1993.

4.2.2 Gene Mapping

"The chromosomal location of the polymorphic loci used for forensic testing shall be submitted to or recorded in the Yale Gene Library or the International Human Gene Mapping Workshop."

The AmpFℓSTR Profiler Plus loci D3S1358, vWA, FGA, amelogenin, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820 have been mapped and the chromosomal locations have been published.[1, 2, 3, 4, 5, 6, 7, 8, 9] They are listed in Table 1-1 on page 1-6.

4.2.3 Detection

"The molecular basis for detecting the polymorphic loci shall be documented in the scientific or technical literature."

4.2.3.2 "For PCR this includes the primers and probes if used."

Primer sequences that amplify the polymorphic region of the amelogenin locus and the repeat regions of the D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D7S820, and D13S317 loci have been published.[1–10] Some of these published sequences have been modified for inclusion in the AmpFℓSTR Profiler Plus PCR Amplification Kit.

1. Kimpton *et al.*, 1992.
2. Li *et al.*, 1993.
3. Mills *et al.*, 1992.
4. Nakahori *et al.*, 1991.
5. Sharma and Litt, 1992.
6. Straub *et al.*, 1993.
7. Barber and Parkin, 1996.
8. Hudson *et al.*, 1995.
9. Green *et al.*, 1991.
10. D5S818, Cooperative Human Linkage Center (CHLC) accession number 512, GenBank accession number G09017; D13S317, Cooperative Human Linkage Center (CHLC) accession number 415, GenBank accession number G08446; D7S820, Cooperative Human Linkage Center (CHLC) accession number 511, GenBank accession number G08616.

4.2.4 Polymorphism

"The type of polymorphism detected shall be known."

The primers for the amelogenin locus flank a six-base pair deletion within intron 1 of the X homologue. Amplification results in 107-bp and 113-bp products from the X and Y chromosomes, respectively, including 3´ A nucleotide addition. The remaining AmpFℓSTR Profiler Plus loci are all tetranucleotide short tandem repeat loci. The differences among the alleles of a particular locus result predominantly from variations in length based on the number of repeat units present.

All of the alleles in the AmpFℓSTR Profiler Plus Allelic Ladder, along with some population database samples, alleles containing partial repeat units, and nonhuman primate DNA samples have been sequenced at Applied Biosystems. In addition, other groups in the forensic community have sequenced alleles at some of these loci.[1, 2, 3, 4, 5, 6] Among the various sources of sequence data on the AmpFℓSTR Profiler Plus loci, there is consensus on the repeat patterns and structure of the STRs (see Table 1-1 on page 1-6).

1. Nakahori *et al.*, 1991.
2. Möller *et al.*, 1994.
3. Barber *et al.*, 1995.
4. Möller and Brinkmann, 1995.
5. Barber *et al.*, 1996.
6. Barber and Parkin, 1996.

4.4 Specific Developmental Validation of PCR-based DNA Procedures

4.4.1 Amplification The following statements are in response to subtitle 4.4.1, *Amplification.* They describe the specific developmental validation of the AmpFℓSTR Profiler Plus PCR Amplification Kit.

4.4.1.1 *"The PCR primers must be of known sequence."*

The primer sequences for the ten AmpFℓSTR Profiler Plus loci are based on known sequences from each locus.[1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12]

4.4.1.2 *"Conditions and measures necessary to protect pre-amplification samples from contamination by post PCR materials should be determined."*

Conditions and measures necessary to prevent contamination of pre-amplification samples with PCR products are explained in Chapter 2, "Laboratory Setup," of this user's manual. Such conditions have been described in several publications[13, 14, 15] and at professional meetings.

1. Kimpton *et al.*, 1992.
2. Li *et al.*, 1993.
3. Mancuso *et al.*, 1989.
4. Mills *et al.*, 1992.
5. Nakahori *et al.*, 1991.
6. Oldroyd *et al.*, 1995.
7. Sharma and Litt, 1992.
8. Urquhart *et al.*, 1995.
9. Straub *et al.*, 1993.
10. Barber and Parkin, 1996.
11. Kimpton *et al.*, 1993.
12. D5S818, D13S317, D7S820, Cooperative Human Linkage Center (CHLC).
13. Cone and Fairfax, 1993.
14. Kwok and Higuchi, 1989.
15. Prince and Andrus, 1992.

4.4.1.3 *"The reaction conditions such as thermocycling parameters and critical reagent concentrations (primers, polymerase and salts) needed to provide the required degree of specificity must be determined."*

The concentration of each component of the AmpFℓSTR Profiler Plus PCR Amplification Kit—Tris-HCl (pH 8.3), KCl, dNTPs, primers, AmpliTaq Gold® DNA Polymerase, $MgCl_2$, bovine serum albumin, and sodium azide—was optimized to give the most reliable performance. The optimal concentration for a particular component was established to be in the middle of a window that meets the reproducible performance characteristics of specificity and sensitivity.

Once the optimal concentration was determined for a single component, the others were tested sequentially until it was determined that each component was at the optimal concentration relative to the concentrations of the other components in the reaction mix. The optimized AmpFℓSTR Profiler Plus PCR Amplification Kit provides the required degree of specificity such that it is specific to primates (with the exception of the amelogenin locus, see page 12-9) and does not produce nonspecific mispriming artifacts.

Thermal cycling parameters were established for amplification of the AmpFℓSTR Profiler Plus PCR Amplification Kit in the DNA Thermal Cycler 480 and GeneAmp PCR Systems 9600 and 2400. Thermal cycling times and temperatures met Applied Biosystems GeneAmp PCR Instrument specifications. Temperature windows were tested around each setpoint to verify that a 2 °C window (DNA Thermal Cycler 480) or 1.5 °C window (GeneAmp PCR System 9600 and 2400) yielded specific PCR product with the desired sensitivity of at least 1 ng of AmpFℓSTR Control DNA 9947A.

4.4.1.4 *"The number(s) of cycles necessary to produce reliable results must be determined."*

AmpFℓSTR Profiler Plus reactions were amplified for 27, 28, 29, and 30 cycles on both the DNA Thermal Cycler 480 and GeneAmp PCR System 9600. While none of the cycle numbers tested produced nonspecific bands, 28 cycles was found to give optimal sensitivity when the amplified products were examined on ABI PRISM instruments. The cycle number was set to obtain reliable, specific amplification of 1 ng of AmpFℓSTR Control DNA 9947A following the conditions outlined in this manual. Additionally, the cycle number was set to avoid detection of low quantities of DNA (35 pg or less).

4.4.1.5 *"Potential for differential amplification must be assessed and addressed."*

Differential amplification can be defined as the difference in the degree of amplification of each locus within a co-amplified system, such that one or more loci may amplify to a lesser extent compared to the other loci. Preferential amplification is used in this manual to describe differences in the amplification efficiency of two alleles at a single locus. Preferential amplification is observed when the peak height ratio between the two alleles at a single locus is less than 70% (see page 9-24).

In assessing potential for differential amplification, four areas were identified that may produce differential amplification among the ten loci of the AmpFℓSTR Profiler Plus kit. These areas were the following: low template copy number, presence of inhibitors in a DNA sample, degraded DNA, and amplification denaturation and annealing temperatures.

Preferential amplification of alleles in systems that distinguish alleles based on length polymorphisms is most likely to be observed when the alleles differ significantly in base pair size. Since most STR loci have small size ranges, the potential for preferential amplification of alleles is low.

DNA samples with FGA alleles in the 300–350 bp range have been observed. One such DNA sample containing FGA alleles separated by 90 bp (240 and 330 bp) was analyzed in some of our studies to determine the potential for preferential amplification.

To determine if the amount of input DNA affected either differential or preferential amplification, varying quantities of two DNA samples were amplified. One nanogram, 0.5 ng, 0.25 ng, 0.125 ng, 0.06 ng, 0.03 ng, and 0.015 ng of AmpFℓSTR Control DNA 9947A and the sample containing the widespread FGA alleles were amplified and then analyzed using the ABI PRISM 377 DNA Sequencer and GeneScan Analysis 2.1 Software.

The results indicate that the AmpFℓSTR Control DNA 9947A does not differentially amplify at any of the ten loci at the quantities tested. The sample with the widespread FGA alleles preferentially amplified the shorter allele at 0.06 ng input DNA, such that the shorter allele (240 bp) was detected and the longer allele (330 bp) was not. However, the shorter allele peak height (30 RFU) was significantly below the recommended minimum threshold of 150 RFU. At 0.25 ng the FGA alleles of this sample both amplified in equal proportions, and at 0.03 ng both alleles were no longer detectable.

The effect of the presence of an inhibitor on the potential for differential and preferential amplification was studied. One nanogram of sample DNA was amplified in the presence of varying concentrations of hematin. PCR products were examined using the ABI PRISM 377 DNA Sequencer and GeneScan Analysis 2.1 Software.

Differential amplification was observed in the presence of high levels of hematin (Figure 9-12 on page 9-29). Moreover, as the concentration of hematin was increased, the overall yield of products was reduced. These results were compared to those obtained when each locus was amplified alone and were similar.

DNA samples were degraded using DNase I and examined for the presence of differential amplification. Results are described in Chapter 9, page 9-30 through page 9-33.

The effects of denaturation and annealing temperatures on the amplification of AmpFℓSTR Profiler Plus loci were examined using 1–2 ng of the AmpFℓSTR Control DNA 9947A.

The denaturation temperatures tested were 92, 94, and 96 °C, all for 1-minute hold times. The annealing temperatures tested were 57, 59, 61, and 63 °C, also for 1-minute hold times. The majority of these were tested on both the DNA Thermal Cycler 480 and the GeneAmp PCR System 9600 and 2400. The PCR products were analyzed using the ABI PRISM 377 DNA Sequencer and GeneScan Analysis 2.1 Software.

Neither preferential nor differential amplification was observed in any of these denaturation temperature experiments.

Of the tested annealing temperatures, 57, 59, and 61 °C did not induce any differential amplification. At 63 °C, the yield of the majority of loci was significantly reduced. This should pose no problem if the thermal cyclers are calibrated routinely and the recommended amplification protocol is followed. Preferential amplification was not observed at any of the tested annealing temperatures.

4.4.1.6 *"Where more than one locus is amplified in one sample mixture, the effects of such amplification on each system (alleles) must be addressed and documented."*

DNA samples were amplified in ten separate reactions containing primers for only one AmpFℓSTR Profiler Plus locus and in an eleventh reaction containing primers for all ten AmpFℓSTR Profiler Plus loci. The samples were taken from a single time point from a sample that had been degraded using the enzyme DNase I.

Amplified samples were then analyzed using the ABI PRISM 377 DNA Sequencer and GeneScan Analysis 2.1 Software. The same result was obtained (genotype and peak height) whether the DNA samples were amplified for each locus alone or co-amplified in the AmpFℓSTR Profiler Plus reaction. (See Figure 9-15 on page 9-32 for an example.)

4.4.2 Detection of PCR Product

The following is in response to Part 4.4.2, *Detection of PCR Product*, which states, "The validation process will identify the panel of positive and negative controls needed for each assay described below."

4.4.2.1

*"When a PCR product is characterized directly, appropriate standards for assessing the alleles shall be established (*e.g.*, size markers)."*

The AmpFℓSTR® Blue, AmpFℓSTR® Green II, and AmpFℓSTR® Yellow Allelic Ladders were developed by Applied Biosystems for accurate characterization of the alleles amplified by the AmpFℓSTR Profiler Plus PCR Amplification Kit. The AmpFℓSTR Blue, Green II, and Yellow Allelic Ladders are combined to create the AmpFℓSTR Profiler Plus Allelic Ladder before running on a gel or injecting into a capillary.

The AmpFℓSTR Profiler Plus Allelic Ladder contains the common alleles at the ten loci as follows: D3S1358 alleles 12–19, vWA alleles 11–21, FGA alleles 18–30, amelogenin (X and Y), D8S11 alleles 8–19, D21S11 alleles 24.2–38, D18S51 alleles 9–26, D5S818 alleles 7–16, D13S317 alleles 8–15, and D7S820 alleles 6–15.

The allelic ladder designations correspond to the number of 4-base pair repeat units present in each allele. The AmpFℓSTR Blue ladder includes a 2-bp variant allele at FGA that is designated as 26.2. The AmpFℓSTR Green II ladder includes several 2-bp variant alleles as follows: nine 2-bp variant alleles at D21S11 designated 24.2, 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, and 35.2, as well as three 2-bp variant alleles at D18S51 designated 10.2, 13.2, and 14.2.

We recommend the GeneScan-500 [ROX] Internal Lane Size Standard for obtaining base pair sizing results. The GeneScan-500 [ROX] standard is designed for sizing DNA fragments in the 35–500 bp range, and contains 16 single-stranded fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bases. This standard has been evaluated extensively as an internal lane size standard and was found to give extremely precise sizing results of AmpFℓSTR Profiler Plus PCR products on the ABI PRISM 310 Genetic Analyzer and the ABI PRISM 377 DNA Sequencer, as reported in Chapter 9.

4.5 Internal Validation of Established Procedures (ASCLD 1986)

4.5.3 The following is in response to Guideline 4.5, *Internal Validation of Established Procedures*, which states, "Prior to implementing a new DNA analysis procedure, or an existing DNA procedure developed by another laboratory that meets the developmental criteria described under Part 4.1, the forensic laboratory must first demonstrate the reliability of the procedure in-house. This internal validation must include the following:"

"Precision (e.g., measurement of fragment lengths) must be determined by repetitive analyses to establish criteria for matching."

Precision for determining accurate and reliable genotypes was determined on the ABI PRISM 310 Genetic Analyzer and the ABI PRISM 377 DNA Sequencer. More information regarding precision measurements can be found in Chapter 9, "Results and Interpretation."

13 Population Genetics

Population Data

Overview

To interpret the significance of a match between genetically typed samples, it is necessary to know the population distribution of alleles at each locus in question. If the genotype of the relevant evidence sample is different from the genotype of the suspect's reference sample, then the suspect is "excluded" as the donor of the biological evidence tested. An exclusion is independent of the frequency of the two genotypes in the population.

If the suspect and evidence samples have the same genotype, then the suspect is "included" as a possible source of the evidence sample. The probability that another, unrelated, individual would also match the evidence sample is equal to the frequency of that genotype in the relevant population.

Population Samples Used in These Studies

The AmpFℓSTR® Profiler Plus™ PCR Amplification Kit was used to generate the population data provided in this section.

African-American

195 samples were provided by Laboratory Corporation of America. Samples had been collected from individuals throughout the United States with no geographical preference.

U.S. Caucasian

200 samples were provided by Laboratory Corporation of America. Samples had been collected from individuals throughout the United States with no geographical preference.

AmpFℓSTR Profiler Plus Allele Frequencies

Table 13-1 shows the AmpFℓSTR Profiler Plus allele frequencies in both populations, listed as percentages.

Table 13-1 AmpFℓSTR Profiler Plus allele frequencies

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
D3S1358		
9	0.26*	*
10	*	*
11	0.26*	0.25*
12	0.51*	0.25*
13	*	0.50*
14	11.80	11.25
15	27.95	28.25
15.2	0.26*	*
16	32.31	22.25
17	21.80	22.25
18	4.62	14.50
19	0.26*	0.50*
vWA		
11	0.26*	*
12	*	*
13	1.54	*
14	7.70	8.50
15	22.05	8.25
16	26.92	19.75
17	16.92	25.00
18	13.85	25.75
19	8.46	11.00
20	2.05	1.50
21	*	0.25*
22	0.26*	*

Table 13-1 AmpFℓSTR Profiler Plus allele frequencies *(continued)*

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
FGA		
16.2	0.26*	*
17	0.26*	*
18	0.51*	1.50
19	4.62	6.25
19.2	0.26*	*
20	4.36	16.25
20.2	*	0.75
21	13.33	17.75
21.2	0.26*	*
22	18.97	16.50
22.2	0.26*	0.50*
23	18.97	14.00
24	16.41	13.25
25	12.31	11.25
26	4.10	1.50
26.2	*	*
27	4.10	0.50*
28	0.77*	*
29	0.26*	*
30	*	*
D8S1179		
8	*	1.75
9	0.51*	1.00*
10	3.08	8.00
11	4.36	6.25
12	10.51	14.25
13	19.74	34.75
14	33.33	18.75
15	21.03	13.00
16	6.41	2.00
17	1.03*	0.25*

Table 13-1 AmpFℓSTR Profiler Plus allele frequencies *(continued)*

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
18	*	*
19	*	*
D21S11		
24.2	*	0.50*
25	*	*
26	0.26*	*
27	5.90	3.75
28	21.80	16.25
28.2	*	*
29	20.00	20.75
29.2	*	0.50*
29.3	0.26*	*
30	16.15	26.25
30.2	2.56	2.50
31	8.97	5.50
31.2	5.90	10.50
32	0.77*	1.25*
32.2	6.92	7.25
33	0.51*	0.25*
33.1	0.26*	*
33.2	4.10	4.00
34	0.26*	*
34.2	*	0.75*
35	3.59	*
35.2	*	*
36	1.54	*
38	0.26*	*
D18S51		
9	*	*
10	0.51*	0.50*
10.2	0.51*	*
11	1.54	2.00

Table 13-1 AmpFℓSTR Profiler Plus allele frequencies *(continued)*

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
12	5.64	14.25
13	4.10	15.00
13.2	0.51*	*
14	6.67	16.75
14.2	0.51*	*
15	17.69	14.25
16	17.44	14.00
17	16.41	10.50
18	11.03	6.00
19	10.26	3.75
20	4.87	1.50
21	1.03*	1.00*
22	1.03*	0.25*
23	0.26*	0.25*
24	*	*
25	*	*
26	*	*
D5S818		
7	0.26*	0.25*
8	5.13	0.50*
9	2.05	2.25
10	7.44	6.75
11	25.39	39.25
12	32.56	33.25
13	24.87	16.50
14	2.05	1.00*
15	0.26*	*
16	*	0.25*

Table 13-1 AmpFℓSTR Profiler Plus allele frequencies *(continued)*

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
D13S317		
5	*	0.25*
8	3.59	11.50
9	2.31	7.75
10	2.31	6.75
11	27.18	31.25
12	44.36	28.25
13	14.10	9.75
14	6.15	4.25
15	*	0.25*
D7S820		
6	0.51*	*
6.3	*	0.25*
7	*	2.50
8	17.95	17.50
9	11.80	13.00
10	33.59	24.00
11	22.82	23.00
12	9.49	16.00
13	3.59	2.75
14	0.26*	0.75*
15	*	0.25*

***** A minimum allele frequency of 1.3% is suggested by the National Research Council in forensic calculations using either the AmpFℓSTR Profiler Plus African-American or U.S. Caucasian database. See following discussion.

Analysis across both databases of 790 total chromosomes revealed a total of 11 different D3S1358 alleles, 11 different vWA alleles, 18 different FGA alleles, 10 different D8S1179 alleles, 20 different D21S11 alleles, 17 different D18S51 alleles, 10 different D5S818 alleles, 9 different D13S317 alleles, and 11 different D7S820 alleles.

In addition to the alleles that were observed and recorded in the Applied Biosystems databases, other known alleles (listed in Table 1-3 on

page 1-8) have either been published or reported to us by other laboratories.

Independence of allelic frequencies within a locus can be expressed by the Hardy-Weinberg (HW) relationship. Approximation of HW expectations in a sample population allows estimation of genotypic frequencies (HW proportions) from observed allelic frequencies using the HW equation (expanded binomial square law).[1, 2]

Several biostatistical tests were used to survey HW relationships at the AmpFℓSTR Profiler Plus STR loci in each sample population. Independence was found between alleles within each locus, as *p* values >0.05 were obtained from the homozygosity test,[3, 4, 5] likelihood-ratio test,[6, 7] and Guo-Thompson exact test.[8]

Additionally, allele frequency data was analyzed for independence based on the total number of observed distinct homozygous and heterozygous genotype classes.[4] Observed values were within two standard errors of expected values for each locus. These sets of data demonstrate that appropriate estimations of AmpFℓSTR Profiler Plus genotype frequencies are generated from allele frequencies observed in the Applied Biosystems African-American and U.S. Caucasian databases.

Existence of random association (linkage equilibrium) between all nine STR loci was established through two separate statistical tests. Results of the first test, which considers the observed variance of the number of heterozygous loci,[9, 10] indicate that in both population samples, all AmpFℓSTR Profiler Plus loci are independently inherited.

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1. Hartl and Clark, 1989.
2. Weir, 1996.
3. Chakraborty *et al.*, 1988.
4. Nei and Roychoudhury, 1974.
5. Nei, 1978.
6. Edwards *et al.*, 1992.
7. Weir, 1992.
8. Guo and Thompson, 1992.
9. Brown *et al.*, 1980.
10. Budowle *et al.*, 1995.

Pairwise interclass correlation tests (Karlin *et al.*, 1981) were performed between every possible two-locus combination across the African-American and U.S. Caucasian databases. Mendelian behavior between the nine STR loci was observed. AmpFℓSTR Profiler Plus multilocus genotype frequency estimates may be derived through direct multiplication of each single-locus genotype frequency (the "product rule") estimated from the Applied Biosystems African-American and U.S. Caucasian databases.

Some alleles of the AmpFℓSTR Profiler Plus loci occur at a low frequency (less than five times in either database). For these alleles, a minimum frequency of 0.013 (five divided by 2n, where n equals the number of individuals in the database) was assigned for the AmpFℓSTR Profiler Plus African-American and U.S. Caucasian databases, as suggested in the 1996 report of the Committee on DNA Forensic Science (National Research Council, 1996). These databases are summarized in Table 13-1 on pages 13-2 through 13-6. The minimum reportable genotype frequency at each locus is then 1.69×10^{-4}, giving a minimum combined multilocus genotype frequency of 1.12×10^{-34} for both the African-American and U.S. Caucasian databases.

Probability of Identity

Table 13-2 shows the Probability of Identity (P_I) values of the AmpFℓSTR Profiler Plus loci individually and combined.

Table 13-2 Probability of Identity values for the AmpFℓSTR Profiler Plus STR loci

Locus	African-American	U.S. Caucasian
D3S1358	0.102	0.078
vWA	0.058	0.065
FGA	0.035	0.036
D8S1179	0.075	0.067
D21S11	0.033	0.045
D18S51	0.028	0.030
D5S818	0.097	0.140
D13S317	0.131	0.074
D7S820	0.081	0.061
Combined	1.48×10^{-11}	1.04×10^{-11}

The P_I value is the probability that two individuals selected at random will have an identical AmpFℓSTR Profiler Plus genotype (Sensabaugh, 1982). The P_I values for the populations described in this section are then approximately $1/6.8 \times 10^{10}$ (African-American) and $1/9.6 \times 10^{10}$ (U.S. Caucasian).

Of 18,915 and 19,900 pairs of AmpFℓSTR Profiler Plus profiles represented by the African-American and U.S. Caucasian databases, respectively, no nine-locus matches were observed.

Linkage disequilibrium between the AmpFℓSTR Profiler Plus loci and the AmpFℓSTR® Green I loci (TH01, TPOX, and CSF1PO) was not detected. The combination of these 12 AmpFℓSTR® loci offers an average probability of identity of approximately $1/1.18 \times 10^{14}$ (African-American) and $1/2.52 \times 10^{14}$ (U.S. Caucasian).

Probability of Paternity Exclusion

Table 13-3 shows the Probability of Paternity Exclusion (P_E) values of the AmpFℓSTR Profiler Plus STR loci individually and combined.

Table 13-3 Probability of paternity exclusion for the AmpFℓSTR Profiler Plus STR loci

Locus	African-American	U.S. Caucasian
D3S1358	0.5260	0.5797
vWA	0.6394	0.6170
FGA	0.7202	0.7173
D8S1179	0.5930	0.6128
D21S11	0.7281	0.6835
D18S51	0.7518	0.7414
D5S818	0.5375	0.4554
D13S317	0.4725	0.5948
D7S820	0.5742	0.6307
Combined	0.999989	0.999982

The P_E value is the probability, averaged over all possible mother-child pairs, that a random alleged father will be excluded from paternity after DNA typing of the AmpFℓSTR Profiler Plus STR loci (Chakraborty and Stivers, 1996).

Technical Support

A

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For Support On This Product	Dial 1-800-831-6844, and...	
ABI PRISM® 3700 DNA Analyzer	**Press**	**FAX**
	8	650-638-5981

For Support On This Product	Dial 1-800-831-6844, and...
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PCR and Sequence Detection	**Press**: **5**, or call 1-800-762-4001, and press 1 for PCR, or 2 for Sequence Detection; **FAX**: 240-453-4613
FMAT	**Telephone**: 1-800-899-5858, and press 1, then press 6; **FAX**: 508-383-7855
Peptide and Organic Synthesis	**Press**: **31**; **FAX**: 650-638-5981
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