AmpF*ℓ*STR® SEfiler Plus™

PCR Amplification Kit

User Guide

AmpFℓSTR®
SEfiler Plus™

PCR Amplification Kit

User Guide

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Preface

How to Use This Guide

Purpose of This Guide

The *Applied Biosystems AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit User Guide* provides information about the Applied Biosystems instruments, chemistries, and software associated with the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit.

Pull-Out Chapters

This guide is designed to allow users to pull out chapters 2, 3, and 4. The pull-out chapters have title and back pages, which indicate the chapter and number title.

Text Conventions

This guide uses the following conventions:

- **Bold** text indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow symbol (▸) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File ▸ Open ▸ Spot Set**.

  Right-click the sample row, then select **View Filter ▸ View All Runs**.

User Attention Words

Two user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note: – Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! – Provides information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

Examples of the user attention words appear below:

Note: The Calibrate function is also available in the Control Console.

IMPORTANT! To verify your client connection to the database, you need a valid user ID and password.

Safety

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word–**IMPORTANT, CAUTION, WARNING, DANGER**–implies a particular level of observation or action, as defined below.

Definitions

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD.** Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" on page vii.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

The MSDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain MSDSs:

1. Go to **docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field of the MSDS Search page:
   a. Enter the chemical name, part number, or other information that you expect to appear in the MSDS of interest.
   b. Select the language of your choice.
   c. Click **Search**.

3. To view, download, or print the document of interest:
   a. Right-click the document title.
   b. Select:
      - **Open** – To view the document
      - **Save Target As** – To download a PDF version of the document to a destination that you choose
      - **Print Target** – To print the document
4. To have a copy of an MSDS sent by fax or e-mail, in the Search Results page:
   a. Select **Fax** or **Email** below the document title.
   b. Click **RETRIEVE DOCUMENTS** at the end of the document list.
   c. Enter the required information.
   d. Click **View/Deliver Selected Documents Now**.

Note: For the MSDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Chemical Waste Hazards

CAUTION **HAZARDOUS WASTE.** Refer to Material Safety Data Sheets and local regulations for handling and disposal.

WARNING **CHEMICAL WASTE HAZARD.** Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

WARNING **CHEMICAL STORAGE HAZARD.** Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological Hazard Safety

WARNING **BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

www.cdc.gov

How to Obtain More Information

Related Documentation

To obtain any of the following documents, go to **www.appliedbiosystems.com**, then click the links for **Support ▸ Product and Service Literature**.

Document	Part Number
Applied Biosystems 3130/3100xl Genetic Analyzers Using Data Collection Software v3.0 User Bulletin	4363787
Applied Biosystems 3130/3130xl Genetic Analyzers Getting Started Guide	4352715
Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide	4352716
Applied Biosystems 3130/3130xl Genetic Analyzers Quick Reference Card	4362825
Applied Biosystems 3130/3130xl Genetic Analyzers AB Navigator Software Administrator Guide	4359472
ABI PRISM® 3100/3100-Avant Data Collection v2.0 User Guide	4347102
ABI PRISM® 3100/3100-Avant Genetic Analyzers Using Data Collection Software v2.0 User Bulletin	4350218
ABI PRISM® 3100 Genetic Analyzer User Manual (Data Collection v1.1)	4315834
ABI PRISM® 3100-Avant Genetic Analyzer User Guide (Data Collection v1.0)	4333549
ABI PRISM® 3100/3100-Avant Genetic Analyzers Protocols for Processing AmpFℓSTR® PCR Amplification Kit PCR Products User Bulletin	4332345
ABI PRISM® 310 Genetic Analyzer User Guide (Windows NT)	4317588
New Features and Installation Procedures for GeneMapper® ID Software V3.2 User Bulletin	4352543
GeneMapper® ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial	4335523
GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide	4338775
Quantifiler® Kits: Quantifiler® Human DNA Quantification Kit and Quantifiler® Y Human Male DNA Quantification Kit User's Manual	4344790
GeneMapper® ID Software v3.2.1 Patch User Bulletin	4382255

Note: For additional documentation, see "How to Obtain Support" on page xii.

Send Us Your Comments

Applied Biosystems welcomes your comments and suggestions for improving its user documents. You can e-mail your comments to:

techpubs@appliedbiosystems.com

IMPORTANT! The e-mail address above is only for submitting comments and suggestions relating to documentation. To order documents, download PDF files, or for help with a technical question, go to **www.appliedbiosystems.com**, then click the link for **Support**. (See "How to Obtain Support" below).

How to Obtain Support

For the latest services and support information for all locations, go to **www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

Overview

1

This chapter covers:

Product Overview 1-2
Workflow Overview 1-8
Instrument and Software Overview. 1-9
Materials and Equipment 1-11

Product Overview

Purpose

The AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit is a short tandem repeat (STR) multiplex assay that amplifies 11 tetranucleotide repeat loci, including the SE33 locus required specifically for the German DNA database. The kit simultaneously coamplifies the seven loci of the European Standard Set (ESSL) (D3S1358, vWA, D8S1179, TH01, FGA, D21S11, and D18S51), the Amelogenin locus, the highly polymorphic SE33 (ACTBP2) locus, and the D2S1338, D16S539, and D19S433 loci. The AmpFℓSTR SEfiler Plus Kit has been developed to deliver improvements in performance over the original SEfiler Kit. Changes to the kit include modified PCR cycling conditions for enhanced sensitivity, a new buffer formulation to improve performance with inhibited samples, improvements in synthesis and purification of the amplification primers, and a redeveloped allelic ladder.

Product Description

The SEfiler Plus kit contains all the necessary reagents for the amplification of human genomic DNA.

The reagents are designed for use with the following Applied Biosystems instruments:

- Applied Biosystems 3130/3130*xl* Genetic Analyzer
- ABI PRISM® 3100/3100-*Avant* Genetic Analyzer
- ABI PRISM® 310 Genetic Analyzer
- Silver 96-Well GeneAmp® PCR System 9700
- Gold-plated silver block GeneAmp® PCR System 9700

About the Primers

The AmpFℓSTR® SEfiler Plus™ kit contains the same loci and primer sequences as the SEfiler™ kit but uses improved synthesis and purification processes to minimize the presence of dye-labeled artifacts. Modifications made in the production of VIC® and PET® dye-labeled primers have greatly minimized the occurrence of the 120 VIC dye artifact as well as the PET dye-labeled artifacts observed at approximately 105 to 115 bp.

Loci Amplified by the Kit

Table 1-1 shows the loci amplified by the AmpFℓSTR® SEfiler Plus™ kit, their chromosomal locations, and the corresponding fluorescent marker dyes. The AmpFℓSTR® SEfiler Plus™ Allelic Ladder is used to genotype the analyzed samples. The alleles contained in the allelic ladder, and the genotype of the AmpFℓSTR Control DNA 007, are also listed in the table.

Table 1-1 AmpFℓSTR® SEfiler Plus™ Kit loci and alleles

Locus Designation	Chromosome Location	Dye Label	AmpFℓSTR® Allelic Ladder Alleles	AmpFℓSTR® Control DNA 007 Genotype
D2S1338	2q35–37.1	6-FAM™	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28	20, 23
D3S1358	3p	6-FAM	12, 13, 14, 15, 16, 17, 18, 19	15, 16
D8S1179 (in some references, designated as D6S502)	8	VIC	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	12, 13
D16S539	16q24-qter	6-FAM	5, 8, 9, 10, 11, 12, 13, 14, 15	9, 10
D18S51	18q21.3	PET	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27	12, 15

Table 1-1 AmpFℓSTR® SEfiler Plus™ Kit loci and alleles

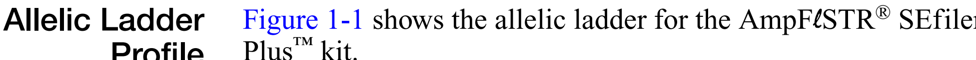
Locus Designation	Chromosome Location	Dye Label	AmpFℓSTR® Allelic Ladder Alleles	AmpFℓSTR® Control DNA 007 Genotype
D19S433	19q12–13.1	NED	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2	14, 15
D21S11	21q11.2–q21	PET	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38	28, 31
FGA	4q28	NED™	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2	24, 26

Table 1-1 AmpFℓSTR® SEfiler Plus™ Kit loci and alleles

Locus Designation	Chromosome Location	Dye Label	AmpFℓSTR® Allelic Ladder Alleles	AmpFℓSTR® Control DNA 007 Genotype
SE33 (ACTBP2)	6	VIC	4.2, 6.3, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 20.2, 21, 21.1, 21.2, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, 35, 35.2, 36, 37	17, 25.2
TH01	11p15.5	NED	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3	7, 9.3

Table 1-1 AmpFℓSTR® SEfiler Plus™ Kit loci and alleles

Locus Designation	Chromosome Location	Dye Label	AmpFℓSTR® Allelic Ladder Alleles	AmpFℓSTR® Control DNA 007 Genotype
vWA	12p12-pter	6-FAM	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24	14, 16
Amelogenin	X: p22.1–22.3 Y: p11.2	VIC	X, Y	X, Y



Allelic Ladder Profile

Figure 1-1 shows the allelic ladder for the AmpF*ℓ*STR® SEfiler Plus™ kit.

Figure 1-1 GeneMapper® *ID* Software plot of the AmpF*ℓ*STR® SEfiler Plus™ Allelic Ladder

Control DNA 007 Profile

Figure 1-2 shows amplification of Control DNA 007 using the AmpFℓSTR® SEfiler Plus™ kit.

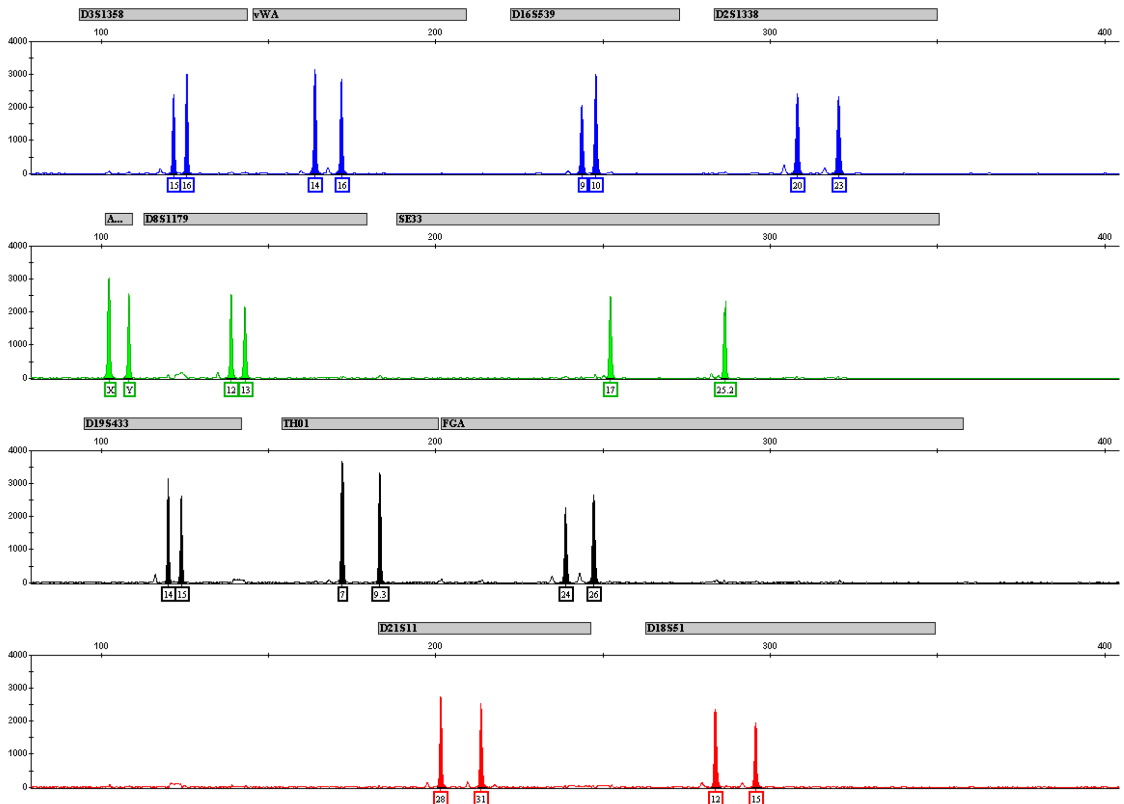


Figure 1-2 500 pg of Control DNA 007 amplified with the AmpFℓSTR SEfiler Plus kit and analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer

Workflow Overview

Extract and Quantify DNA

Quantifiler™ Total Human DNA Quantification Kit

PCR Amplify DNA

AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit

GeneAmp® PCR System 9700 Thermal Cycler*

* amplification should be done on silver or gold-plated silver blocks

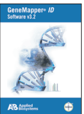
Perform Electrophoresis

ABI PRISM®
310 Genetic Analyzer

ABI PRISM®
3100/3100-*Avant*
Genetic Analyzer

Applied Biosystems
3130/3130*xl*
Genetic Analyzer

Analyze Data



GeneMapper® *ID* Software

Instrument and Software Overview

This section provides information about the data collection and analysis software versions required to run the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit on specific instruments.

Data Collection and Analysis Software

The data collection software provides instructions to firmware running on the instrument and displays instrument status and raw data in real time. As the instrument records sample fluorescence on the detection system hardware, the data collection software collects the data and stores it. Information about each sample is stored in a sample file (.fsa), which is then analyzed by the analysis software.

Instrument and Software Compatibility

Instrument	Operating System	Data Collection Software	Analysis Software
3130/3130*xl*‡	Windows XP	3.0	GeneMapper® *ID* v3.2.1
3100/3100-*Avant*	Windows NT®	1.1 (3100) 1.0 (3100-*Avant*)	GeneMapper *ID* v3.2.1
	Windows 2000	2.0	GeneMapper *ID* v3.2.1
310	Windows XP	3.1	GeneMapper *ID* v3.2.1
	Windows NT and Windows 2000	3.0	GeneMapper *ID* v3.2.1

‡ Applied Biosystems performed validation studies for the SEfiler Plus kit using these configurations.

About Multicomponent Analysis

Applied Biosystems fluorescent multi-color dye technology allows the analysis of multiple loci, including loci that have alleles with overlapping size ranges. Alleles for overlapping loci are distinguished by labeling locus-specific primers with different colored dyes.

Multicomponent analysis is the process that separates the five different fluorescent dye colors into distinct spectral components. The four dyes used in the AmpFℓSTR SEfiler Plus PCR Amplification Kit to label samples are 6-FAM™, VIC®, NED™, and PET®dyes. The fifth dye, LIZ®, is used to label the GeneScan™ 600 LIZ® Size Standard.

How Multicomponent Analysis Works

Each of the fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection on the Applied Biosystems and ABI PRISM® instruments, the fluorescence signals are separated by a diffraction grating according to their wavelengths and projected onto a charge-coupled device (CCD) camera in a predictably spaced pattern. The 6-FAM dye emits at the shortest wavelength and is displayed as blue, followed by the VIC dye (green), NED dye (yellow), PET dye (red), and LIZ dye (orange).

Although each of these dyes emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra between the dyes (Figure 1-3). The goal of multicomponent analysis is to correct for spectral overlap.

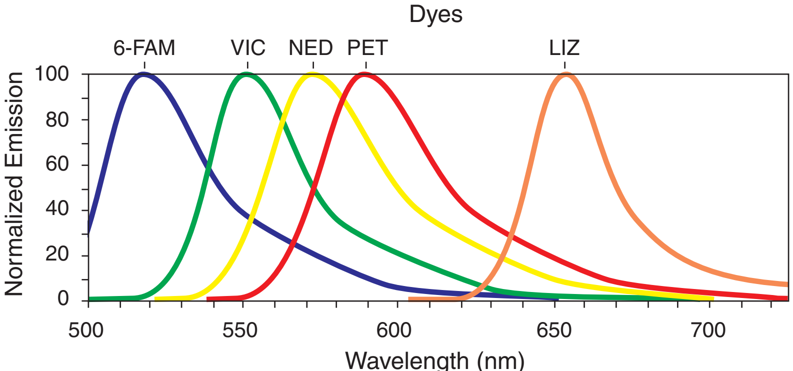


Figure 1-3 Emission spectra of the five dyes used in the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit

Materials and Equipment

Kit Contents and Storage

The AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit (PN 4382699) contains materials sufficient to perform 200 reactions at a 25-µL reaction volume.

IMPORTANT! The fluorescent dyes attached to the primers are light-sensitive. Protect the primer set from light when not in use. Amplified DNA, AmpFℓSTR® SEfiler Plus™ Allelic Ladder, and GeneScan™ 600 LIZ® Size Standard should also be protected from light. Keep freeze-thaw cycles to a minimum.

Table 1-2 AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit contents

Reagent	Contents	Quantity	Storage
AmpFℓSTR® SEfiler Plus™ Primer Set	Forward and reverse primers to amplify human DNA target	1 tube, 0.50 mL	–15 to –25 °C on receipt, 2 to 8 °C after initial use
AmpFℓSTR® SEfiler Plus™ Master Mix	Two tubes of Master Mix containing enzyme, salts, dNTPs, carrier protein, and 0.05% sodium azide	2 tubes, 0.50 mL/tube	–15 to –25 °C on receipt, 2 to 8 °C after initial use
AmpFℓSTR® SEfiler Plus™ Allelic Ladder	Allelic ladder containing amplified alleles (refer to "AmpFℓSTR® SEfiler Plus™ Kit loci and alleles" on page 1-3 for a list of alleles included in the ladder)	1 tube, 50 µL	–15 to –25 °C on receipt, 2 to 8 °C after initial use
AmpFℓSTR® Control DNA 007	0.10 ng/µL human male 007 DNA in 0.02% sodium azide and buffer (refer to "AmpFℓSTR® SEfiler Plus™ Kit loci and alleles" on page 1-3 for profile)	1 tube, 0.3 mL	2 to 8 °C

Standards for Samples

For the AmpFℓSTR® SEfiler Plus™ Kit, the panel of standards needed for PCR amplification, PCR product sizing, and genotyping are:

- **Control DNA 007** – A positive control for evaluating the efficiency of the amplification step and STR genotyping using the AmpFℓSTR® SEfiler Plus™ Allelic Ladder.
- **GeneScan™ 600 LIZ® Size Standard** – A size standard used for obtaining sizing results in the 60 to 400 nt range during use with the AmpFℓSTR SEfiler Plus kit. The GeneScan 600 LIZ Size Standard contains 36 single-stranded fragments of 20, 40, 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, 460, 480, 500, 514, 520, 540, 560, 580, and 600 nucleotides. This standard has been evaluated as an internal size standard, and it yields precise sizing results for AmpFℓSTR SEfiler Plus PCR products. Order the GeneScan 600 LIZ Size Standard (PN 4366589) separately.
- **AmpFℓSTR® SEfiler Plus™ Allelic Ladder** – An allelic ladder developed by Applied Biosystems for accurate characterization of the alleles amplified by the SEfiler Plus kit. The AmpFℓSTR SEfiler Plus Allelic Ladder contains most alleles reported for the 11 autosomal loci. Refer to "Loci Amplified by the Kit" on page 1-3 for a list of the alleles included in the SEfiler Plus kit.

Equipment and Materials Not Included

Tables 1-3 and 1-4 list required and optional equipment and materials not supplied with the SEfiler Plus kit. Unless otherwise indicated, many of the items are available from major laboratory suppliers (MLS).

Table 1-3 Equipment

Equipment	Source
Applied Biosystems 3130/3100*xl* Genetic Analyzer	Contact your local Applied Biosystems sales representative
ABI PRISM® 3100/3100-*Avant* Genetic Analyzer	
ABI PRISM® 310 Genetic Analyzer	
GeneAmp® PCR System 9700 with the Silver 96-Well block	N8050001
GeneAmp® PCR System 9700 with the Gold-plated silver block	4314878
Silver 96-Well sample block	N8050251

Table 1-3 Equipment *(continued)*

Equipment	Source
Gold-plated Silver 96-Well sample block	4314443
Tabletop centrifuge with 96-well plate adapters (optional)	Major Laboratory Supplier (MLS)

Table 1-4 User-supplied materials‡

Material	Source
AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit	4382699
3130/3100*xl* Analyzer materials	
96-Well Plate Septa	4315933
Reservoir Septa	4315932
3130*xl*/3100 Genetic Analyzer Capillary Array, 36-cm	4315931
3130/3100-*Avant* Genetic Analyzer Capillary Array, 36-cm	4333464
POP-4™ Polymer for 3130/3130*xl* Genetic Analyzers	4352755
3100/3100-*Avant* Genetic Analyzer Autosampler Plate Kit, 96-well	4316471
GeneScan™ 600 LIZ® Size Standard	4366589
Running Buffer, 10×	402824
DS-33 Matrix Standard Kit (Dye Set G5)	4345833
MicroAmp™ Optical 96-Well Reaction Plate	N8010560
Hi-Di™ Formamide	4311320
For a complete list of parts and accessories for the 3130/3130*xl* instrument, refer to Appendix A of the *Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide* (PN 4352716).	

Table 1-4 User-supplied materials‡ *(continued)*

Material	Source
3100/3100-*Avant* Analyzer materials	
96-Well Plate Septa	4315933
Reservoir Septa	4315932
3130*xl*/3100 Genetic Analyzer Capillary Array, 36-cm	4315931
3130/3100-*Avant* Genetic Analyzer Capillary Array, 36-cm	4333464
POP-4™ Polymer for 3100/3100-*Avant* Genetic Analyzers	4316355
3100/3100-*Avant* Genetic Analyzer Autosampler Plate Kit, 96-well	4316471
GeneScan™ 600 LIZ® Size Standard	4366589
Running Buffer, 10×	402824
DS-33 Matrix Standard Kit (Dye Set G5)	4345833
MicroAmp™ Optical 96-Well Reaction Plate	N8010560
250-µL Glass Syringe, (array-fill syringe)	4304470
5.0-mL Glass Syringe, (polymer-reserve syringe)	628-3731
For a complete list of parts and accessories for the 3100/3100-*Avant* instrument, refer to Appendix B of the *ABI PRISM® 3100 Genetic Analyzer and 3100-Avant Genetic Analyzer User Reference Guide* (PN 4335393).	
310 Analyzer materials	
310 Genetic Analyzer Capillary, 47-cm	402839
0.5-mL Sample Tray	5572
96-Well Tray Adaptor (for 9700 thermal cycler trays)	4305051
GeneScan™ 600 LIZ® Size Standard	4366589
Running Buffer, 10×	402824
Genetic Analyzer Septa Retainer Clips for 96-Tube Sample Tray	402866
Genetic Analysis Sample Tubes (0.5-mL)	401957

Table 1-4 User-supplied materials‡ *(continued)*

Material	Source
Septa for 0.5-mL Sample Tubes	401956
DS-33 Matrix Standard Set [6FAM™, VIC®, NED™, PET®, and LIZ® dyes] for ABI PRISM® 310/377 systems	4318159
MicroAmp™ 8-Tube Strip, 0.2-mL	N8010580
MicroAmp™ 96-Well Base (holds 0.2-mL reaction tubes)	N8010531
MicroAmp™ 96-Well Full Plate Cover	N8010550
MicroAmp™ 96-Well Tray/Retainer Set	403081
POP-4™ Polymer for the 310 Genetic Analyzer	402838
For a complete list of parts and accessories for the 310 instrument, refer to Appendix B of the *ABI PRISM® 310 Genetic Analyzer User Guide* (PN 4317588).	
PCR Amplification	
MicroAmp™ 96-Well Tray	N8010541
MicroAmp® Reaction Tube with Cap, 0.2-mL	N8010540
MicroAmp™ 8-Tube Strip, 0.2-mL	N8010580
MicroAmp™ 8-Caps Strip	N8010535
MicroAmp™ 96-Well Tray/Retainer Set	403081
MicroAmp™ 96-Well Base	N8010531
MicroAmp™ Optical 96-Well Reaction Plate	N8010560
Other user-supplied materials	
Hi-Di™ Formamide, 25-mL	4311320
Aerosol-resistant pipette tips	MLS
Microcentrifuge tubes	MLS
Pipettors	MLS
Tape, labeling	MLS

Table 1-4 User-supplied materials‡ *(continued)*

Material	Source
Tube, 50-mL Falcon	MLS
Tube decapper, autoclavable	MLS
Deionized water, PCR grade	MLS
Tris-HCL, pH 8.0	MLS
EDTA, 0.5 M	MLS
Vortex	MLS

‡ For the Material Safety Data Sheet (MSDS) of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Chapter 2

PCR Amplification

PCR Amplification 2

This chapter covers:

PCR Work Areas 2-2
Required User-Supplied Materials and Reagents 2-4
Quantifying DNA 2-5
Preparing the Reactions. 2-7
Performing PCR 2-9
Amplification Using Bloodstained FTA Cards 2-10

PCR Work Areas

Work Area Setup and Lab Design

Many resources are available for the appropriate design of a PCR laboratory.

- If you are using the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit for forensic DNA testing, refer to *Forensic Laboratories: Handbook for Facility Planning, Design, Construction and Moving*, National Institute of Justice, 1998 (www.nij.org/publications).
- If you are using the SEfiler Plus kit for parentage DNA testing, refer to the *Guidance for Standards for Parentage Relationship Testing Laboratories*, American Association of Blood Banks, 7th edition, 2004.

The sensitivity of the SEfiler Plus kit (and other PCR-based tests) enables amplification of minute quantities of DNA, necessitating precautions to avoid contamination of samples yet to be amplified (Kwok and Higuchi, 1989).

Also take care while handling and processing samples to prevent contamination by human DNA. Wear gloves at all times and change them frequently. Close sample tubes when not in use. Limit aerosol dispersal by handling sample tubes and reagents carefully.

Note: Applied Biosystems does not intend these references for laboratory design to constitute all precautions and care necessary for using PCR technology.

PCR Setup Work Area

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

- Calculator
- Gloves, disposable
- Marker pen, permanent
- Microcentrifuge
- Microcentrifuge tubes, 1.5-mL, or 2.0-mL, or other appropriate clean tube (for Master Mix preparation)
- Microcentrifuge tube rack
- Pipette tips, sterile, disposable hydrophobic filter-plugged
- Pipettors
- Tube decapper, autoclavable
- Vortex

Amplified DNA Work Area

The following GeneAmp® PCR Systems should be placed in the Amplified DNA Work Area.

- Silver block 96-Well GeneAmp® PCR System 9700
- Gold-plated silver block GeneAmp® PCR System 9700

Required User-Supplied Materials and Reagents

Kit Contents and Storage

Each AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit contains materials sufficient to perform 200 reactions at a 25-µL reaction volume. See “Kit Contents and Storage” on page 1-12 for details on SEfiler Plus kit contents.

IMPORTANT! The fluorescent dyes attached to the primers are light-sensitive. Protect the primer set from light when not in use. Amplified DNA, AmpFℓSTR® SEfiler Plus™ Allelic Ladder, and GeneScan™ 600 LIZ® Size Standard should also be protected from light. Minimize freeze-thaw cycles.

User-Supplied Reagents

In addition to the SEfiler Plus kit reagents, the use of low TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) is recommended. You can prepare the buffer as described in the following table or order it from Teknova (Cat # T0223).

To prepare low TE buffer

<table>
<tr><td>1.</td><td>Mix together:
10 mL of 1 M Tris-HCl, pH 8.0
0.2 mL of 0.5 M EDTA, pH 8.0
990 mL glass-distilled or deionized water
WARNING CHEMICAL HAZARD. EDTA. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Note: Adjust the volumes accordingly for specific needs.</td></tr>
<tr><td>2.</td><td>Aliquot and autoclave the solutions.</td></tr>
<tr><td>3.</td><td>Store at room temperature.</td></tr>
</table>

Quantifying DNA

Importance of Quantitation

Quantifying the amount of DNA in a sample before amplification allows you to determine whether or not sufficient DNA is present to permit amplification and to calculate the optimum amount of DNA to add to the reaction. The optimum amount of DNA for the SEfiler Plus Kit is 0.50 to 0.75 ng in a maximum input volume of 10 µL.

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

- Fluorescence intensity that exceeds the linear dynamic range for detection by the instrument (“off-scale” data).
  Off-scale data are problematic because:
  - 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, and it results in poor spectral separation (“pull-up”).
- Incomplete A nucleotide addition.

When the total number of allele copies added to the PCR is extremely low, allelic dropout can occur resulting in a partial profile.

Methods for Quantifying DNA

Applied Biosystems provides several kits for quantifying DNA in samples. See the reference cited in Table 2-1 on page 2-6 for details about these kits.

Table 2-1 Methods for quantifying DNA

<table>
<tr><th>Product</th><th>Description</th><th>References</th></tr>
<tr><td>Quantifiler® Y Human Male DNA Quantification Kit (PN 4343906)</td><td rowspan="2">Properties:
• Both Quantifiler® kits have high specificity for human DNA. The Quantifiler® Y kit is highly specific for human male DNA.
• The kit detects single-stranded and degraded DNA.
How it works:
The DNA quantification assay combines two 5′ nuclease assays:
• A target-specific (human DNA or human male DNA) assay, which consists of two primers for amplifying human or human male DNA and one TaqMan® MGB probe labeled with FAM™ dye for detecting the amplified sequence
• An internal PCR control (IPC) assay, which consists of an IPC template DNA (a synthetic sequence not found in nature), two primers for amplifying the IPC template DNA, and one TaqMan MGB probe labeled with VIC® dye for detecting the amplified IPC DNA</td><td rowspan="2">Quantifiler® Human DNA Quantification Kits User's Manual (PN 4344790)</td></tr>
<tr><td>Quantifiler® Human DNA Quantification Kit (PN 4343895)</td></tr>
</table>

Preparing the Reactions

SEfiler Plus Kit Reactions

To prepare the reactions

1.	Calculate the volume of each component needed to prepare the reactions, using the table below. **Component** / **Volume Per Reaction (µL)**: AmpFℓSTR® SEfiler Plus™ Master Mix — 10.0 AmpFℓSTR® SEfiler Plus™ Primer Set — 5.0 **Note:** Include additional reactions in your calculations to provide excess volume for the loss that occurs during reagent transfers.
2.	Prepare reagents. Thaw the AmpFℓSTR® SEfiler Plus™ Master Mix and the AmpFℓSTR® SEfiler Plus™ Primer Set, then vortex the mix and primer set 3 seconds and centrifuge them briefly before opening the tubes. **IMPORTANT!** Thawing is required only during first use of the kit. After first use, reagents are stored at 2 to 8 °C and, therefore, do not require subsequent thawing. Do not refreeze the reagents.
3.	Pipette the required volumes of components into an appropriately sized polypropylene tube.
4.	Vortex the reaction mix for 3 seconds, then centrifuge briefly.
5.	Dispense 15 µL of the reaction mix into each reaction well of a MicroAmp™ Optical 96-Well Reaction Plate or each MicroAmp® Reaction Tube.

To prepare the reactions *(continued)*

<table>
<tr><td>6.</td><td>Prepare the DNA samples:

DNA Sample	To Prepare...
Negative Control	Add 10 μL of low TE buffer.
Your Sample	Dilute a portion of your DNA sample with low TE buffer so that 0.50 to 0.75 ng of total DNA is in a final volume of 10 μL. Add your sample to the reaction mix.
Positive Control	Combine 5 μL of control DNA (0.1 ng/μL) with 5 μL of low TE buffer for a total volume of 10 μL. (The final sample concentration is 0.05 ng/μL.) Add to the reaction mix.

Note: The final reaction volume should be 25 μL.</td></tr>
<tr><td>7.</td><td>Centrifuge the plate at 3,000 rpm for about 20 seconds in a tabletop centrifuge with plate holders to remove any bubbles.</td></tr>
<tr><td>8.</td><td>Amplify the DNA in a Silver block 96-Well GeneAmp® PCR System 9700, or a Gold-plated silver block GeneAmp® PCR System 9700.</td></tr>
</table>

Performing PCR

To run PCR

1. Set the thermal cycling conditions.

   IMPORTANT! If using the Gold-plated Silver or Silver 96-Well GeneAmp PCR System 9700, select the **9600 Emulation mode**.

Initial Incubation Step	Cycle (30 cycles)			Final Extension	Final Hold
	De-nature	Anneal	Extend		
HOLD	CYCLE			HOLD	HOLD
95 °C 11 min	94 °C 20 sec	59 °C 2 min	72 °C 1 min	60 °C 60 min	4 °C ∞

2. Load the plate into the thermal cycler, then close the heated cover.

PHYSICAL INJURY HAZARD. During instrument operation, the temperature of the heated cover can be as high as 108 °C, and the temperature of the sample block can be as high as 100 °C. Keep hands away from the heated cover and sample block.

3. Start the run.

4. Store the amplified DNA.

If you are storing the DNA for...	Store at...
<2 weeks	2 to 8 °C
>2 weeks	–15 to –25 °C

   IMPORTANT! Protect the amplified products from light.

Amplification Using Bloodstained FTA Cards

FTA™-treated DNA collection cards can be useful for the collection, storage, and processing of biological samples. You can place a small punch disk of the bloodstained card directly into an amplification tube, purify it, and amplify it without transferring the evidence. Applied Biosystems studies indicate that a 1.2-mm bloodstained disk contains approximately 5 to 20 ng DNA. An appropriate cycle number for this high quantity of DNA is 25 cycles. It is recommended that each laboratory determine the cycle number based on individual validation studies.

In the example shown in Figure 2-1, a 1.2-mm disk of a bloodstained FTA card was purified using three washes with FTA Purification Reagent and two washes with 1✕ TE buffer. After drying at room temperature overnight, the punch was then amplified directly in the MicroAmp® tube for 25 cycles.

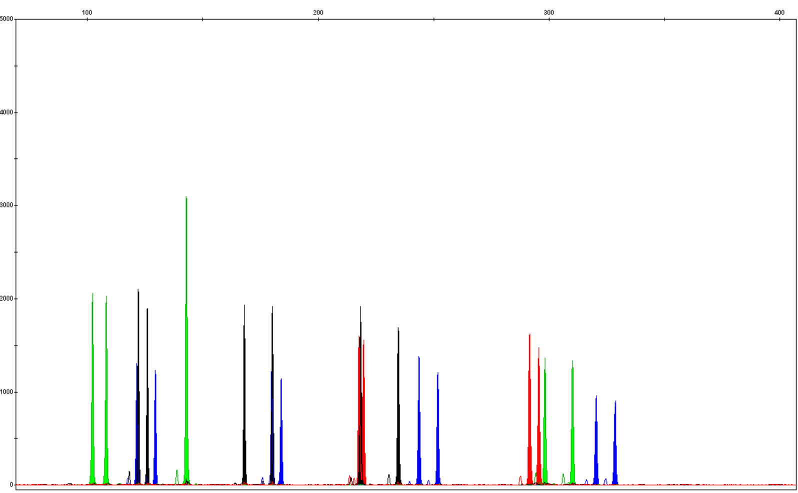


Figure 2-1 AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit results from a 1.2-mm FTA bloodstain disk (25-cycle amplification), analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer

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Chapter 3

Electrophoresis

Performing Electrophoresis 3

This chapter covers:

Allelic Ladder Requirements 3-2
Setting-Up the 3100/3100-*Avant* or 3130/3130*xl* Instrument for Electrophoresis 3-3
Preparing Samples for Electrophoresis on the 3100/3100-*Avant* or 3130/3130*xl* Instrument 3-5
Setting-Up the 310 Instrument for Electrophoresis 3-7
Preparing Samples for Electrophoresis on the 310 Instrument. . . .3-8

Allelic Ladder Requirements

To accurately genotype samples, use an Allelic Ladder sample that is run with the samples. Applied Biosystems recommends that for:

- ABI PRISM® 310 Genetic Analyzer – Run at least one allelic ladder for every 10 sample injections.
- ABI PRISM® 3100 or Applied Biosystems 3130 series instruments – Run at least one allelic ladder for each set of 16 samples.
  - Applied Biosystems 3130*xl* or ABI PRISM® 3100 systems – One ladder per injection; one injection = 16 samples (15 samples + 1 allelic ladder)
  - Applied Biosystems 3130 or ABI PRISM® 3100-*Avant* – One ladder for every 4 injections; one injection = 4 samples

IMPORTANT! Variation in laboratory temperature can cause changes in fragment migration speed, which can, in turn, cause sizing variation. Applied Biosystems recommends the above frequency of allelic ladder injections to account for normal variation in run speed. However, during internal validation studies, each laboratory should verify the required allelic ladder injection frequency to ensure accurate genotyping of all samples in each laboratory environment.

It is critical to genotype using an allelic ladder that is run under the same conditions as the samples because:

- Size values obtained for the same sample can differ between instrument platforms because of different polymer matrices and electrophoretic conditions.
- Slight procedural and reagent variations between single and multiple capillaries result in greater size variation than that found between samples injected in the same capillary in a single run.

Setting-Up the 3100/3100-*Avant* or 3130/3130*xl* Instrument for Electrophoresis

Reagents and Parts

Table 1-4 on page 1-14 lists the required materials not supplied with the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit.

IMPORTANT! The fluorescent dyes attached to the primers are light-sensitive. Protect the primer set from light when not in use. Amplified DNA, AmpFℓSTR® SEfiler Plus™ Allelic Ladder, and GeneScan™ 600 LIZ® Size Standard should also be protected from light. Minimize freeze-thaw cycles.

Electrophoresis Setup Software and Reference Documents

Table 3-1 lists data collection software and the run modules that you can use to analyze SEfiler Plus kit products. For details on the procedures, refer to the documents listed in the table.

Table 3-1 SEfiler Plus kit: data collection software and reference documents for use with the 3100/3100-*Avant* or 3130/3130*xl* instruments

Operating System	Data Collection Software	Run Module	References
Windows XP	3.0 (3130/3130*xl* Analyzer)‡	• HIDFragmentAnalysis36_POP4_1 • Dye Set G5	*Applied Biosystems 3130/3130xl Genetic Analyzers Using Data Collection Software v3.0, Protocols for Processing AmpFℓSTR® PCR Amplification Kit PCR Products User Bulletin* (PN 4363787).
Windows 2000	2.0	• HIDFragmentAnalysis36_POP4_1 • Dye Set G5	*ABI PRISM® 3100/3100-Avant Genetic Analyzers Using Data Collection Software v2.0, Protocols for Processing AmpFℓSTR® PCR Amplification Kit PCR Products User Bulletin* (PN 4350218)

Operating System	Data Collection Software	Run Module	References
Windows NT®	1.1 (3100 Analyzer)	Run Module: GeneScan36vb_DyeSetG5Module Analysis Module: GS600Analysis.gsp	*ABI PRISM® 3100/3100-Avant Genetic Analyzers Protocols for Processing AmpFℓSTR® PCR Amplification Kit PCR Products User Bulletin* (PN 4332345)
	1.0 (3100-*Avant* Analyzer)	Run Module: GeneScan36Avb_DyeSetG5Module Analysis Module: GS600Analysis.gsp	

‡ Applied Biosystems performed validation studies for the SEfiler Plus kit using this configuration.

Preparing Samples for Electrophoresis on the 3100/3100-*Avant* or 3130/3130*xl* Instrument

Preparing the Samples

Prepare the samples for electrophoresis on the 3100/3100-*Avant* or 3130/3130*xl* instrument immediately before loading.

To prepare samples for electrophoresis

1. Calculate the volume of Hi-Di™ Formamide and GeneScan™ 600 LIZ® Internal Size Standard needed to prepare the samples, using the table below.

Reagent	Volume per reaction (µL)
GeneScan™ 600 LIZ® Size Standard	0.6
Hi-Di™ Formamide	8.4

Note: Include additional samples in your calculations to provide excess volume for the loss that occurs during reagent transfers.

IMPORTANT! The volume of size standard indicated in the table is a suggested amount. Determine the appropriate amount of size standard based on your results/experiments.

CHEMICAL HAZARD. Hi-Di Formamide. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

2. Pipette the required volumes of components into an appropriately sized polypropylene tube.

3. Vortex the tube, then centrifuge briefly.

To prepare samples for electrophoresis *(continued)*

4.	Into each well of a MicroAmp™ Optical 96-Well Reaction Plate, add: • 9 µL of the formamide:size standard mixture • 1 µL of PCR product or Allelic Ladder **Note:** For blank wells, add 10 µL of Hi-Di™ formamide.
5.	Seal the reaction plate with appropriate septa, then briefly centrifuge the plate to ensure that the contents of each well are mixed and collected at the bottom.
6.	Heat the reaction plate in a thermal cycler for 3 minutes at 95 °C. **WARNING** **PHYSICAL INJURY HAZARD.** During instrument operation, the temperature of the heated cover can be as high as 108 °C, and the temperature of the sample block can be as high as 100 °C. Keep hands away from the heated cover and sample block.
7.	Immediately place the plate on ice for 3 minutes.
8.	Prepare the plate assembly on the autosampler.
9.	Start the electrophoresis run.

Setting-Up the 310 Instrument for Electrophoresis

Reagents and Parts

Table 1-4 on page 1-14 lists the required materials not supplied with the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit.

IMPORTANT! The fluorescent dyes attached to the primers are light-sensitive. Protect the primer set from light when not in use. Amplified DNA, AmpFlSTR® SEfiler Plus™ Allelic Ladder, and GeneScan™ 600 LIZ® Size Standard should also be protected from light. Minimize freeze-thaw cycles.

Electrophoresis Setup Software and Reference Documents

Table 3-2 lists data collection software and the run modules that you can use to analyze SEfiler Plus kit products. For details on the procedures, refer to the documents listed in the table.

Table 3-2 SEfiler Plus kit: data collection software and reference documents for use with the 310 instrument

Operating System	Data Collection Software	Run Module	References
Windows XP	3.1‡	GS STR POP4 (1ml) G5 v2.md5	*ABI PRISM® 310 Genetic Analyzer User's Manual (Windows)* (PN 4317588) *ABI PRISM® 310 Protocols for Processing AmpFℓSTR® PCR Amplification Kit Products with Microsoft Windows NT Operating System: User Bulletin* (PN 4341742)
Windows NT® and Windows 2000	3.0	GS STR POP4 (1ml) G5 v2.md5	*ABI PRISM® 310 Genetic Analyzer User's Manual (Windows)* (PN 4317588) *ABI PRISM® 310 Protocols for Processing AmpFℓSTR® PCR Amplification Kit Products with Microsoft Windows NT Operating System: User Bulletin* (PN 4341742)

‡ Applied Biosystems conducted concordance studies for the SEfiler Plus kit using this configuration.

Preparing Samples for Electrophoresis on the 310 Instrument

Preparing the Samples

Prepare the samples for electrophoresis on the 310 instrument immediately before loading.

To prepare samples for electrophoresis

1. Calculate the volume of Hi-Di™ Formamide and GeneScan™ 600 LIZ® Internal Size Standard needed to prepare the samples, using the table below.

Reagent	Volume per reaction (µL)
GeneScan™ 600 LIZ® Size Standard	1.0
Hi-Di™ Formamide	24.0

Note: Include additional samples in your calculations to provide excess volume for the loss that occurs during reagent transfers.

IMPORTANT! The volume of size standard indicated in the table is a suggested amount. Determine the appropriate amount of size standard based on your results/experiments.

CHEMICAL HAZARD. Hi-Di Formamide. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

2. Pipette the required volumes of components into an appropriately sized polypropylene tube.

3. Vortex the tube, then centrifuge briefly.

To prepare samples for electrophoresis *(continued)*

4.	Into each 0.2-mL or 0.5-mL sample tube, add: • 25 µL of the formamide:size standard mixture • 1.5 µL of PCR product or Allelic Ladder
5.	Seal the tubes with appropriate septa, then briefly centrifuge the tubes to ensure that the contents of each tube are mixed and collected at the bottom.
6.	Heat the tubes in a thermal cycler for 3 minutes at 95 °C. **WARNING** **PHYSICAL INJURY HAZARD.** During instrument operation, the temperature of the heated cover can be as high as 108 °C, and the temperature of the sample block can be as high as 100 °C. Keep hands away from the heated cover and sample block.
7.	Immediately place the tubes on ice for 3 minutes.
8.	Place the sample tray on the autosampler.
9.	Start the electrophoresis run.

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Chapter 4

Analyzing Data

Analyzing Data 4

This chapter covers:

Overview of GeneMapper® *ID* Software 4-2
Setting Up GeneMapper® *ID* Software v3.2.1 for Analyzing AmpFℓSTR® SEfiler Plus™ Kit Data 4-3
Analyzing and Editing Sample Files with GeneMapper® *ID* Software 4-17

Overview of GeneMapper® *ID* Software

What Does GeneMapper *ID* Software Do?

GeneMapper® *ID* Software is an automated genotyping software solution for forensic, paternity, and database data analysis and other genotyping needs.

After electrophoresis, the data collection software stores information for each sample in a .fsa file. Using GeneMapper *ID* v3.2.1 software, you can then analyze and interpret the data.

Instruments

Refer to "Instrument and Software Overview" on page 1-10 for a list of compatible instruments.

Before You Start

When using GeneMapper *ID* Software version 3.2.1 to perform Human Identification (HID) analysis with AmpFℓSTR® kits, consider that:

- HID analysis requires at least one allelic ladder sample per run folder. Your laboratory can use multiple ladder samples in an analysis as long as it performs the appropriate validation studies.

  For multiple ladder samples, the GeneMapper *ID* Software calculates allelic bin offsets by using an average of all ladders that use the same panel within a run folder.
- Allelic ladder samples in an individual run folder are considered to be from a single run.

  When the software imports multiple run folders into a project, only the ladder(s) within their respective run folders are used for calculating allelic bin offsets and subsequent genotyping.
- Allelic ladder samples need to be identified as "Allelic Ladder" in the Sample Type column in a project. Failure to apply this setting for ladder samples results in failed analysis.
- Allelic bin definitions are stored in the AmpFLSTR_SEfiler_Plus_Panels_v1 in the Panel Manager.
- Injections containing the allelic ladder must be analyzed with the same analysis method and parameter values that are used for samples to ensure proper allele calling.

When using GeneMapper *ID-X* Software version 1.0 to perform Human Identification (HID) analysis with AmpFℓSTR® kits, refer to the *GeneMapper® ID-X Software Version 1.0 Human Identification Analysis Getting Started Guide* (PN 4375574).

- Alleles not found in the AmpFℓSTR® Allelic Ladders do exist. Off-ladder alleles may contain full and/or partial repeat units. An off-ladder allele is an allele that occurs outside the ±0.5-nt bin window of any known allelic ladder allele or virtual bin.

Note: If a sample allele peak is called as an off-ladder allele, the sample result needs to be verified according to the laboratory's protocol.

Setting Up GeneMapper® *ID* Software v3.2.1 for Analyzing AmpFℓSTR® SEfiler Plus™ Kit Data

Overview

Before you can analyze sample (.fsa) files using GeneMapper *ID* Software v3.2.1 for the first time, you need to:

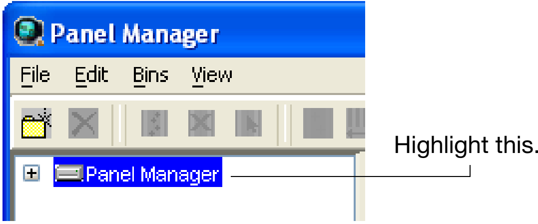
- Import panels and bins into the Panel Manager, as explained in "Importing Panels and Bins" on page 4-4.
- Import an analysis method as explained in "Importing an HID Analysis Method" on page 4-9.
- Define custom views of analysis tables.
- Refer to Chapter 1 of the *GeneMapper® ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial* (PN 4335523) for more information.
- Define custom views of plots.
- Refer to Chapter 1 of the *GeneMapper® ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial* (PN 4335523) for more information.

Note: For details about GeneMapper *ID* features, refer to the *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide* (PN 4338775), *GeneMapper® ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial* (PN 4335523). Also, refer to the *Installation Procedures and New Features for GeneMapper® ID Software v3.2 User Bulletin* (PN 4352543).

Note: For details about GeneMapper *ID-X*, refer to *GeneMapper® ID-X Software Version 1.0 Human Identification Analysis Getting Started Guide* (PN 4375574).

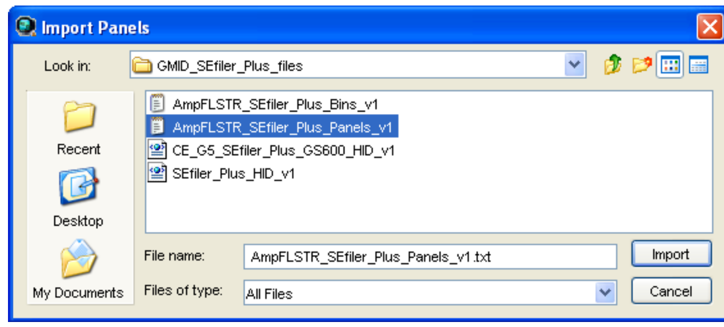
Importing Panels and Bins

To import the SEfiler Plus kit panels and bin sets from the Applied Biosystems web site into the GeneMapper *ID* software database:

1.	a. Download the GMID_SEfiler_Plus_files.zip file containing panels and bins from: www.appliedbiosystems.com ▸ Support ▸ Software Downloads ▸ Select GeneMapper® ID Software v3.2 ▸ Updaters & Patches. b. Unzip the file.
2.	Start the GeneMapper *ID* software, then log in with the appropriate user name and password. **IMPORTANT!** If you need log on instructions, refer to the *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide* (PN 4338775), page 2-7.
3.	Select **Tools** ▸ **Panel Manager** to open the Panel Manager.
4.	Find, then open the folder containing the panels and bins: a. Select **Panel Manager** in the navigation pane.  b. Select **File** ▸ **Import Panels** to open the Import Panels dialog box. c. Navigate to, then open the GMID_SEfiler_Plus_files folder that you unzipped in step .

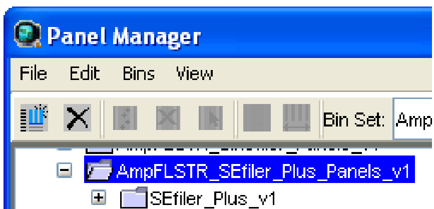
5. Select **AmpFLSTR_SEfiler_Plus_Panels_v1**, then click **Import**.

Note: Importing this file creates a new folder in the navigation pane of the Panel Manager, AmpFLSTR_SEfiler_Plus_Panels_v1. This folder contains the panels and associated markers.



6. Import AmpFLSTR_SEfiler_Plus_Bins_v1:

   a. Select the **AmpFLSTR_SEfiler_Plus_Panels_v1** folder in the navigation pane.

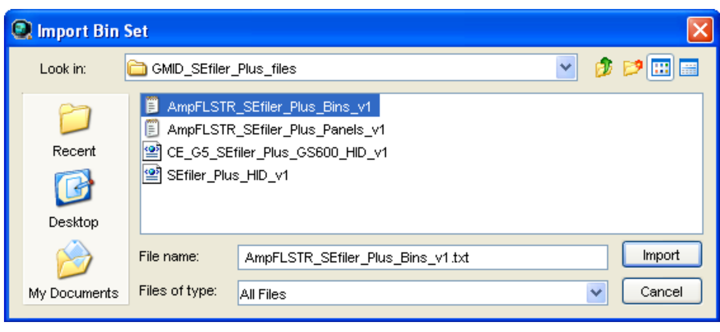


   b. Select **File ▸ Import Bin Set** to open the Import Bin Set dialog box.

   c. Navigate to, then open the **GMID_SEfiler_Plus_files** folder.

   d. Select **AmpFLSTR_SEfiler_Plus_Bins_v1**, then click **Import**.

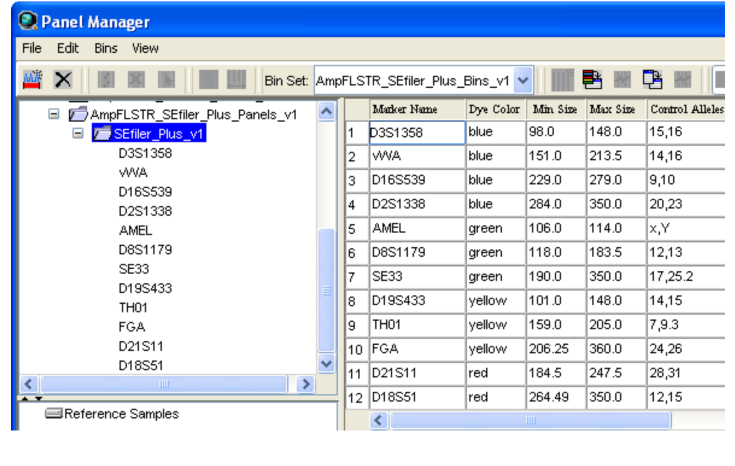
   Note: Importing this file associates the bin set with the panels in the AmpFLSTR_SEfiler_Plus_Panels_v1 folder.

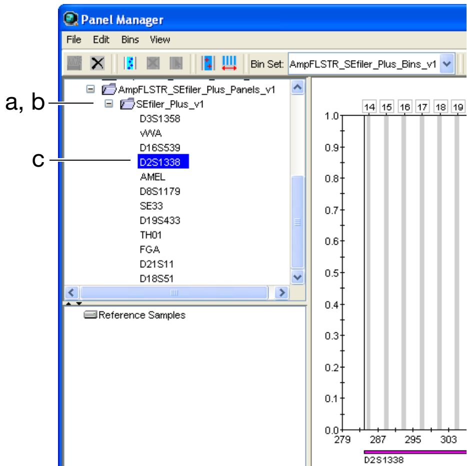


7. View the imported panels in the navigation pane:

   a. Double-click **AmpFLSTR_SEfiler_Plus_Panels_v1** folder to view the SEfiler_Plus_v1 folder.

   b. Double-click the **SEfiler_Plus_v1** folder to display the panel information in the right pane and the markers below it.



8.	View the markers and display the Bin view in the navigation pane: a. Select the **SEfiler_Plus_v1** folder to display its list of markers in the right pane. b. Double-click the **SEfiler_Plus_v1** folder to display its list of markers below it. c. Select **D2S1338** to display the Bin view for the marker in the right pane. 
9.	Click **Apply**, then **OK** to add the SEfiler Plus panel and bin set to the GeneMapper *ID* database. **IMPORTANT!** If you close the Panel Manager without clicking **OK**, the panels and bins are not imported into the GeneMapper *ID* database.

Importing an HID Analysis Method

The analysis method for the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit uses the HID Advanced Mode Peak Detection Algorithm. This analysis method provides users with the same analysis parameters available in GeneScan® Software v3.7.1 for the Windows operating system.

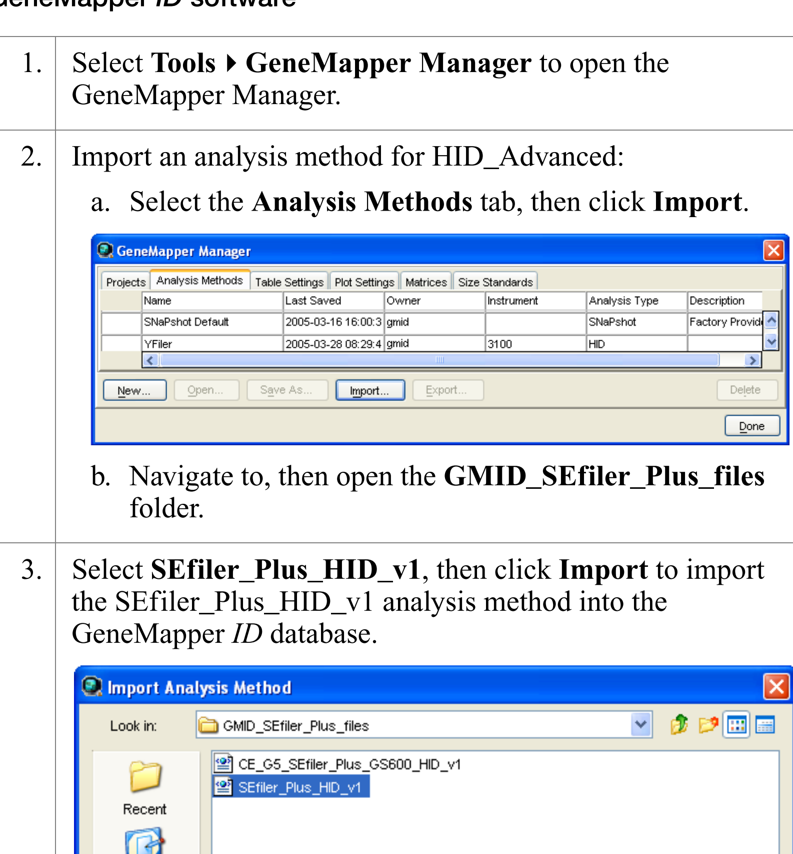
Use the following procedure to import the analysis method for the SEfiler Plus kit from the folder that you downloaded from the Applied Biosystems web site into the GeneMapper *ID* software database. Refer to step 1a on page 4-4 for downloading instructions.

To import the HID Advanced Mode analysis method into GeneMapper *ID* software

1. Select **Tools ▸ GeneMapper Manager** to open the GeneMapper Manager.

2. Import an analysis method for HID_Advanced:

   a. Select the **Analysis Methods** tab, then click **Import**.



   b. Navigate to, then open the **GMID_SEfiler_Plus_files** folder.

3. Select **SEfiler_Plus_HID_v1**, then click **Import** to import the SEfiler_Plus_HID_v1 analysis method into the GeneMapper *ID* database.

To import the HID Advanced Mode analysis method into GeneMapper *ID* software *(continued)*

4.	To view the settings in the SEfiler_Plus_HID_v1 analysis method: a. Select the **Analysis Methods** tab. b. Select **SEfiler_Plus_HID_v1** in the Name column, then click **Open**. Table 4-1 on page 4-10 shows the settings for each tab of the Analysis Method Editor - HID.

Table 4-1 SEfiler_Plus_HID_v1 Advanced Mode analysis method settings

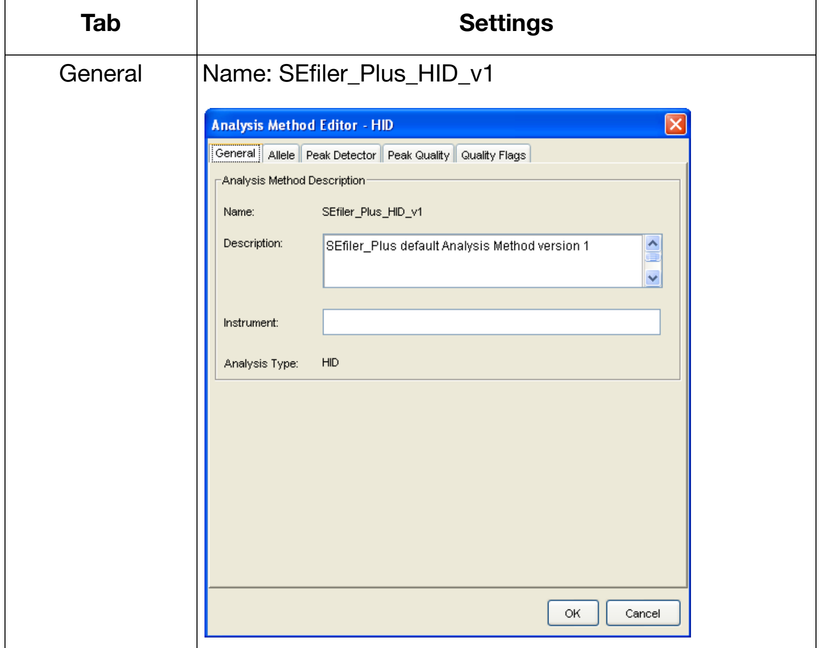
Tab	Settings
General	Name: SEfiler_Plus_HID_v1 

Table 4-1 SEfiler_Plus_HID_v1 Advanced Mode analysis method settings *(continued)*

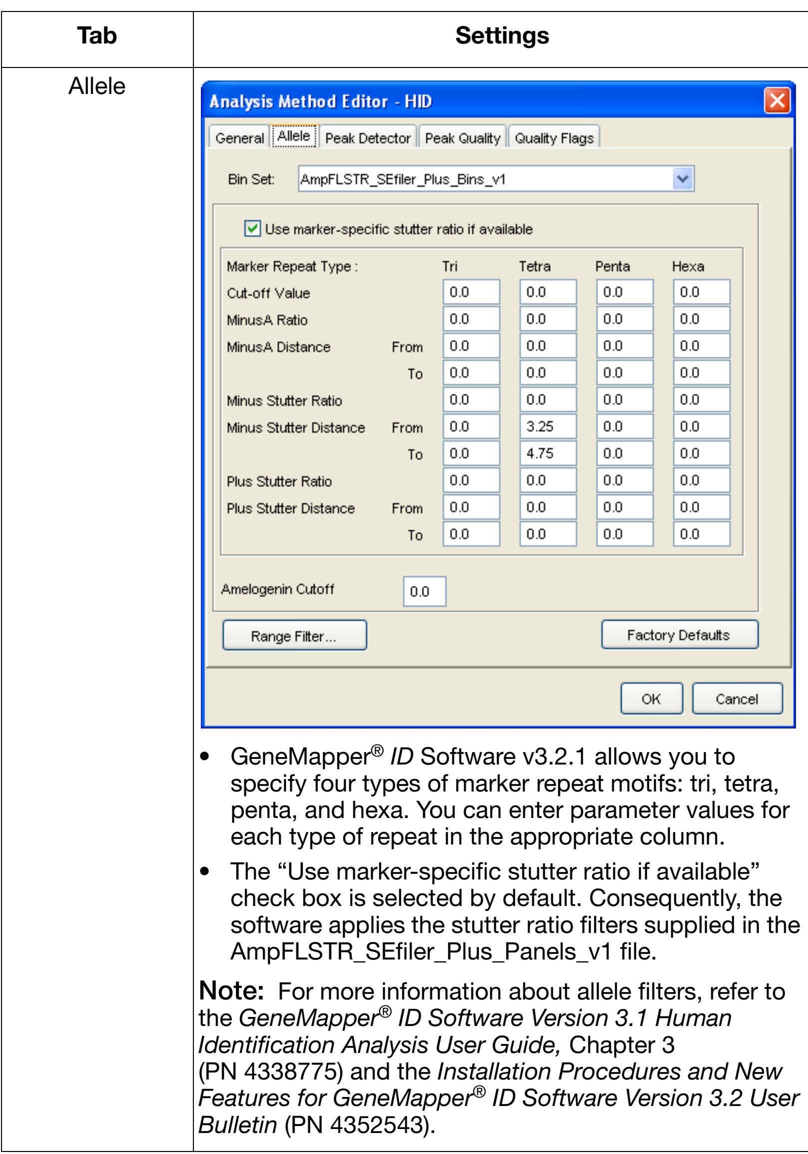
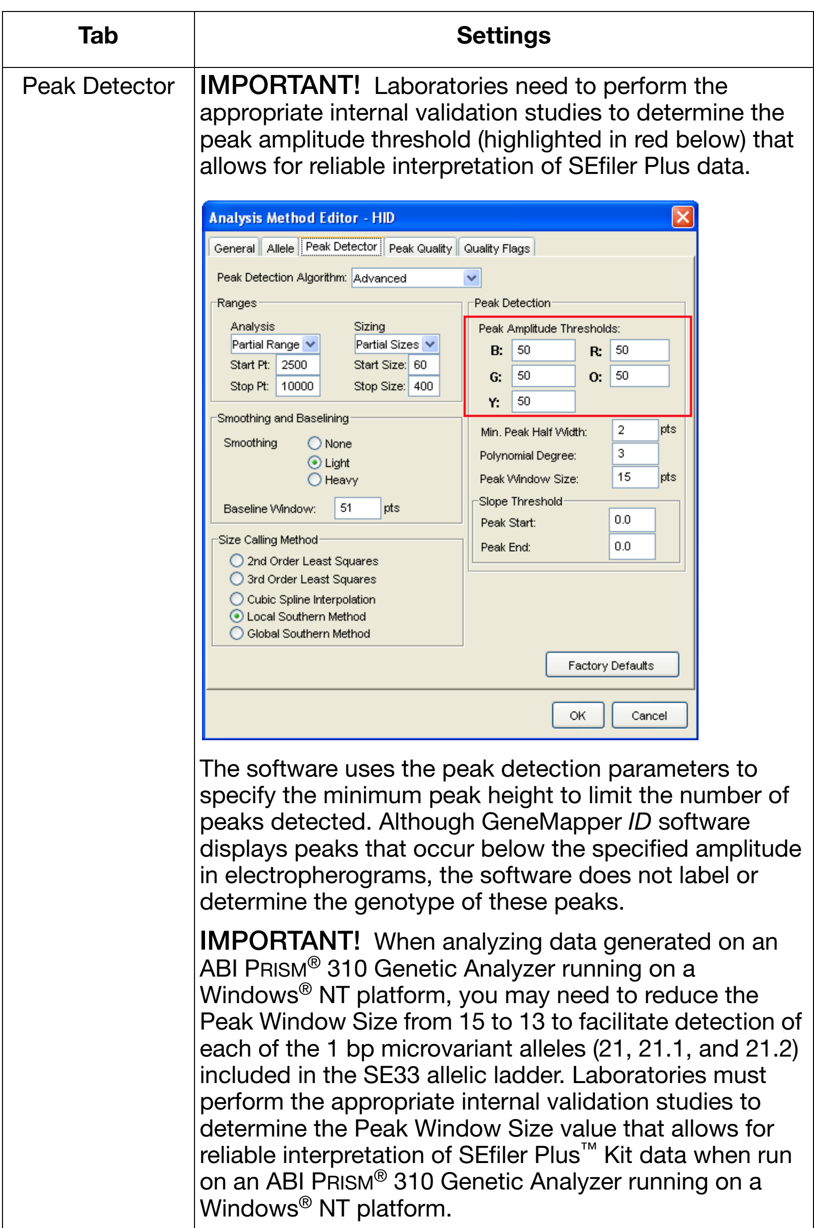
Tab	Settings
Allele	 • GeneMapper® *ID* Software v3.2.1 allows you to specify four types of marker repeat motifs: tri, tetra, penta, and hexa. You can enter parameter values for each type of repeat in the appropriate column. • The "Use marker-specific stutter ratio if available" check box is selected by default. Consequently, the software applies the stutter ratio filters supplied in the AmpFLSTR_SEfiler_Plus_Panels_v1 file. **Note:** For more information about allele filters, refer to the *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide,* Chapter 3 (PN 4338775) and the *Installation Procedures and New Features for GeneMapper® ID Software Version 3.2 User Bulletin* (PN 4352543).

Table 4-1 SEfiler_Plus_HID_v1 Advanced Mode analysis method settings *(continued)*

<table>
<tr><th>Tab</th><th>Settings</th></tr>
<tr><td>Peak Detector</td><td>

IMPORTANT! Laboratories need to perform the appropriate internal validation studies to determine the peak amplitude threshold (highlighted in red below) that allows for reliable interpretation of SEfiler Plus data.



The software uses the peak detection parameters to specify the minimum peak height to limit the number of peaks detected. Although GeneMapper *ID* software displays peaks that occur below the specified amplitude in electropherograms, the software does not label or determine the genotype of these peaks.

IMPORTANT! When analyzing data generated on an ABI PRISM® 310 Genetic Analyzer running on a Windows® NT platform, you may need to reduce the Peak Window Size from 15 to 13 to facilitate detection of each of the 1 bp microvariant alleles (21, 21.1, and 21.2) included in the SE33 allelic ladder. Laboratories must perform the appropriate internal validation studies to determine the Peak Window Size value that allows for reliable interpretation of SEfiler Plus™ Kit data when run on an ABI PRISM® 310 Genetic Analyzer running on a Windows® NT platform.

</td></tr>
</table>

Table 4-1 SEfiler_Plus_HID_v1 Advanced Mode analysis method settings *(continued)*

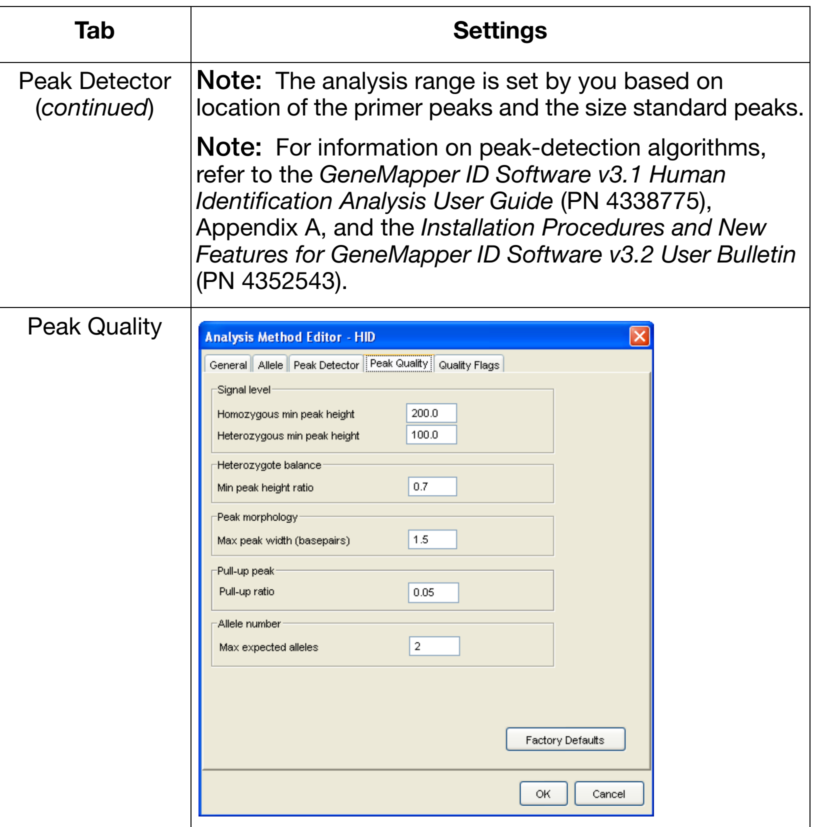
Tab	Settings
Peak Detector (*continued*)	**Note:** The analysis range is set by you based on location of the primer peaks and the size standard peaks. **Note:** For information on peak-detection algorithms, refer to the *GeneMapper ID Software v3.1 Human Identification Analysis User Guide* (PN 4338775), Appendix A, and the *Installation Procedures and New Features for GeneMapper ID Software v3.2 User Bulletin* (PN 4352543).
Peak Quality	

Table 4-1 SEfiler_Plus_HID_v1 Advanced Mode analysis method settings *(continued)*

Tab	Settings
Quality Flags and PQV Thresholds	Analysis Method Editor - HID General · Allele · Peak Detector · Peak Quality · Quality Flags Quality weights are between 0 and 1. Quality Flag Settings Spectral Pull-up 0.8 · Control Concordance 1.0 Broad Peak 0.8 · Low Peak Height 0.3 Out of Bin Allele 0.8 · Off-scale 0.8 Overlap 0.8 · Peak Height Ratio 0.3 PQV Thresholds Pass Range: · Low Quality Range: Sizing Quality: From 0.75 to 1.0 · From 0.0 to 0.25 Genotype Quality: From 0.75 to 1.0 · From 0.0 to 0.25 Factory Defaults OK · Cancel

Importing an HID Size Standard

The size standard for the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit uses the following GS600 peaks in its sizing algorithm: 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, and 400.

Use the following procedure to import the size standard for the SEfiler Plus kit from the folder that you downloaded from the Applied Biosystems web site, into the GeneMapper *ID* software database. Refer to step 1a on page 4-4 for downloading instructions.

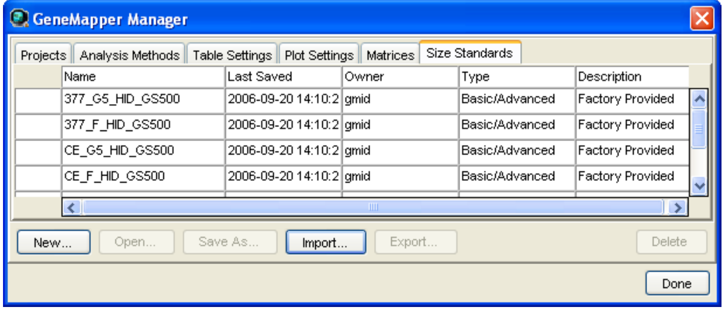
To import an HID Size Standard

1.	Select **Tools** ▸ **GeneMapper Manager** to open the GeneMapper Manager.

To import an HID Size Standard *(continued)*

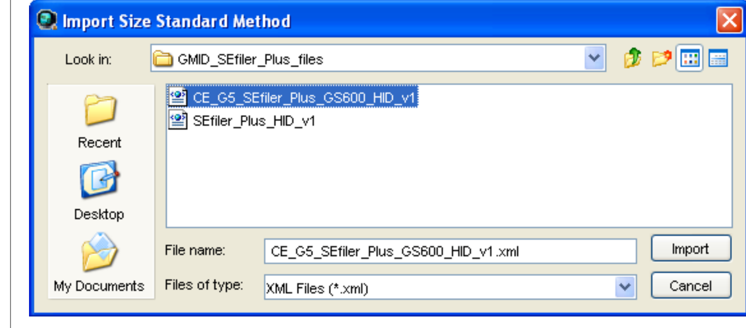
2. Import a Size Standard:

   a. Select the **Size Standards** tab, then click **Import**.



   b. Navigate to, then open the **GMID_SEfiler_Plus_files** folder.

3. Select **CE_G5_SEfiler_Plus_GS600_HID_v1**, then click **Import** to import the SEfiler_Plus_HID_v1 analysis method into the GeneMapper *ID* database.



Analyzing and Editing Sample Files with GeneMapper® *ID* Software

Analyzing a Project

1. In the Project window, select **File ▸ Add Samples to Project**, then navigate to the disk or directory containing the sample files.

2. Apply analysis settings to the samples in the project.

Parameter	Advanced Analysis Method
Sample Type	Select the sample type.
Analysis Method	**SEfiler_Plus_HID_v1**
Panel	**AmpFLSTR_SEfiler_Plus_Panels_v1**
Size Standard‡	**CE_G5_SEfiler_Plus_GS600_HID**§
Matrix	Select a matrix for 310 instruments only.

‡ For more information about how the Size Caller works, refer to the *ABI PRISM® GeneScan® Analysis Software for the Windows NT® Operating System Overview of the Analysis Parameters and Size Caller User Bulletin* (PN 4335617).

§ The following fragments are defined for the CE_G5_HID_GS600 size standard provided with the AmpFℓSTR® SEfiler Plus kit: 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, and 400. For additional information about size standards, refer to the *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide* (PN 4338775), Appendix D.

3. Click ▶ (Analyze), enter a name for the project (in the Save Project dialog), then click **OK** to start analysis.
   - The status bar displays progress of analysis:
     – As a completion bar extending to the right with the percentage indicated
     – With text messages on the left
   - The table displays the row of the sample currently being analyzed in green (or red if analysis failed for the sample).
   - The Genotypes tab becomes available after analysis.

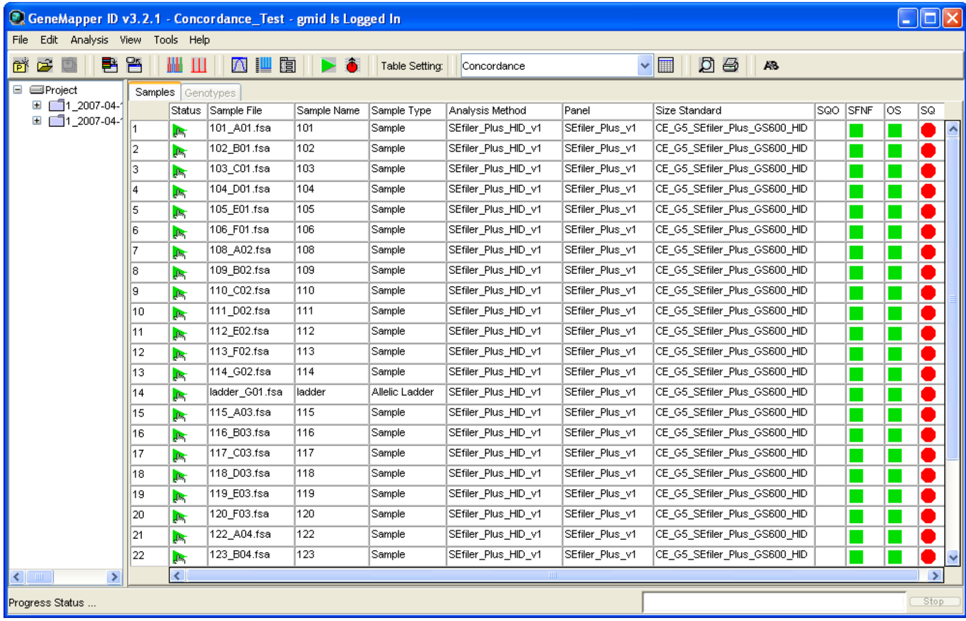


Figure 4-1 Project Window before analysis

For more information about any of these tasks, refer to the *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide* (PN 4338775).

Examining and Editing a Project

You can display electropherogram plots from the Samples and Genotypes tabs of the Project window to examine the data. These procedures start with the Samples tab of the Project window (assuming the analysis is complete).

For more information about any of these tasks, refer to:

- *Installation Procedures and New Features for GeneMapper® ID Software v3.2 User Bulletin* (PN 4352543)
- *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide* (PN 4338775)
- *GeneMapper® ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial* (PN 4335523)

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5

Experiments and Results

This chapter covers:

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Overview

Experiments Using AmpFℓSTR® SEfiler Plus™ Kit

This chapter provides results of the developmental validation experiments performed by Applied Biosystems using the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit (SEfiler Plus kit).

Importance of Validation

Validation of a DNA typing procedure for human identification applications is an evaluation of the procedure's efficiency, reliability, and performance characteristics. By challenging the procedure with samples commonly encountered in forensic and parentage laboratories, the validation process uncovers attributes and limitations that are critical for sound data interpretation in casework (Sparkes, Kimpton, Watson *et al.,* 1996; Sparkes, Kimpton, Gilbard *et al.,* 1996; and Wallin *et al.,* 1998).

Experiments

Experiments to evaluate the performance of the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit were performed at Applied Biosystems. These experiments were performed according to the DNA Advisory Board (DAB) Quality Assurance Standards, effective October 1, 1998 (DNA Advisory Board, 1998). The DAB standards describe the quality assurance requirements that a laboratory should follow to ensure the quality and integrity of the data and competency of the laboratory.

Additional validation was performed according to the revised guidelines from the Scientific Working Group on DNA Analysis Methods (SWGDAM, July 10, 2003). Based on these guidelines, Applied Biosystems has performed experiments that comply with guidelines 1.0 and 2.0 and its associated subsections. This DNA methodology is not novel (Moretti *et al.,* 2001; Frank *et al.,* 2001; Wallin *et al.,* 2002; and Holt *et al.,* 2000).

This chapter discusses many of the experiments performed by Applied Biosystems and provides examples of results obtained. Applied Biosystems chose conditions that produced maximum PCR product yield and that met reproducible performance standards. It is the opinion of Applied Biosystems that although these experiments are not exhaustive, they are appropriate for a manufacturer.

IMPORTANT! Each laboratory using the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit must perform internal validation studies.

Developmental Validation

SWGDAM Guideline 1.2.1

"Developmental validation is the demonstration of the accuracy, precision, and reproducibility of a procedure by the manufacturer, technical organization, academic institution, government laboratory, or other party." (SWGDAM, July 2003)

SWGDAM Guideline 2.10.1

"The reaction conditions needed to provide the required degree of specificity and robustness must be determined. These include thermocycling parameters, the concentration of primers, magnesium chloride, DNA polymerase, and other critical reagents." (SWGDAM, July 2003)

PCR Components

Applied Biosystems examined the concentration of each component of the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit. The concentration for each individual component was established to be in the window that meets the reproducible performance characteristics of specificity and sensitivity. For example, various magnesium chloride concentrations were tested on the Applied Biosystems 3130*xl* Genetic Analyzer. The amplification of 0.50 ng of the control DNA 007 is shown in Figure 5-1 on page 5-4. Applied Biosystems observed that the performance of the multiplex is most robust within a ±20% window of magnesium chloride concentration.

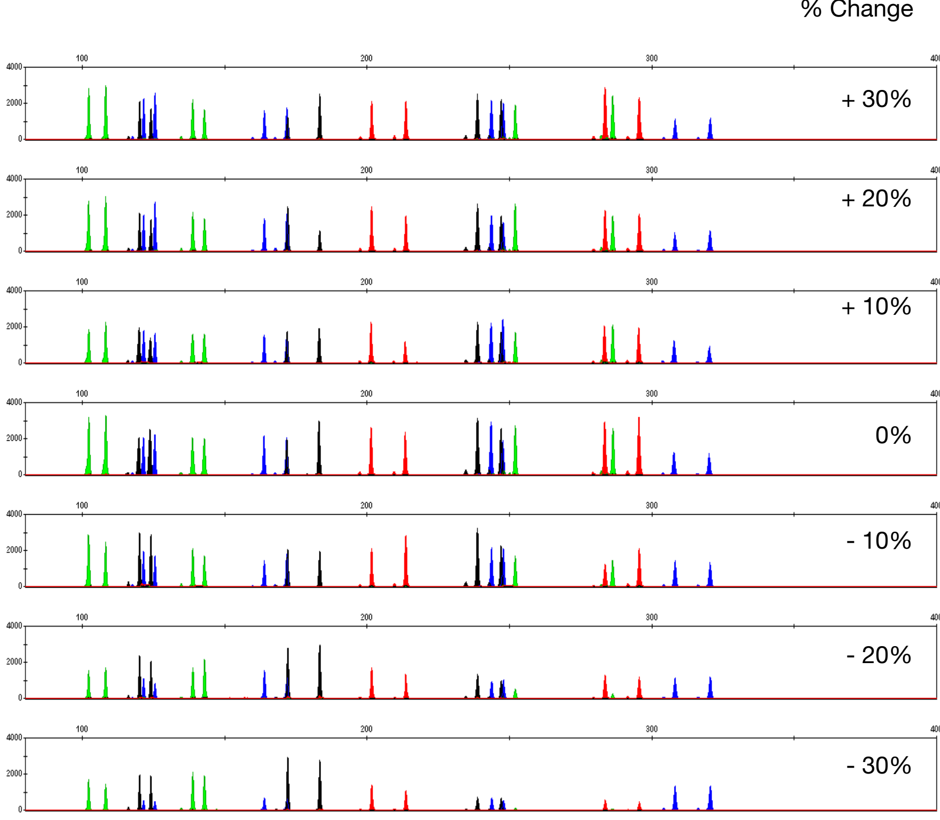

Figure 5-1 0.50 ng of control DNA 007 amplified with the AmpFℓSTR® SEfiler Plus™ kit in the presence of varying concentrations of magnesium chloride and analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer

Thermal Cycler Parameters

Thermal cycling parameters were established for amplification of the SEfiler Plus kit. Thermal cycling times and temperatures of GeneAmp® PCR systems were verified. Varying annealing and denaturation temperature windows were tested to verify that a specific PCR product with the desired sensitivity of at least 0.50 ng of AmpFℓSTR® Control DNA 007 was produced.

For example, annealing temperatures were tested at 55, 57, 59, 61, and 63 °C (Figure 5-2 on page 5-5) for 2-minute hold times in the Silver 96-Well GeneAmp® PCR System 9700. The PCR products were analyzed using the Applied Biosystems 3130*xl* Genetic Analyzer.

Of the tested annealing temperatures, 55 to 61 °C produced robust profiles. At 63 °C the yield of the majority of loci was significantly reduced. Routine thermal cycler calibration is recommended when you follow the amplification protocol. No preferential amplification was observed at the standard annealing temperature of 59 °C.

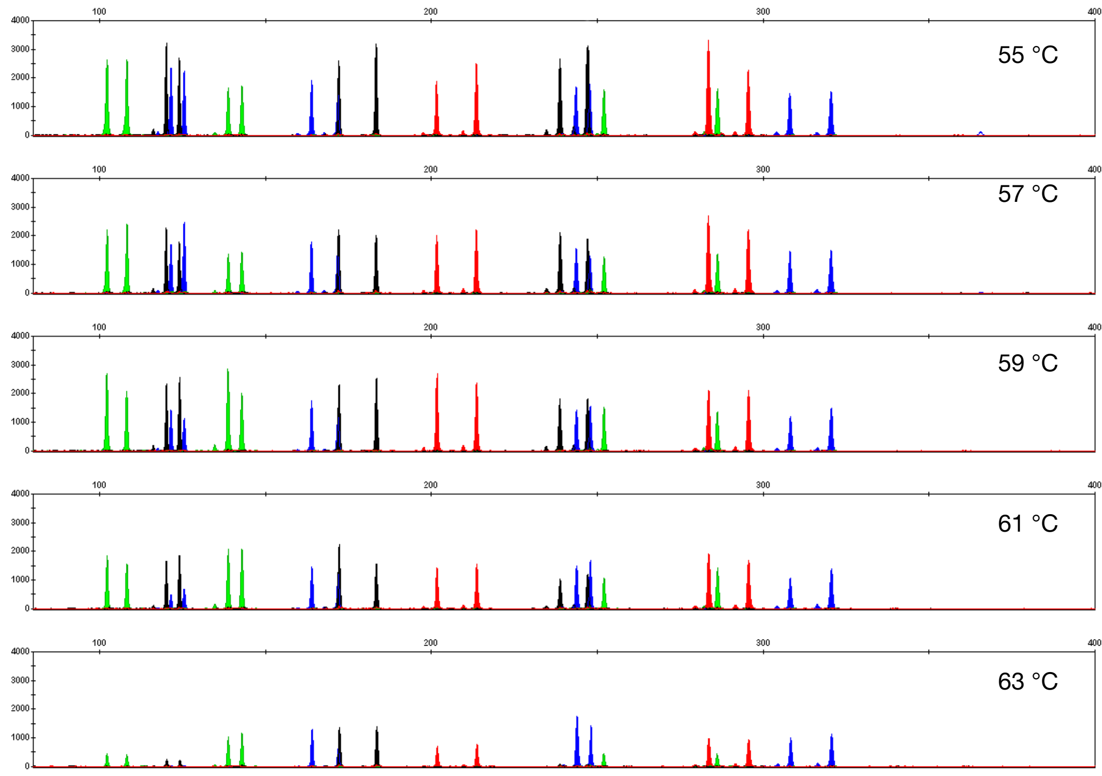

Figure 5-2 Electropherograms obtained from amplification of 0.50 ng of control DNA 007 at annealing temperatures of 55 °C, 57 °C, 59 °C, 61 °C, and 63 °C, analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer, Y-axis scale (0 to 4,000 RFUs)

PCR Cycle Number

SEfiler Plus kit reactions were amplified for 28, 29, 30, 31, and 32 cycles on the Silver 96-Well GeneAmp® PCR System 9700 using 0.50 ng from three DNA samples. As expected, the amount of PCR product increased with the number of cycles. A full profile was generated at 28 cycles and off-scale data were collected for several allele peaks at 32 cycles (Figure 5-3).

Although none of the cycle numbers tested produced nonspecific peaks, 30 cycles was found to give optimal sensitivity when the amplified products were examined on Applied Biosystems 3130*xl* Genetic Analyzers.

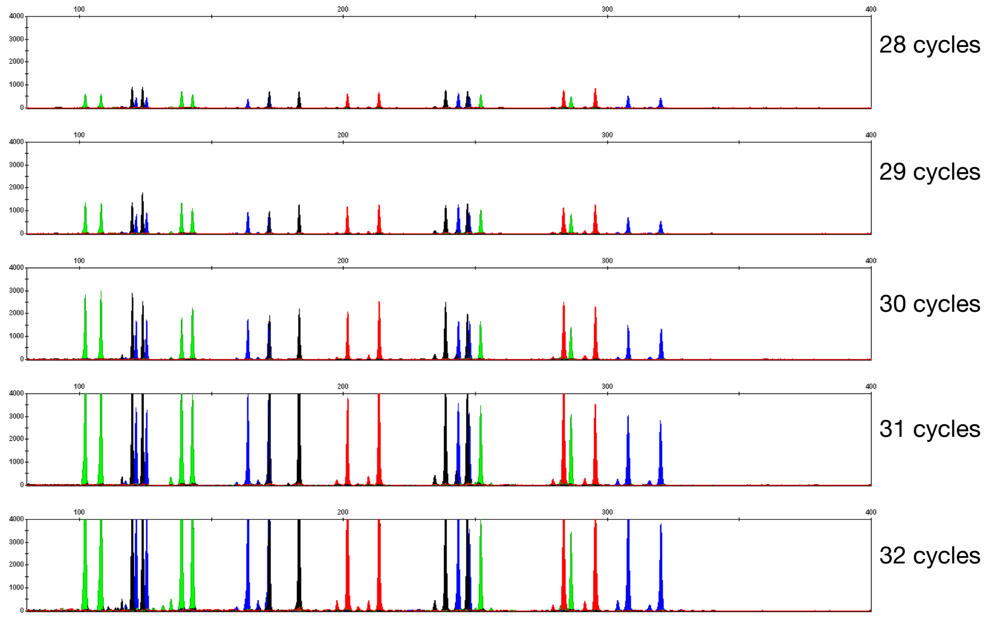

Figure 5-3 Representative AmpFℓSTR® SEfiler Plus™ kit profiles obtained from amplification of 0.50 ng DNA template using 28, 29, 30, 31, and 32 cycles, analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer, Y-axis scale (0 to 4,000 RFUs)

Accuracy, Precision, and Reproducibility

SWGDAM Guideline 2.9

"The extent to which a given set of measurements of the same sample agree with their mean and the extent to which these measurements match the actual values being measured should be determined." (SWGDAM, July 2003)

Accuracy

Laser-induced fluorescence detection of length polymorphism at short tandem repeat loci is not a novel methodology (Holt *et al.,* 2000; and Wallin *et al.,* 2002). However, accuracy and reproducibility of AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit profiles have been determined from various sample types.

Figure 5-4 on page 5-8 shows the size differences that are typically observed between sample alleles and allelic ladder alleles on the Applied Biosystems 3130*xl* Genetic Analyzer with POP-4™ polymer. The x-axis in Figure 5-4 on page 5-8 represents the nominal nucleotide sizes for the AmpFℓSTR® SEfiler Plus™ Allelic Ladder. The dashed lines parallel to the x-axis represent the ±0.25-nt windows. The y-axis represents the deviation of each sample allele size from the corresponding allelic ladder allele size. All sample alleles are within ±0.5 nt from a corresponding allele in the allelic ladder.

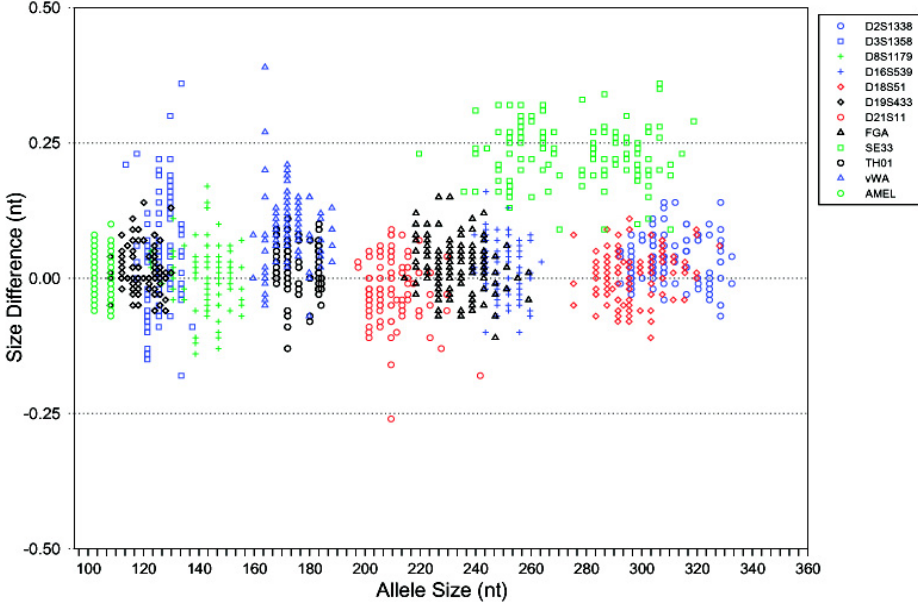

Figure 5-4 Size deviation of 77 samples analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer

Precision and Size Windows

Sizing precision allows for determining accurate and reliable genotypes. Sizing precision was measured on the Applied Biosystems 3130*xl* Genetic Analyzer. The recommended method for genotyping is to employ a ±0.5-nt "window" around the size obtained for each allele in the AmpFℓSTR® SEfiler Plus™ Allelic Ladder. A ±0.5-nt window allows for the detection and correct assignment of alleles. Any sample allele that sizes outside the specified window could be:

- An "off-ladder" allele, that is, an allele of a size that is not represented in the AmpFℓSTR® SEfiler Plus™ Allelic Ladder

  or

- 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 injections on a capillary instrument or in several lanes of one gel.

Table 5-1 on page 5-10 shows typical precision results obtained from five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder on the Applied Biosystems 3130*xl* Genetic Analyzer (36-cm capillary and POP-4™ polymer). The internal size standard that was used was GeneScan™ 600 LIZ® Size Standard. The results were obtained within a set of injections on a single capillary array.

Sample alleles may occasionally size outside the ±0.5-nt 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 5-4 on page 5-8 illustrates the tight clustering of allele sizes obtained on the Applied Biosystems 3130*xl* Genetic Analyzer, where the standard deviation in sizing is typically less than 0.15 nt. The instance of a sample allele sizing outside of the ±0.5-nt window because of measurement error is relatively rare when the standard deviation in sizing is approximately 0.15 nt or less (Smith, 1995).

For sample alleles that do not size within a ±0.5-nt window, the PCR product must be rerun to distinguish between a true off-ladder allele versus 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.

The GeneMapper® *ID* software v3.2.1 automatically flags sample alleles that do not size within the specified window around an allelic ladder allele.

Although the precision within a 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 polymer mixture, run temperature, and electrophoresis conditions. Variations in sizing can occur between runs on the same instrument and between runs on different instruments because of these factors.

Applied Biosystems strongly recommends that the allele sizes be compared to the sizes obtained for known alleles in the AmpFℓSTR® SEfiler Plus™ Allelic Ladder from the same run and then be converted to genotypes (as described in “Before You Start” on

page 4-2.). See Table 5-1 for the results of five runs of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder. For more information on precision and genotyping, see Lazaruk *et al.,* 1998 and Mansfield *et al.,* 1998.

In Table 5-1, the mean size for all the alleles in each run (16 capillaries) was calculated. The mean range shown in the table is the lowest and highest mean-size values of the five runs. Similarly, the standard deviation for the allele sizing was calculated for all the alleles in each run. The standard deviation range shown in Table 5-1 is the lowest and highest standard deviation values of the five runs.

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
Amelogenin		
X	102.16–102.18	0.028–0.034
Y	108.14–108.15	0.015–0.038
D16S539		
5	227.25–227.29	0.027–0.047
8	239.36–239.42	0.034–0.044
9	243.51–243.56	0.027–0.045
10	247.63–247.69	0.029–0.042
11	251.67–251.74	0.026–0.043
12	255.63–255.69	0.029–0.045
13	259.56–259.63	0.024–0.041
14	263.57–263.63	0.031–0.045
15	267.59–267.64	0.027–0.041

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
D18S51		
7	263.07–263.14	0.025–0.05
9	271.19–271.24	0.031–0.048
10	275.24–275.3	0.024–0.039
10.2	277.24–277.3	0.023–0.047
11	279.32–279.37	0.023–0.048
12	283.36–283.41	0.03–0.048
13	287.38–287.43	0.025–0.037
13.2	289.34–291.43	0.027–0.039
14	291.37–291.43	0.027–0.039
14.2	293.35–293.4	0.033–0.045
15	295.38–295.43	0.026–0.037
16	299.37–299.43	0.032–0.043
17	303.31–303.37	0.023–0.04
18	307.29–307.34	0.027–0.044
19	311.32–311.37	0.032–0.055
20	315.46–315.5	0.024–0.036
21	319.75–319.81	0.034–0.048
22	323.93–324	0.03–0.046
23	327.97–328.04	0.03–0.049
24	332.06–332.13	0.023–0.04

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
25	336.12–336.18	0.027–0.036
26	340.15–340.19	0.023–0.042
27	344.27–344.29	0.022–0.038
D19S433		
9	99.6–99.68	0.03–0.05
10	103.76–103.85	0.04–0.044
11	107.9–107.97	0.03–0.045
12	111.99–112.06	0.029–0.053
12.2	114.03–114.11	0.024–0.039
13	115.97–116.06	0.029–0.036
13.2	117.96–118.04	0.027–0.044
14	119.89–119.98	0.034–0.045
14.2	121.87–121.95	0.033–0.045
15	123.8–123.9	0.033–0.039
15.2	125.79–125.9	0.03–0.04
16	127.76–127.86	0.03–0.044
16.2	129.75–129.86	0.027–0.047
17	131.72–131.83	0.035–0.057
17.2	133.72–133.84	0.034–0.044

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
D21S11		
24	185.16–185.24	0.022–0.041
24.2	187.22–187.29	0.026–0.041
25	189.25–189.33	0.021–0.039
26	193.32–193.41	0.026–0.041
27	197.41–197.51	0.026–0.048
28	201.41–201.48	0.025–0.036
28.2	203.36–203.44	0.031–0.046
29	205.34–205.42	0.031–0.046
29.2	207.37–207.46	0.021–0.035
30	209.34–209.43	0.026–0.039
30.2	211.31–211.4	0.025–0.036
31	213.32–213.41	0.033–0.038
31.2	215.34–215.43	0.028–0.044
32	217.4–217.51	0.026–0.041
32.2	219.44–219.51	0.037–0.043
33	221.48–221.57	0.037–0.044
33.2	223.44–223.51	0.031–0.041
34	225.57–225.64	0.029–0.045
34.2	227.5–227.57	0.028–0.046
35	229.56–229.65	0.035–0.044

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder ***(continued)***

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
35.2	231.51–231.59	0.038–0.044
36	233.5–233.58	0.029–0.046
37	237.58–237.68	0.032–0.044
38	241.59–241.67	0.03–0.04
D2S1338		
15	288.11–288.2	0.035–0.039
16	292.09–292.18	0.039–0.041
17	296.05–296.13	0.03–0.038
18	300–300.08	0.034–0.041
19	303.91–303.98	0.031–0.038
20	307.84–307.92	0.03–0.049
21	311.86–311.93	0.044–0.05
22	315.97–316.07	0.028–0.048
23	320.22–320.28	0.033–0.041
24	324.3–324.37	0.03–0.047
25	328.36–328.44	0.031–0.046
26	332.38–332.5	0.027–0.053
27	336.44–336.53	0.029–0.038
28	340.76–340.83	0.027–0.042

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
D3S1358		
12	109.27–109.27	0.027–0.041
13	113.51–113.52	0.009–0.033
14	117.47–117.48	0.02–0.035
15	121.31–121.34	0.027–0.037
16	125.44–125.46	0.027–0.044
17	129.56–129.58	0.027–0.052
18	133.57–133.58	0.024–0.044
19	137.5–137.51	0.033–0.037
D8S1179		
8	122.59–122.62	0.026–0.04
9	126.58–126.6	0.025–0.045
10	130.58–130.6	0.027–0.041
11	134.62–134.63	0.024–0.03
12	138.69–138.7	0.012–0.039
13	142.91–142.93	0.024–0.037
14	147.06–147.07	0.023–0.038
15	151.18–151.2	0.023–0.032
16	155.33–155.35	0.028–0.039
17	159.49–159.5	0.028–0.042
18	163.58–163.6	0.027–0.047

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
19	167.64–167.65	0.02–0.042
FGA		
17	210.09–210.1	0.032–0.038
18	214.14–214.15	0.027–0.042
19	218.28–218.3	0.026–0.032
20	222.4–222.42	0.025–0.043
21	226.47–226.5	0.033–0.046
22	230.54–230.57	0.029–0.048
23	234.63–234.65	0.033–0.041
24	238.71–238.73	0.033–0.044
25	242.86–242.89	0.029–0.037
26	247.03–247.06	0.029–0.044
26.2	249.1–249.13	0.034–0.039
27	251.1–251.14	0.03–0.037
28	255.11–255.13	0.038–0.043
29	259.08–259.11	0.027–0.035
30	263.15–263.18	0.034–0.049
30.2	264.97–265	0.036–0.049
31.2	269.03–269.06	0.035–0.048
32.2	273.09–273.11	0.031–0.045
33.2	277.17–277.2	0.036–0.047

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
42.2	313.41–313.48	0.039–0.053
43.2	317.67–317.72	0.028–0.046
44.2	321.89–321.94	0.039–0.042
45.2	326.03–326.07	0.039–0.048
46.2	330.04–330.06	0.037–0.045
47.2	334.09–334.13	0.024–0.05
48.2	338.19–338.22	0.034–0.041
50.2	346.3–346.35	0.032–0.043
51.2	350.39–350.44	0.031–0.04
SE33		
4.2	201.24–201.27	0.026–0.046
6.3	210.31–210.34	0.037–0.046
8	215.36–215.4	0.03–0.042
9	219.5–219.54	0.027–0.05
11	227.65–227.7	0.032–0.051
12	231.71–231.76	0.036–0.042
13	235.79–235.83	0.031–0.041
14	239.87–239.91	0.031–0.049
15	244.04–244.08	0.033–0.048
16	248.19–248.26	0.04–0.052
17	252.26–252.31	0.029–0.047

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
18	256.26–256.3	0.035–0.045
19	260.2–260.24	0.031–0.053
20	264.24–264.3	0.034–0.047
20.2	266.23–266.28	0.034–0.05
21	268.3–268.36	0.034–0.049
21.1	269.3–269.37	0.029–0.054
21.2	270.26–270.31	0.033–0.051
22.2	274.34–274.38	0.034–0.052
23.2	278.42–278.46	0.032–0.049
24.2	282.47–282.51	0.044–0.053
25.2	286.47–286.53	0.039–0.05
26.2	290.49–290.53	0.04–0.049
27.2	294.48–294.54	0.036–0.045
28.2	298.48–298.53	0.028–0.043
29.2	302.43–302.48	0.027–0.051
30.2	306.38–306.44	0.035–0.05
31.2	310.39–310.46	0.036–0.052
32.2	314.48–314.56	0.042–0.052
33.2	318.75–318.82	0.033–0.049
34.2	322.93–323	0.036–0.048

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
35	325.01–325.07	0.038–0.054
35.2	327.05–327.09	0.033–0.044
36	329.1–329.15	0.039–0.051
37	333.21–333.22	0.042–0.05
TH01		
4	159.72–159.79	0.004–0.049
5	163.79–163.85	0.028–0.048
6	167.84–167.9	0.027–0.05
7	171.88–171.96	0.023–0.039
8	175.94–176	0.029–0.039
9	179.98–180.05	0.021–0.04
9.3	183.12–183.2	0.027–0.044
10	184.09–184.14	0.022–0.036
11	188.19–188.27	0.024–0.035
13.3	199.45–199.52	0.035–0.044
vWA		
11	151.27–151.32	0.025–0.034
12	155.37–155.41	0.023–0.042
13	159.52–159.56	0.006–0.042
14	163.79–163.81	0.02–0.045
15	167.7–167.74	0.018–0.045

Table 5-1 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® SEfiler Plus™ Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
16	171.76–171.8	0.025–0.045
17	175.8–175.84	0.025–0.031
18	179.81–179.85	0.024–0.045
19	183.9–183.95	0.026–0.043
20	187.99–188.04	0.029–0.045
21	192.05–192.08	0.016–0.037
22	196.12–196.16	0.026–0.035
23	200.09–200.13	0.027–0.047
24	204.4–204.45	0.024–0.036

Extra Peaks in the Electropherogram

Causes of Extra Peaks

Peaks other than the target alleles may be detected on the electropherogram. Causes for extra peaks include stutter products, incomplete 3´ A nucleotide addition (at the n-1 position), dye artifacts, and mixed DNA samples (see DAB Standard 8.1.2.2).

Stutter Products

A stutter is a well-characterized PCR artifact that refers to the appearance of a minor peak that is one repeat unit smaller (or, less frequently, one repeat unit larger) than the major STR product (Butler, 2005; Mulero *et al.*, 2006). 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. Peak heights were measured for amplified samples (n = 229) at the loci used in the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit. All data were generated on the Applied Biosystems 3130*xl* Genetic Analyzer.

Some conclusions from these measurements and observations are:

- For each SEfiler Plus kit locus, the percent stutter generally increases with allele length, as shown in Figure 5-5 to Figure 5-8 on pages 5-22 through 5-24.
- Smaller alleles display a lower level of stutter relative to the longer alleles within each locus.
- Each allele within a locus displays a percent stutter that is consistent.
- The highest observed percent stutter for each locus is included as the filtering step in the GeneMapper® *ID* software v3.2.1. These values are shown in Table 5-2 on page 5-25. Peaks in the stutter position that are above the highest observed percent stutter are not filtered. Peaks in the stutter position that have not been filtered and remain labeled can be further evaluated. For evaluation of mixed samples, see Figure 5-17 on page 5-45.
- The measurement of percent stutter for peaks that are off-scale may be unusually high.

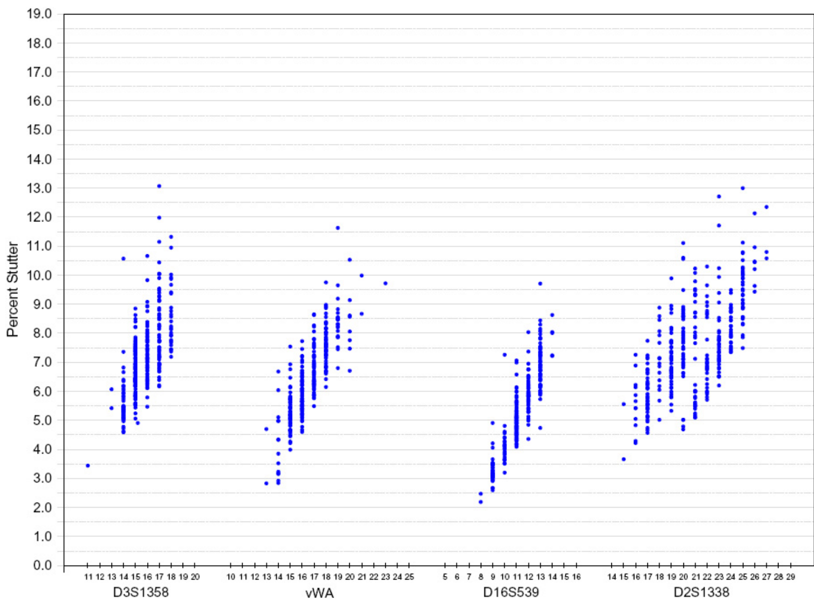

Figure 5-5 Stutter percentages for the D3S1358, vWA, D16S539, and D2S1338 loci

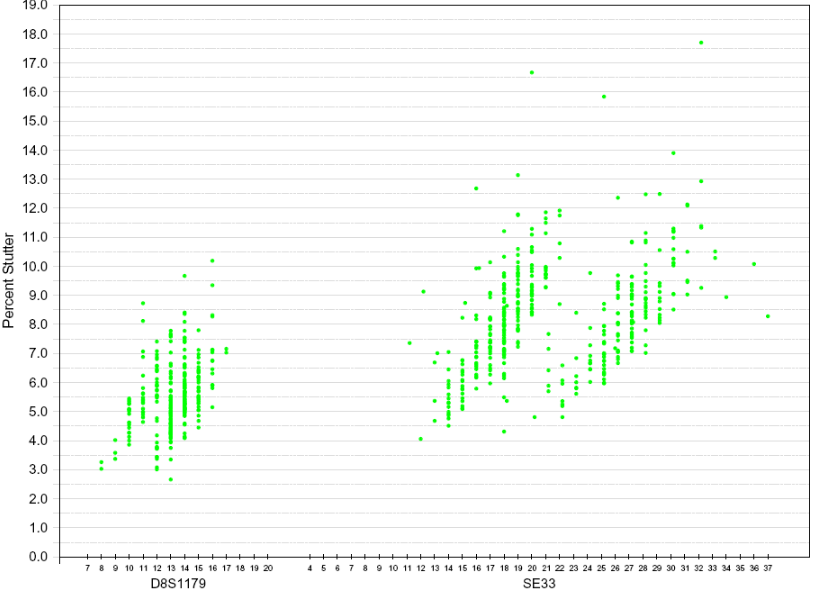

Figure 5-6 Stutter percentages for D8S1179 and SE33 loci

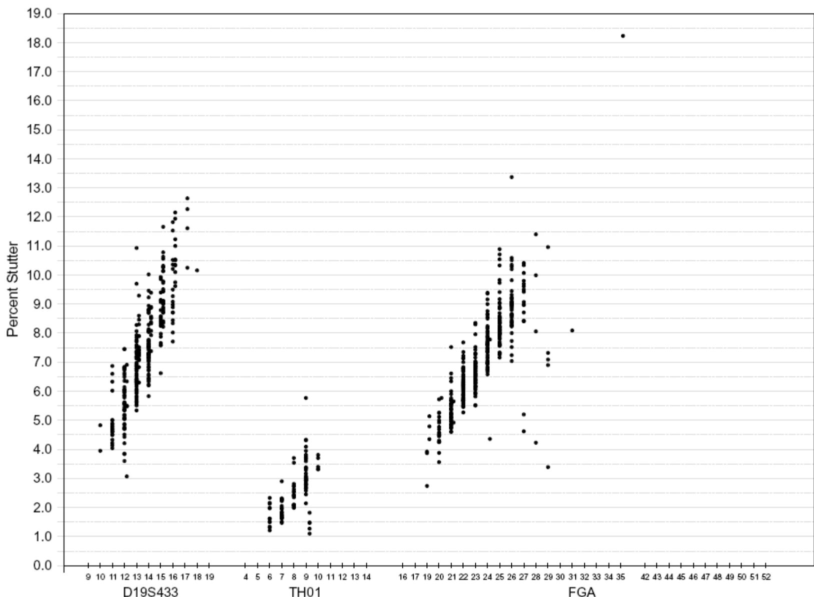

Figure 5-7 Stutter percentages for the D19S433, TH01, and FGA loci

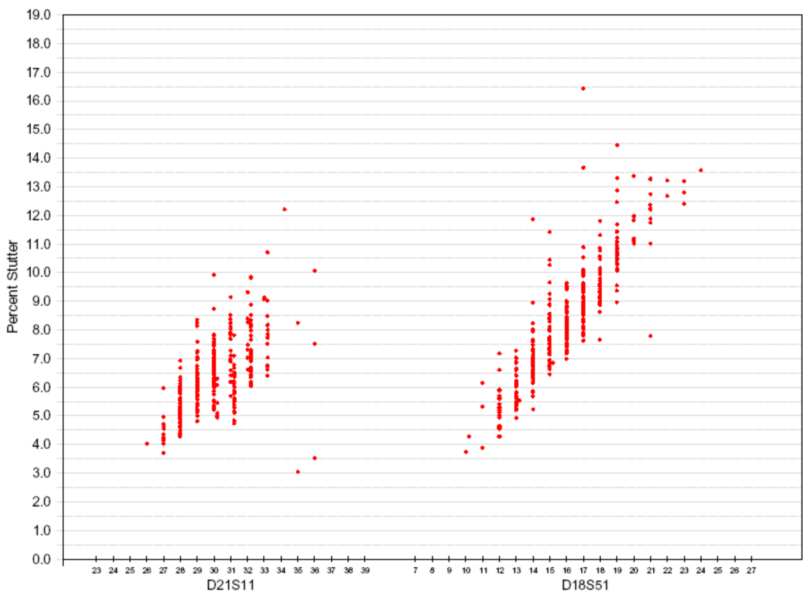

Figure 5-8 Stutter percentages for the D21S11 and D18S51 loci

Table 5-2 Marker-specific stutter percentages (ratios used in GeneMapper *ID* v3.2.1 AmpFLSTR_SEfilerPlus_panels_v1) for SEfiler Plus Kit loci

Locus	% Stutter
D21S11	12.22
D18S51	16.43
D19S433	12.64
TH01	5.77
FGA	18.23
D3S1358	13.07
vWA	11.63
D16S539	9.71
D2S1338	13.00
D8S1179	10.19
SE33	17.70

Addition of 3´ A Nucleotide

Many DNA polymerases can catalyze the addition of a single nucleotide (predominately adenosine) to the 3´ ends of double-stranded PCR products (Clark, 1988; Magnuson *et al.,* 1996). This nontemplate addition results in a PCR product that is one nucleotide longer than the actual target sequence. 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 SEfiler Plus kit includes two design features that promote maximum +A addition:

- The primer sequences have been optimized to encourage +A addition.
- The final extension step is 60 °C for 60 minutes.

This final extension step gives the DNA polymerase additional time to complete +A addition to all double-stranded PCR products. STR systems (where each allele is represented by two peaks that are one nucleotide apart) that have not been optimized for +A addition may have “split peaks.”

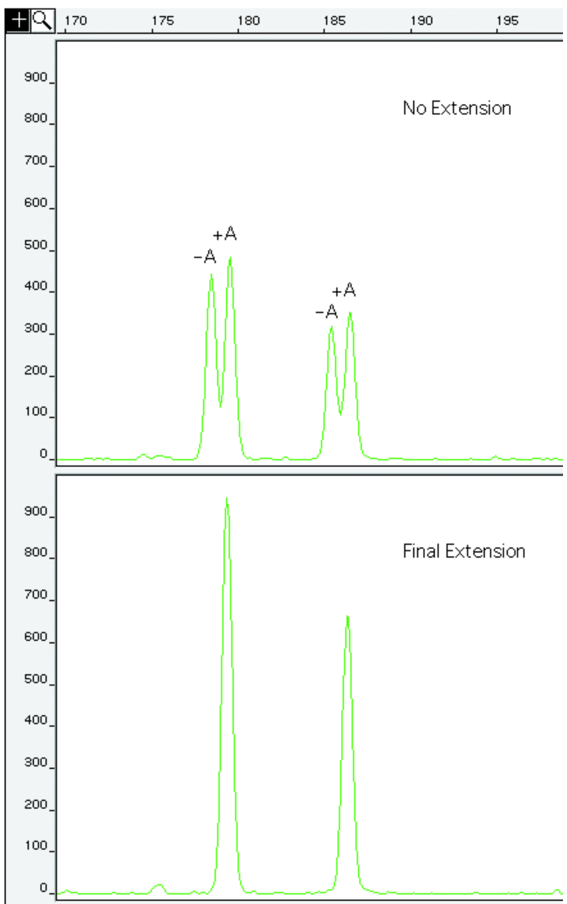

Figure 5-9 Omitting the final extension step results in split peaks due to incomplete A nucleotide addition. Data are from an ABI PRISM® 310 Genetic Analyzer using another AmpFℓSTR® kit.

Lack of complete +A nucleotide addition may be observed in SEfiler Plus kit results when the amount of input DNA is greater than the recommended protocols, because more time is needed for the enzyme to add the +A nucleotide to all molecules as more PCR product is generated. Amplification of too much input DNA may also result in off-scale data.

Artifacts

Artifacts and anomalies are seen in all molecular biological systems. Artifacts are typically reproducible. Anomalies are nonreproducible, intermittent occurrences that are not consistently observed in a system, for example, spikes and baseline noise. Artifacts have been seen in data produced on genetic analyzers when using the SEfiler Plus kit. Low-level artifacts in the calling region may appear in the green (115 nt) and yellow (94 nt) dyes, depending on the sensitivity of the instrument.

Figure 5-10 on page 5-28 and Figure 5-11 on page 5-29 show examples of baseline noise and artifacts in an electropherogram while using the SEfiler Plus kit. Genotyping may result in the detection of these artifacts as off-ladder (OL) alleles. This occurs if the recommended amount of input DNA is exceeded and off-scale data are obtained. You should consider possible noise and artifacts when interpreting data from the SEfiler Plus kit on the Applied Biosystems 3130/3130*xl*, ABI PRISM® 3100/3100-*Avant*, and ABI PRISM® 310 Genetic Analyzers.

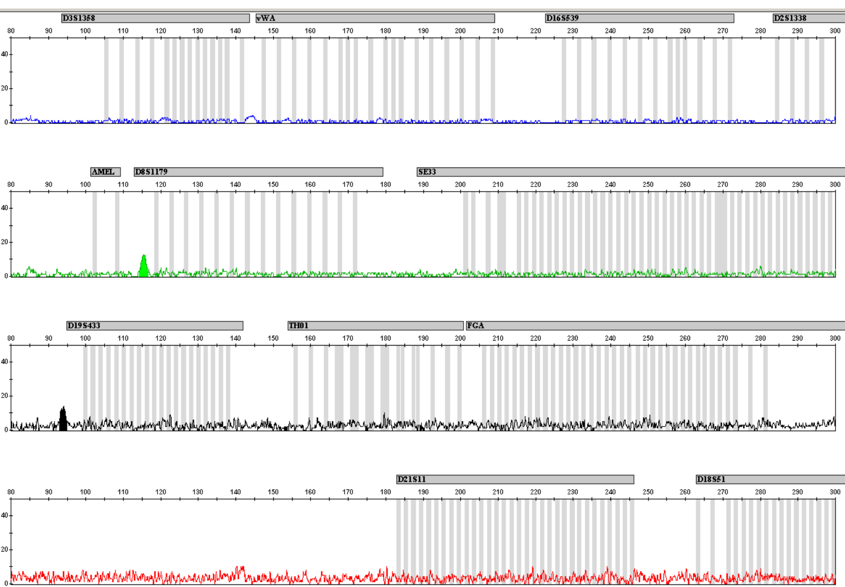

Figure 5-10 Examples of baseline noise and reproducible artifacts in data produced on the Applied Biosystems 3130*xl* Genetic Analyzer, Y-axis scale (0–50 RFUs)

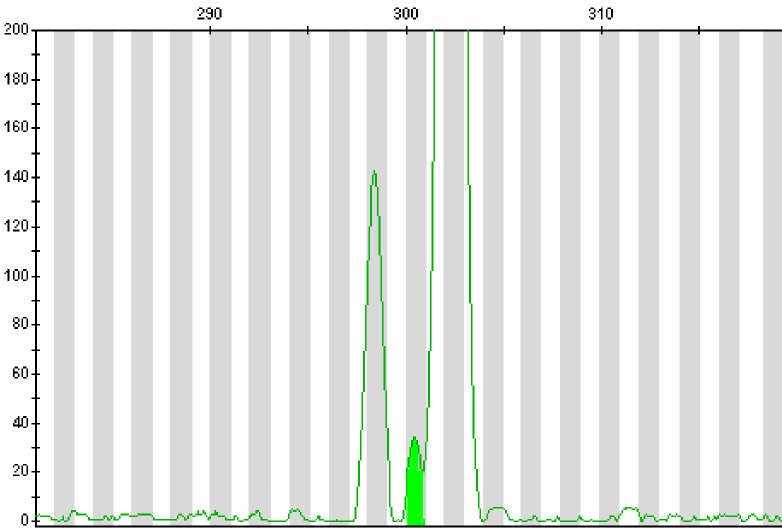

Figure 5-11 Examples of a -2 nt reproducible artifact in the SE33 locus. Data produced on the Applied Biosystems 3130*xl* Genetic Analyzer

Note that a high degree of magnification (y-axis) is used in Figure 5-10 on page 5-28 and Figure 5-11 on page 5-29 to show the artifacts.

Characterization of Loci

SWGDAM Guideline 2.1

"The basic characteristics of a genetic marker must be determined and documented." (SWGDAM, July 2003)

This section describes basic characteristics of the 11 loci and the sex-determining marker, amelogenin, that are amplified with the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit. These loci have been extensively characterized by other laboratories.

Nature of the Polymorphisms

The primers for the amelogenin locus flank a six-nucleotide deletion within intron 1 of the X homologue. Amplification results in 107-nt and 113-nt products from the X and Y chromosomes, respectively. (Sizes are the actual nucleotide size according to sequencing results, including 3´ A nucleotide addition.) The remaining SEfiler Plus kit loci, except the SE33 locus, are all tetranucleotide short tandem repeat (STR) loci. The length differences among alleles of a particular locus result from differences in the number of 4-nt repeat units.

The SE33 locus is highly polymorphic. The SE33 locus not only possesses structural variation, it also exhibits length and sequence polymorphism (Möller, Schurenkamp *et al.,* 1995). Among the sequence polymorphisms Type I contains the known regular four nt repeat AAAG; while Type II has an additional hexanucleotide unit, AAAAAG. These result in additional interalleles in the SE33 locus differing by 1 to 3 nt (Urquhart *et al.,* 1993).

All the alleles in the AmpFℓSTR® SEfiler Plus™ Allelic Ladder, including microvariants, have been subjected to DNA sequencing at Applied Biosystems. In addition, other groups have sequenced alleles at some of these loci (Nakahori *et al.,* 1991; Puers *et al.,* 1993; Möller *et al.,* 1994; Barber *et al.,* 1995; Möller and Brinkmann, 1995; Barber *et al.,* 1996; Barber and Parkin, 1996; Brinkmann *et al.,* 1998; Momhinweg *et al.,* 1998; Watson *et al.,* 1998). Among the various sources of sequence data on the AmpFℓSTR SEfiler Plus kit loci, there is consensus on the repeat patterns and structure of the STRs.

Inheritance

The Centre d'Etude du Polymorphisme Humain (CEPH) has collected DNA from 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 (Begovich *et al.,* 1992).

Because the oligonucleotide sequences between the SEfiler and SEfiler Plus kits are identical, no changes in the pattern of inheritance are reported.

Mapping

The SEfiler Plus kit loci have been mapped, and the chromosomal locations have been published (Nakahori *et al.,* 1991; Edwards *et al.,* 1992; Kimpton *et al.,* 1992; Mills *et al.,* 1992; Sharma and Litt, 1992; Li *et al.,* 1993; Straub *et al.,* 1993; Barber and Parkin, 1996).

Species Specificity

SWGDAM Guideline 2.2

"For techniques designed to type human DNA, the potential to detect DNA from forensically relevant nonhuman species should be evaluated." (SWGDAM, July 2003)

The AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit provides the required specificity for detecting human alleles.

Nonhuman Studies

Nonhuman DNA may be present in forensic casework samples. The data from SEfiler Plus kit experiments on nonhuman DNA sources are shown in Figure 5-12.

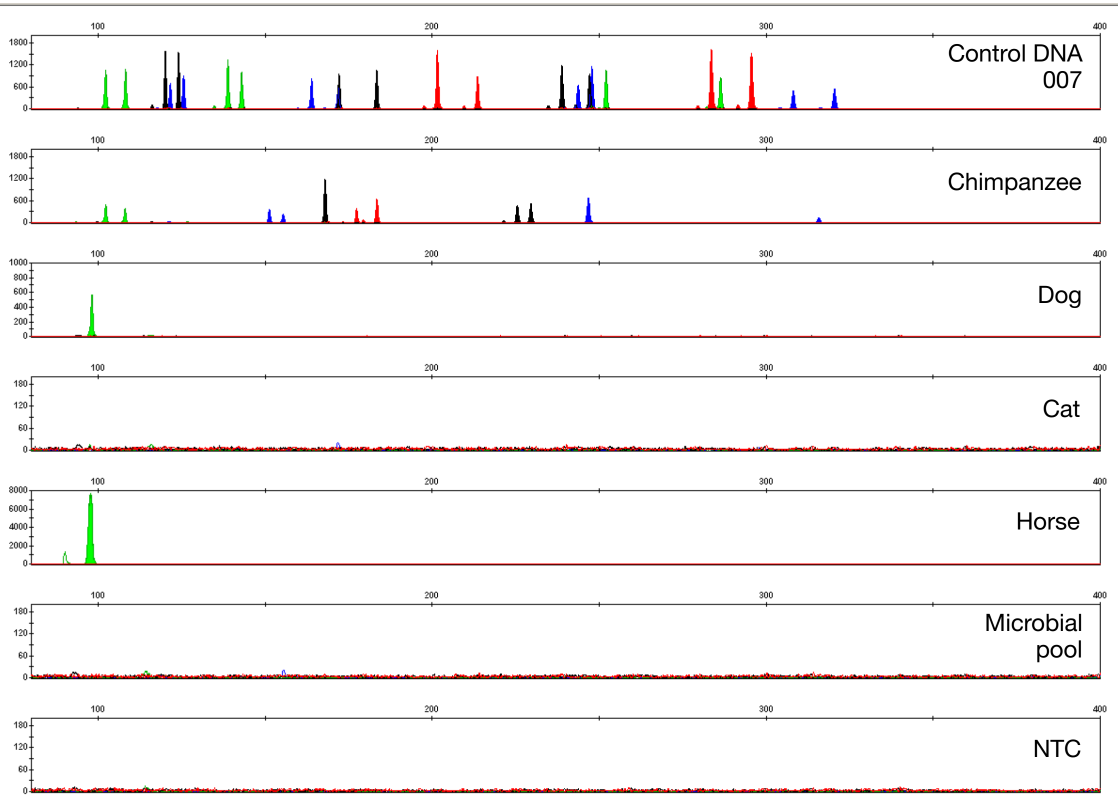

Figure 5-12 Representative electropherograms from a species-specificity study including positive and non-template controls (NTC)

Figure 5-12 on page 5-32 shows amplification for: control DNA 007 (0.50 ng, panel 1), chimpanzee (0.50 ng, panel 2), dog (2.5 ng. panel 3), cat (2.5 ng, panel 4), horse (2.5 ng, panel 5), microbial DNA pool (equivalent to 10^5 copies of *Candida albicans*, *Neisseria gonorrhoeae*, *E. coli 0157:H7*, *Bacillus subtilis,* and *Lactobacillus rhamnosus*, panel 6), and the negative control (panel 7). The extracted DNA samples were amplified with the SEfiler Plus kit and analyzed using the Applied Biosystems 3130*xl* Genetic Analyzer.

- Primates: gorilla, chimpanzee, orangutan, and macaque (0.50 ng each)
- Non-primates: mouse, dog, sheep, rabbit, cat, horse, hamster, rat, chicken, and cow (2.5 ng each)
- Microorganisms: *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Bacillus subtilis,* and *Lactobacillus rhamnosus* (equivalent to 10^5 copies)

The chimpanzee and gorilla DNA samples produced partial profiles within the 70 to 283 nucleotide region.

The microorganisms, chicken, hamster, mouse, rabbit, and rat did not yield detectable products. Dog, horse, sheep, and cow produced a 98-bp fragment near the amelogenin locus in the VIC® dye.

Sensitivity

SWGDAM Guideline 2.3

"When appropriate, the range of DNA quantities able to produce reliable typing results should be determined."
(SWGDAM, July 2003)

Importance of Quantitation

The optimal amount of input DNA added to the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit should be between 0.50 and 0.75 ng. The DNA sample should be quantitated before amplification using a system such as the Quantifiler® Human DNA Quantification Kit (PN 4343895). The final DNA concentration should be 0.05 to 0.075 ng/µL so that 0.50 to 0.75 ng of DNA is added to the PCR reaction in a volume of 10 µL. If the sample contains degraded or inhibited DNA, amplification of additional DNA may be beneficial. In Figure 5-13 on page 5-35, the control DNA 007 was serially diluted from 1 ng to 0.062 ng. Full profiles (24 PCR products) were consistently obtained at 0.125 ng, but occasional partial profiles that are missing anywhere from 1 to 3 alleles were observed at 0.062 ng.

Effect of DNA Quantity on Results

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

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

  Off-scale data is a problem because:

  – 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. This inaccuracy results in poor spectral separation ("pull-up").

- Incomplete +A nucleotide addition

  The sample can be reamplified using less DNA.

When the total number of allele copies added to the PCR is extremely low, unbalanced amplification of the alleles may occur due to stochastic fluctuation.

Individual laboratories may find it useful to determine an appropriate minimum peak height threshold based on their own results and instruments using low amounts of input DNA.

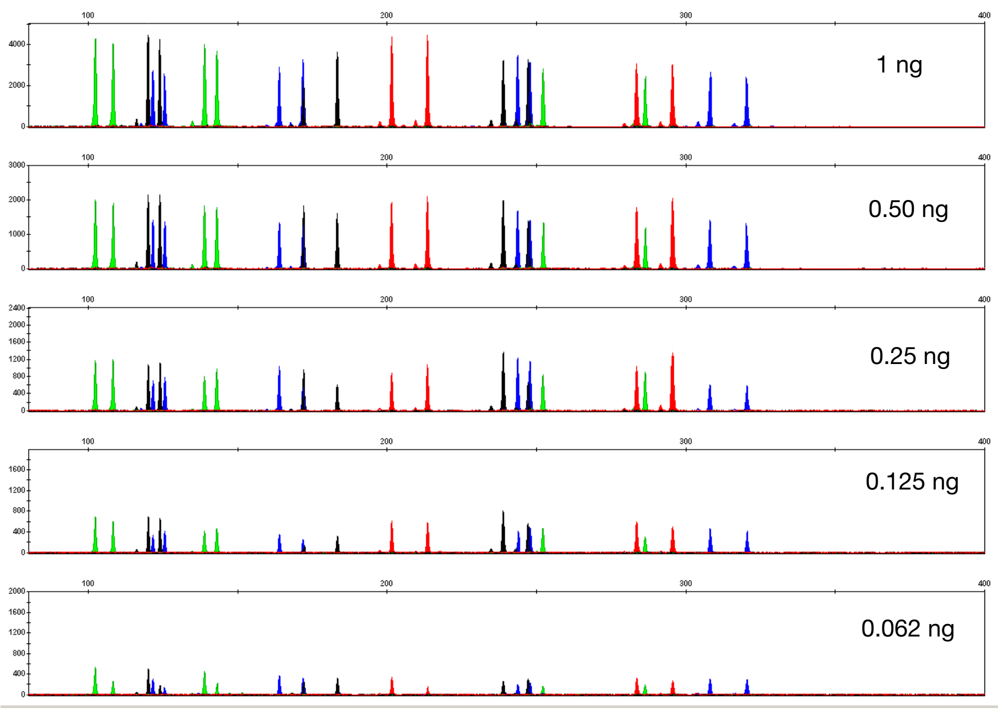

Figure 5-13 Effect of amplifying 1 ng, 0.50 ng, 0.25 ng, 0.125 ng, and 0.062 ng of control DNA 007

Note that the y-axis scale is magnified for the lower amounts of DNA, analyzed using the Applied Biosystems 3130*xl* Genetic Analyzer.

Stability

SWGDAM Guideline 2.4

"The ability to obtain results from DNA recovered from biological samples deposited on various substrates and subjected to various environmental and chemical insults has been extensively documented. In most instances, assessment of the effects of these factors on new forensic DNA procedures is not required. However, if substrates and/or environmental and/or chemical insults could potentially affect the analytical process, then the process should be evaluated using known samples to determine the effects of such factors." (SWGDAM, July 2003)

Degraded DNA

As the average size of degraded 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 Raji DNA was sonicated and incubated with increasing doses of DNase I (0 to 6 Units) for 20 minutes (Bender *et al.,* 2004). The DNA was examined by agarose gel analysis to determine the average size of the DNA fragments at each time point.

One nanogram of degraded DNA was amplified using the AmpFℓSTR SEfiler Plus Kit. As the DNA became increasingly degraded, the loci became undetectable according to size. Preferential amplification was not observed. The loci failed to robustly amplify in the order of decreasing size as the extent of degradation progressed.

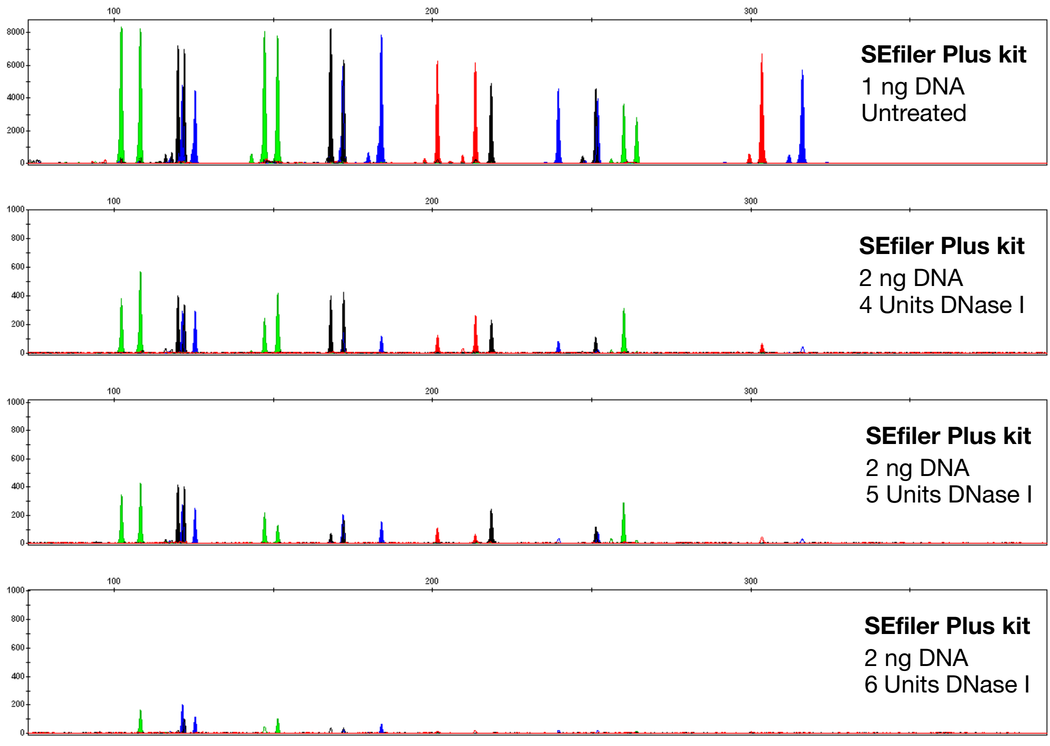

Figure 5-14 Amplification of Raji DNA samples sonicated and incubated with increasing doses of DNase I. Panels 1, 2, 3, and 4 correspond to 0, 4, 5, and 6 units of DNase I.

Effect of Inhibitors — Hematin

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

To examine the effects of hematin on the amplification results obtained by the SEfiler Plus kit, male DNA 007 (1 ng input DNA for the SEfiler™ and SEfiler Plus™ kits) was amplified with increasing concentrations of hematin for 30 cycles of amplification. The concentrations of hematin used were 0 µM, 30 µM, 45 µM, and 55 µM (see Table 5-3).

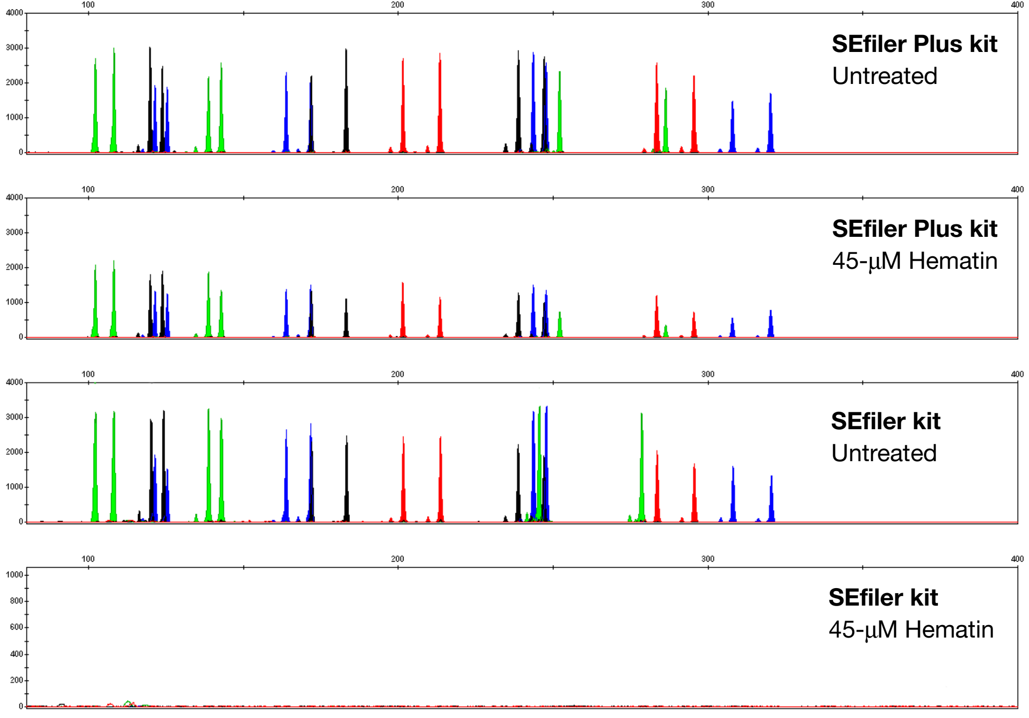

Figure 5-15 Amplification with AmpFℓSTR® SEfiler Plus™ and SEfiler™ kits in the presence and absence of hematin. Panels 1 and 3 correspond to control samples; panels 2 and 4 correspond to samples amplified in the presence of 45 µM of hematin.

Comparison of SEfiler Plus™ and SEfiler™ kit performance in a simulated model of hematin inhibition

Only those peaks >50 RFUs were counted. A complete profile with control 007 DNA yields 24 peaks using the SEfiler Plus kit (see Table 5-3).

Table 5-3 Comparison of SEfiler Plus™ and SEfiler™ kit performance in simulated model of hematin inhibition (n = 3)

Hematin (μM)	SEfiler Plus™ kit	SEfiler™ kit
0	24/24, 24/24, 24/24	24/24, 24/24, 24/24
30	24/24, 24/24, 24/24	2/24, 2/24, 8/24
45	24/24, 21/24, 24/24	0/24, 0/24, 0/24
55	18/24, 23/24, 5/24	0/24, 0/24, 0/24

Effect of Inhibitors — Humic Acid

Traces of humic acid may inhibit the PCR amplification of DNA evidence collected from soil. In this study, Applied Biosystems tested increasing amounts of humic acid in the PCR amplification of 1 ng of control DNA 007 with the SEfiler and SEfiler Plus kits for 30 cycles of amplification (see Figure 5-16). The concentrations of humic acid tested were 0, 20, 40, and 60 ng/µL.

The SEfiler Plus kit efficiently amplified the DNA at concentrations of humic acid that inhibited the amplification of DNA with the SEfiler™ kit (see Figure 5-16 on page 5-40).

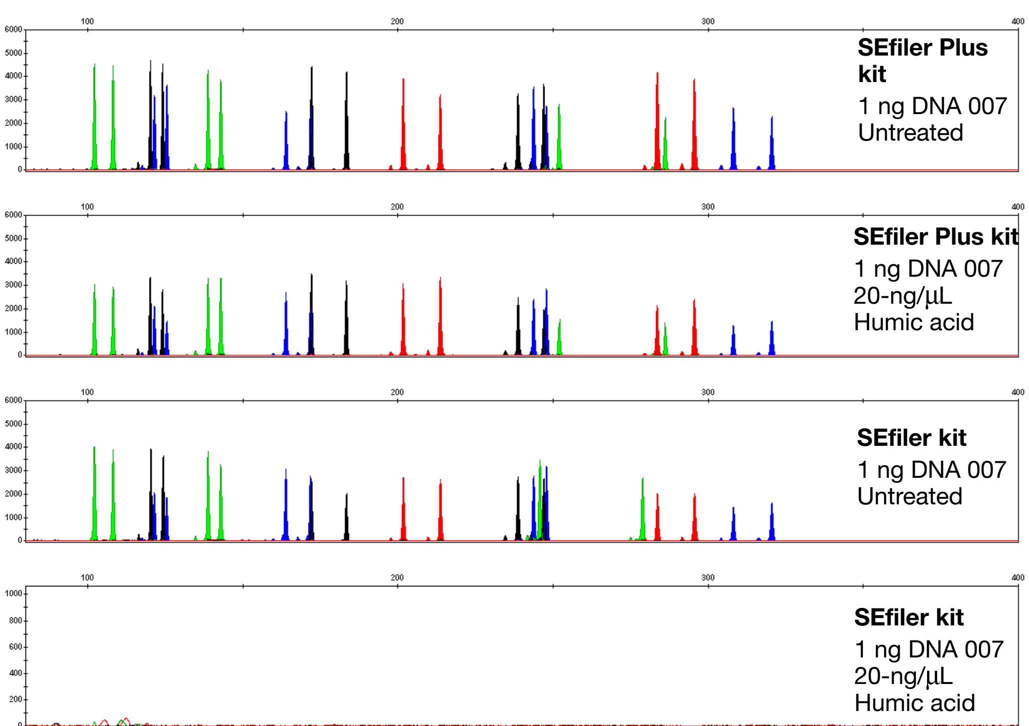

Figure 5-16 Amplification with AmpFℓSTR® SEfiler Plus™ and SEfiler™ kits in the presence and absence of humic acid. Panels 1 and 3 correspond to control samples; panels 2 and 4 correspond to samples amplified in the presence of 20 ng/µL humic acid.

Comparison of SEfiler Plus™ and SEfiler™ kit performance in a simulated model of humic acid inhibition

Only those peaks >50 RFUs were counted. A complete profile with control 007 DNA yields 24 peaks using the SEfiler Plus kit (see Table 5-4).

Table 5-4 Comparison of SEfiler Plus™ and SEfiler™ kit performance in simulated model of humic acid inhibition (n = 3)

Humic Acid (ng/µL)	SEfiler Plus™ kit	SEfiler™ kit
0	24/24, 24/24, 24/24	24/24, 24/24, 24/24
20	24/24, 24/24, 24/24	0/24, 0/24, 0/24
40	20/24, 23/24, 24/24	0/24, 0/24, 0/24
60	9/24, 8/24, 12/24	0/24, 0/24, 0/24

Mixture Studies

SWGDAM Guideline 2.8

"The ability to obtain reliable results from mixed source samples should be determined." (SWGDAM, July 2003)

Evidence samples may contain DNA from more than one individual. The possibility of multiple contributors should be considered when interpreting the results. Applied Biosystems recommends that individual laboratories assign a minimum peak height threshold based on validation experiments performed in each laboratory to avoid typing when stochastic effects are likely to interfere with accurate interpretation of mixtures.

Mixture Studies

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. Mixed samples can be distinguished from single-source samples by:

- The presence of more 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
- 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, median, minimum, and maximum peak height ratios observed for alleles in the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit loci in unmixed population database samples are shown in Table 5-5:

Table 5-5 Peak height ratios for 0.50 ng of input DNA

Locus	Number of Observations (n)	Mean	Median	Minimum	Maximum
Amelogenin	160	82.5	84.2	50.0	99.7
D16S539	170	84.1	86.7	50.9	99.6
D18S51	200	83.5	86.0	44.4	99.9
D19S433	185	83.3	84.1	55.9	99.9
D21S11	202	82.6	83.9	35.3	99.9
D2S1338	203	80.7	82.2	37.8	100.0
D3S1358	154	83.5	84.5	43.8	99.7
D8S1179	177	83.3	84.3	51.3	99.8
FGA	193	83.4	85.4	45.6	99.9
SE33	214	82.3	84.5	43.6	100.0
TH01	176	82.2	84.7	33.0	99.9
vWA	194	83.4	83.8	54.1	99.8

If an unusually low peak height ratio is observed for one locus, and there are no other indications that the sample is a mixture, the sample may be reamplified and reanalyzed to determine if the imbalance is reproducible. Possible causes of imbalance at a locus are:

- Degraded DNA
- Presence of inhibitors
- Extremely low amounts of input DNA
- A mutation in one of the primer binding sites

- Presence of an allele containing a rare sequence that does not amplify as efficiently as the other allele

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 (see 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 or not 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 sample(s).

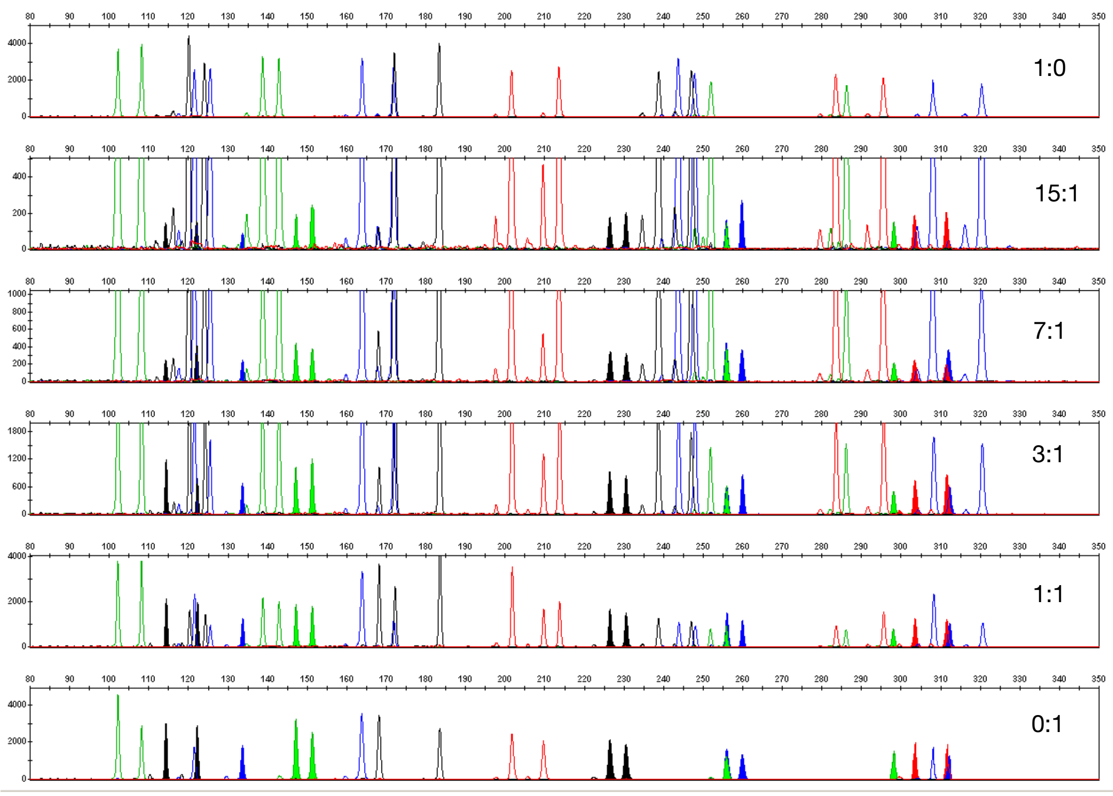

Figure 5-17 Amplification of DNA mixtures at various ratios

Limit of Detection of the Minor Component

Mixtures of two DNA samples were examined at various ratios (0:1, 1:1, 3:1, 7:1, 15:1, 1:0). The total amount of genomic input DNA mixed at each ratio was 1 ng. The samples were amplified in a GeneAmp® PCR System 9700, then electrophoresed and detected using an Applied Biosystems 3130*xl* Genetic Analyzer.

The results of the mixed DNA samples are shown in Figure 5-17 on page 5-45, where samples A and B were mixed according to the ratios provided. The minor component allele calls at non-overlapping loci are highlighted. The amplification of the minor contributor at 3:1 and 7:1 (0.875:0.125 ng) mixture ratios was readily typeable. 15:1 ratios generally resulted in partial profiles for the minor component.

Table 5-6 shows the profiles of the samples in Figure 5-17 on page 5-45.

Table 5-6 Genotypes of mixed DNA samples

Allele	Profile Sample A (Control DNA 007)	Profile Sample B
D3S1358	15, 16	15, 18
vWA	14, 16	14
D16S539	9,10	12, 13
D2S1338	20,23	20, 21
Amelogenin	X, Y	X, Y
D8S1179	12, 13	14, 15
SE33	17,25.2	18, 28.2
D19S433	14, 15	12.2, 14.2
TH01	7, 9.3	6, 9.3
FGA	24, 26	21, 22
D21S11	28, 31	28, 30
D18S51	12, 15	17, 19

Population Data

SWGDAM Guideline 2.7

"The distribution of genetic markers in populations should be determined in relevant population groups." (SWGDAM, July 2003)

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 estimated by the frequency of that genotype in the relevant population(s).

The AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit contains loci for which extensive population data are available. For additional information, see the population data and additional studies section of the *AmpFℓSTR® SEfiler™ PCR Amplification Kit User's Manual* (PN 4323291).

Mutation Rate

Estimating Germline Mutations

Estimation of spontaneous or induced germline mutation at genetic loci may be achieved through comparison of the genotypes of offspring to those of their parents. From such comparisons, the number of observed mutations are counted directly.

In previous studies, genotypes of 10 STR loci amplified by the AmpFℓSTR® SGM Plus® PCR Amplification Kit were determined for a total of 146 parent-offspring allelic transfers (meioses) at the Forensic Science Service, Birmingham, England. One length-based STR mutation was observed at the D18S11 locus; mutation was not detected at any of the other nine STR loci. The D18S11 mutation was represented by an increase of one 4-nt repeat unit, a 17 allele was inherited as an 18 (single-step mutation). The maternal/paternal source of this mutation could not be distinguished.

Additional Mutation Studies

Additional studies (Edwards *et al.,* 1991; Edwards *et al.,* 1992; Weber and Wong, 1993; Hammond *et al.,* 1994; Brinkmann *et al.,* 1995; Chakraborty *et al.,* 1996; Chakraborty *et al.,* 1997; Brinkmann *et al.,* 1998; Momhinweg *et al.,* 1998; Szibor *et al.,* 1998) of direct mutation rate counts produced:

- Larger sample sizes for some of the AmpFℓSTR SEfiler Plus kit loci.
- Methods for modifications of these mutation rates (to infer mutation rates indirectly for those loci where these rates are not large enough to be measured directly and/or to account for those events undetectable as Mendelian errors).

Probability of Identity

Table of Probability of Identity

Table 5-7 shows the Probability of Identity (PI) values of the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit loci individually and combined.

Table 5-7 Probability of identity values for the AmpFℓSTR® SEfiler Plus™ kit STR loci

Locus	African-American	U.S. Caucasian
D2S1338	0.025	0.038
D3S1358	0.114	0.099
D8S1179	0.079	0.072
D16S539	0.074	0.085
D18S51	0.038	0.056
D19S433	0.045	0.126
D21S11	0.051	0.057
FGA	0.034	0.044
SE33	0.019	0.02
TH01	0.103	0.119
vWA	0.067	0.077
Combined	6.47×10^{-15}	7.46×10^{-14}

The P_I value is the probability that two individuals selected at random will have an identical SEfiler Plus kit genotype (Sensabaugh, 1982). The P_I values for the populations described in this section are then approximately $1/1.54 \times 10^{14}$ (African-American) and $1/1.34 \times 10^{13}$ (U.S. Caucasian).

Probability of Paternity Exclusion

Table of Probability of Paternity of Exclusion

Table 5-8 shows the Probability of Paternity Exclusion (P_E) values of the AmpFℓSTR® SEfiler Plus™ PCR Amplification Kit STR loci individually and combined.

Table 5-8 Probability of Paternity Exclusion values for the AmpFℓSTR® SEfiler Plus™ kit STR loci

Locus	African-American	U.S. Caucasian
D2S1338	0.0745	0.621
D3S1358	0.734	0.65
D8S1179	0.477	0.763
D16S539	0.67	0.42
D18S51	0.725	0.912
D19S433	0.632	0.516
D21S11	0.745	0.734
FGA	0.784	0.676
SE33	0.745	0.792
TH01	0.578	0.734
vWA	0.613	0.705
Combined	0.999997	0.999998

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 SEfiler Plus kit STR loci (Chakraborty and Stivers, 1996).

Troubleshooting

A

In This Appendix

Follow the recommended actions for the observations described in this appendix to understand and eliminate problems you experience during analysis.

Troubleshooting. A-2

Troubleshooting

Table A-1 Troubleshooting causes and recommended actions

Observation	Possible Causes	Recommended Actions
Faint or no signal from both the AmpFℓSTR® Control DNA 007 and the DNA test samples at all loci.	Incorrect volume or absence of either AmpFℓSTR® SEfiler Plus™ Master Mix or AmpFℓSTR® SEfiler Plus™ Primer Set	Repeat amplification.
	No activation of enzyme	Repeat amplification, making sure to hold reactions initially at 95 °C for 11 min.
	Master Mix not vortexed thoroughly before aliquoting	Vortex Master Mix thoroughly.
	AmpFℓSTR® SEfiler Plus™ Primer Set exposed to too much light	Store Primer Set protected from light.
	GeneAmp® PCR System malfunction	Refer to the thermal cycler user's manual and check instrument calibration.
	Incorrect thermal cycler parameters	Check the protocol for correct thermal cycler parameters.
	Tubes not seated tightly in the thermal cycler during amplification	Push reaction tubes firmly into contact with block after first cycle. Repeat test.
	Wrong PCR reaction tube	Use Applied Biosystems MicroAmp Reaction Tubes with Caps for the GeneAmp 9700.
	MicroAmp™ Base used with tray/retainer set and tubes in GeneAmp® System 9700	Remove MicroAmp Base from tray/retainer set and repeat test.

Table A-1 Troubleshooting causes and recommended actions *(continued)*

Observation	Possible Causes	Recommended Actions
Faint or no signal from both the AmpFℓSTR® Control DNA 007 and the DNA test samples at all loci. *(continued)*	Insufficient PCR product electrokinetically injected	For ABI PRISM® 3100/3100-*Avant* or Applied Biosystems 3130/3130*xl* instrument runs: Mix 1.0 µL of PCR product and 9 µL of Hi-Di™ Formamide/GeneScan™ 600 LIZ® solution. **WARNING CHEMICAL HAZARD. Formamide** causes eye, skin, and respiratory tract irritation. It is a possible reproductive and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
	Degraded formamide	Check the storage of formamide; do not thaw and refreeze multiple times. Try Hi-Di™ Formamide. **WARNING CHEMICAL HAZARD. Formamide** causes eye, skin, and respiratory tract irritation. It is a possible reproductive and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Table A-1 Troubleshooting causes and recommended actions *(continued)*

Observation	Possible Causes	Recommended Actions
Positive signal from AmpFℓSTR® Control DNA 007 but partial or no signal from DNA test samples	Quantity of test DNA sample is below assay sensitivity	Quantitate DNA and add 0.5 to 0.75 ng of DNA. Repeat test.
	Test sample contains high concentration of PCR inhibitor (for example, heme compounds, certain dyes)	Quantitate DNA and add minimum necessary volume. Repeat test.
		Wash the sample in a Centricon®-100 centrifugal filter unit. Repeat test.
	Test sample DNA is severely degraded	If possible, evaluate the quality of DNA sample by running an agarose gel. If DNA is degraded, reamplify with an increased amount of DNA.
	Dilution of test sample DNA in H_2O or wrong buffer (for example, wrong EDTA concentration)	Redilute DNA using TE Buffer (with 0.1 mM EDTA).
More than one allele 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 (0.5 to 0.75 ng).
	Mixed sample	See "Stutter Products" on page 5-21.
	Amplification of stutter product (n-4 nt position)	
	Incomplete 3´ A base addition (n-1 nt position)	See "Addition of 3´ A Nucleotide" on page 5-25. Be sure to include the final extension step of 60 °C for 45 min in the PCR.
	Signal exceeds dynamic range of instrument (off-scale data)	Quantitate DNA and reamplify sample, adding 0.5 to 0.75 ng of DNA.
	Poor spectral separation (bad matrix)	Follow the steps for creating a matrix file.
		Confirm that Filter Set G5 modules are installed and used for analysis.

Table A-1 Troubleshooting causes and recommended actions *(continued)*

Observation	Possible Causes	Recommended Actions
Some but not all loci visible on electropherogram	Test-sample DNA is severely degraded	If possible, evaluate the quality of DNA sample by running an agarose gel. If DNA is degraded, reamplify with an increased amount of DNA.
	Test sample contains high concentrations of a PCR inhibitor (for example, heme compounds, certain dyes)	Quantitate DNA and add minimum necessary volume. Repeat test.
		Wash the sample in a Centricon-100 centrifugal filter unit.
Poor peak height balance	Incorrect thermal cycler parameters.	Check the protocol for correct thermal cycler parameters
	GeneAmp PCR System 9700 with Aluminum 96-Well block or third-party thermal cyclers.	Use Applied Biosystems GeneAmp PCR System 9700 with silver or gold-plated silver blocks only.

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