AmpF*l*STR® Identifiler® Plus

PCR Amplification Kit

User's Guide

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Preface

Safety information

Note: For general safety information, see this Preface and Appendix B, "Safety" on page 122. When a hazard symbol and hazard type appear by a chemical name or instrument hazard, see the "Safety" Appendix for the complete alert on the chemical or instrument.

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:

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.

MSDSs The MSDSs for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining MSDSs, see "Obtaining MSDSs" on page 124.

IMPORTANT! For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.

How to use this guide

Purpose of this guide

The Applied Biosystems AmpF*ℓ*STR® Identifiler ® Plus PCR Amplification Kit User Guide provides information about the Applied Biosystems instruments, chemistries, and software associated with the AmpF*ℓ*STR® Identifier ® 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 number and 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.

How to obtain support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the Applied Biosystems web site, 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

<div style="text-align: right">

1

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This chapter covers:

Product overview

Purpose   The AmpF*l*STR® Identifiler® Plus PCR Amplification Kit is a short tandem repeat (STR) multiplex assay that amplifies 15 tetranucleotide repeat loci and the Amelogenin gender-determining marker in a single PCR amplification:

- All thirteen of the required loci for the Combined DNA Index System (CODIS) loci are included in this kit for known-offender databasing in the United States (Budowle *et al.*, 1998).
- Two additional loci, D2S1338 and D19S433, are included. These loci are consistent with the AmpF*l*STR SGM Plus™ PCR Amplification Kit.
- The combination of the 15 loci are consistent with several worldwide database recommendations.

The AmpF*l*STR Identifiler Plus Kit delivers a 16-locus multiplex with the same power of discrimination as, better sensitivity than, and better robustness than the earlier generation of the AmpF*l*STR Identifiler Kit. The kit uses modified PCR cycling conditions for enhanced sensitivity, a new buffer formulation to improve performance with inhibited samples, and an improved process for DNA synthesis and purification of the amplification primers to deliver a much cleaner electrophoretic background.

Product description   The AmpF*l*STR Identifiler Plus Kit uses the same primer sequences as the earlier generation AmpF*l*STR® Identifiler® Kit. The AmpF*l*STR Identifiler 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:

- ABI PRISM® 3100/3100-*Avant* Genetic Analyzer
- Applied Biosystems 3130/3130*xl* Genetic Analyzer
- Applied Biosystems 310 Genetic Analyzer
- GeneAmp® PCR System 9700 with the Silver 96-Well Block
- GeneAmp® PCR System 9700 with the Gold-plated Silver 96-Well Block
- Veriti® 96-Well Thermal Cycler

About the primers   The AmpF*l*STR Identifiler Plus Kit employs the latest improvements in primer synthesis and purification techniques to minimize the presence of dye-labeled artifacts. These improvements result in a much cleaner electropherogram background that enhances the assay's signal-to-noise ratio and simplifies the interpretation of results.

Non-nucleotide linkers are used in primer synthesis for the following loci: CSF1PO, D13S317, D16S539, D2S1338, and TPOX. For these primers, non-nucleotide linkers are placed between the primers and the fluorescent dye during oligonucleotide synthesis (Butler, 2005, Grossman *et al.*, 1994, and Baron *et al.*, 1996). Non-nucleotide linkers enable reproducible positioning of the alleles to facilitate

interlocus spacing. The combination of a five-dye fluorescent system and the inclusion of non-nucleotide linkers allows for simultaneous amplification and efficient separation of the 15 STR loci and Amelogenin during automated DNA fragment analysis.

Loci amplified by the kit

The following table shows the loci amplified, their chromosomal locations, and the corresponding fluorescent marker dyes. The AmpF*l*STR® Identifiler® Plus Allelic Ladder is used to genotype the analyzed samples. The alleles contained in the allelic ladder, and the genotype of the AmpF*l*STR® Identifiler® Plus Control DNA 9947A are also listed in the table.

Table 1   AmpF*l*STR® Identifiler® Plus Kit loci and alleles

Locus designation	Chromosome location	Alleles included in Identifiler® Plus Allelic Ladder	Dye label	Control DNA 9947A
D8S1179	8	8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	6-FAM™	13‡
D21S11	21q11.2-q21	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		30§
D7S820	7q11.21-22	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 11
CSF1PO	5q33.3-34	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 12
D3S1358	3p	12, 13, 14, 15, 16, 17, 18, 19	VIC®	14, 15
TH01	11p15.5	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3		8, 9.3
D13S317	13q22-31	8, 9, 10, 11, 12, 13, 14, 15		11#
D16S539	16q24-qter	5, 8, 9, 10, 11, 12,13, 14, 15		11, 12
D2S1338	2q35-37.1	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28		19, 23
D19S433	19q12-13.1	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2	NED™	14, 15
vWA	12p12-pter	11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24		17, 18
TPOX	2p23-2per	6, 7, 8, 9, 10, 11, 12, 13		8‡‡
D18S51	18q21.3	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		15, 19
Amelogenin	X: p22.1-22.3 Y: p11.2	X, Y	PET®	X
D5S818	5q21-31	7, 8, 9, 10, 11, 12, 13, 14, 15, 16		11§§
FGA	4q28	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		23, 24

‡ For CODIS purposes, profile reported as 13, 13.
§ For CODIS purposes, profile reported as 30, 30.
# For CODIS purposes, profile reported as 11, 11.
‡‡ For CODIS purposes, profile reported as 8, 8.
§§ For CODIS purposes, profile reported as 11, 11.

Allelic ladder profile Figure 1 shows the allelic ladder for the AmpF*l*STR Identifiler Plus Kit. See "Allelic ladder requirements" on page 27 for information on ensuring accurate genotyping.
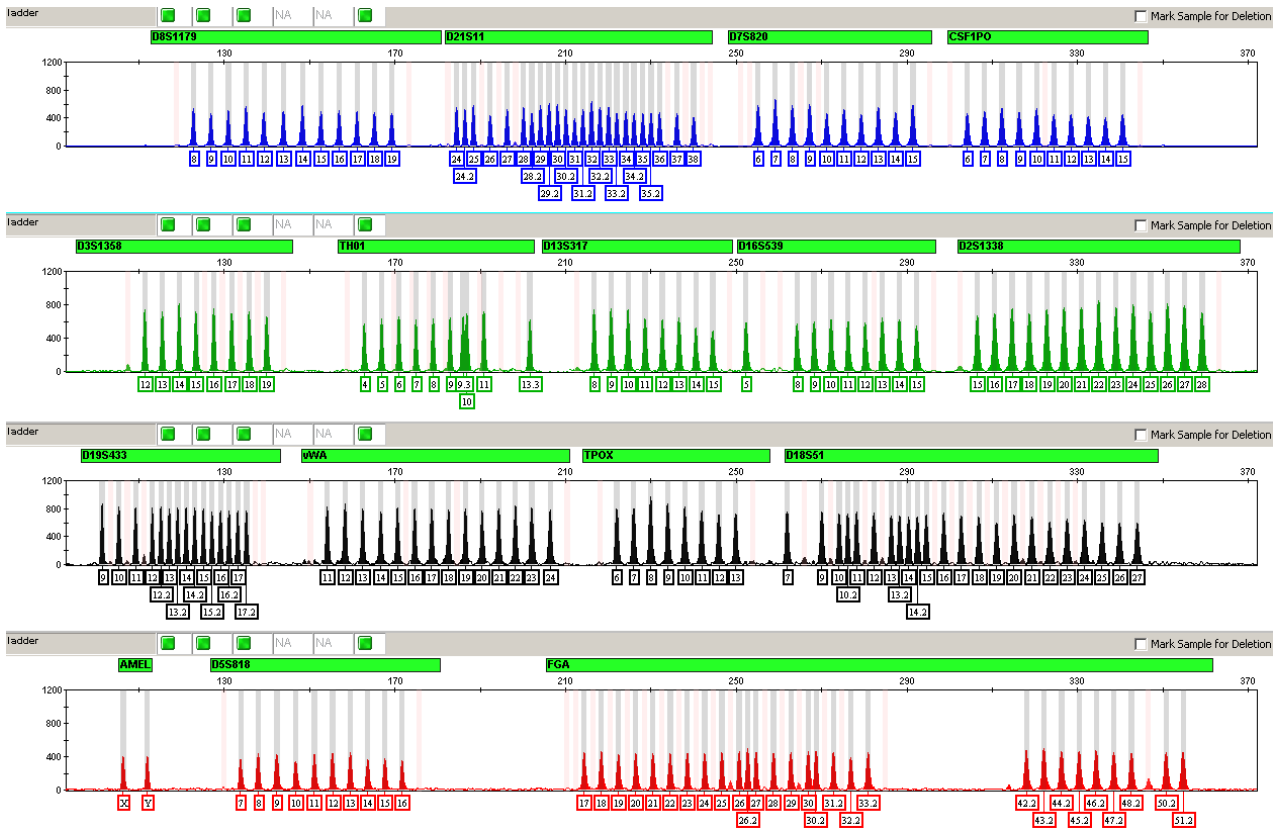
Figure 1   GeneMapper® *ID-X* Software plot of the AmpF*l*STR® Identifiler® Plus Kit Allelic Ladder

Control DNA 9947A profile

Figure 2 shows amplification of Control DNA 9947A using the AmpF*l*STR Identifiler Plus Kit.

Figure 2   1 ng of Control DNA 9947A amplified with the $\mathrm{AmpF}\ell\mathrm{STR}^{\circledR}$ **Identifiler**$^{\circledR}$ **Plus Kit and analyzed on the Applied Biosystems 3130***xl* **Genetic Analyzer**

Workflow overview

Extract DNA

PrepFiler™ Forensic DNA Extraction Kit

Quantify DNA

Quantifiler® Duo DNA Quantification Kit

Perform PCR

AmpF*l*STR® Identifiler® Plus PCR Amplification Kit

GeneAmp® PCR System 9700 Thermal Cycler*

Veriti* 96-Well Thermal Cycler

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* or GeneMapper® *ID-X* Software

Instrument and software overview

This section provides information about the Data Collection Software versions required to run the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit on specific instruments.

Data Collection and GeneMapper® *ID* or *ID-X* 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 measures sample fluorescence with its detection system, the Data Collection Software collects the data and stores it. The Data Collection Software stores information about each sample in a sample file (.fsa), which is then analyzed by the GeneMapper® *ID* or *ID-X* Software.

Instrument and software compatibility

Instrument	Operating system	Data Collection Software	Analysis software
3130/3130*xl*‡	Windows® XP	3.0	• GeneMapper® *ID* Software v3.2.1 *and* • GeneMapper® *ID-X* Software v1.0.1 or higher
3100/3100-*Avant*	Windows NT®	1.1 (3100) 1.0 (3100-*Avant*)	
	Windows 2000	2.0	
310	Windows XP	3.1	
	Window NT and Windows 2000	3.0	

‡ Applied Biosystems conducted validation studies for the AmpF*l*STR® Identifiler® Plus Kit using this configuration.

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 AmpF*l*STR® Identifiler® Plus PCR Amplification Kit to label samples are 6-FAM™, VIC®, NED™, and PET® dyes. The fifth dye, LIZ® dye, is used to label the GeneScan™ 500 LIZ® Size Standard.

How multicomponent analysis works

Each of these 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 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 it 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 3). The goal of multicomponent analysis is to correct for spectral overlap.
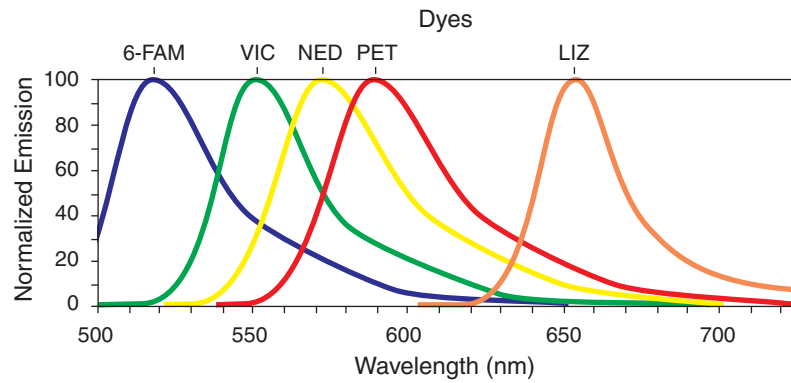
Figure 3   Emission spectra of the five dyes used in the AmpF*ℓ*STR® Identifiler® Plus Kit

Materials and equipment

Kit contents and storage

The AmpF*l*STR® Identifiler® Plus PCR Amplification Kit (PN 4427368) contains materials sufficient to perform 200 amplifications at 25 µL reaction volumes.

IMPORTANT! The fluorescent dyes attached to the primers are light sensitive. Protect the primer set from light when not in use. Amplified DNA, AmpF*l*STR® Identifiler® Plus Allelic Ladder, and GeneScan™ 500 LIZ® Size Standard should also be protected from light. Keep freeze-thaw cycles to a minimum.

Component	Description	200× Volume	Storage
AmpF*l*STR® Identifiler® Plus Primer Set	Contains forward and reverse primers to amplify human DNA targets.	1 tube, 1.0 mL	-15 to -25 °C on receipt, 2 to 8 °C after initial use
AmpF*l*STR® Identifiler® Plus Master Mix	Contains enzyme, salts, dNTPs, carrier protein, and 0.04% sodium azide.	2 tubes, 1.0 mL each	-15 to -25 °C on receipt, 2 to 8 °C after initial use
AmpF*l*STR® Identifiler® Plus Allelic Ladder	Contains amplified alleles. See Table 1 on page 3 for a list of alleles included in the allelic ladder.	1 tube, 50.0 µL	-15 to -25 °C on receipt, 2 to 8 °C after initial use
AmpF*l*STR® Control DNA 9947A	Contains 0.10 ng/µL human female 9947A DNA in 0.05% sodium azide and buffer‡. See Table 1 on page 3 for profile.	1 tube, 0.3 mL	2 to 8 °C

‡ The AmpF*l*STR® Control DNA 9947A is included at a concentration appropriate to its intended use as an amplification control (i.e., to provide confirmation of the capability of the kit reagents to generate a profile of expected genotype). The AmpF*l*STR® Control DNA 9947A is not designed to be used as a DNA quantitation control, and laboratories may expect to see variation from the labelled concentration when quantitating aliquots of the AmpF*l*STR® Control DNA 9947A.

Standards for samples

For the AmpF*l*STR Identifiler Plus Kit, the panel of standards needed for PCR amplification, PCR product sizing, and genotyping are:

- **Control DNA 9947A** – A positive control for evaluating the efficiency of the amplification step and STR genotyping using the AmpF*l*STR® Identifiler® Plus Allelic Ladder.

- **GeneScan™ 500 LIZ® Size Standard** – Standard used for obtaining sizing results. It contains 16 single-stranded labeled fragments of: 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 nucleotides. This standard, which has been evaluated as an internal lane size standard, yields precise sizing results for AmpF*l*STR Identifiler Plus Kit PCR products. Order the GeneScan 500 LIZ Size Standard (PN 4322682) separately.

- **AmpF*l*STR® Identifiler® Plus Allelic Ladder** – Allelic ladder developed by Applied Biosystems for accurate characterization of the alleles amplified by the AmpF*l*STR Identifiler Plus Kit. The AmpF*l*STR® Identifiler® Plus Allelic Ladder contains most of the alleles reported for the 15 autosomal loci. Refer to Table 1 on page 3 for a list of the alleles included in the AmpF*l*STR® Identifiler® Plus Allelic Ladder.

Equipment and materials not included

Tables 2 and 3 list required and optional equipment and materials not supplied with the AmpF*l*STR Identifiler Plus Kit. Unless otherwise noted, many of the items are available from major laboratory suppliers (MLS).

Table 2   Equipment

Equipment	Source
ABI PRISM® 3100/3100-*Avant* Genetic Analyzer	Contact your local Applied Biosystems sales representative
Applied Biosystems 3130/3130*xl* Genetic Analyzer	
Applied Biosystems 310 Genetic Analyzer	
GeneAmp® PCR System 9700 with the Silver 96-Well Block	N8050001
GeneAmp® PCR System 9700 with the Gold-plated Silver 96-Well Block	4314878
Veriti® 96-Well Thermal Cycler	4375786
Silver 96-Well Sample Block	N8050251
Gold-plated Silver 96-Well Sample Block	4314443
Tabletop centrifuge with 96-Well Plate Adapters (optional)	MLS

Table 3   User-supplied materials‡

Item	Source
AmpF*l*STR® Identifiler® Plus PCR Amplification Kit	4427368
3100/3100-*Avant* Genetic Analyzer materials	
96-Well Plate Septa	4315933
Reservoir Septa	4315932
3100/3100-*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™ 500 LIZ® Size Standard	4322682
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 instrument, refer to Appendix B of the *ABI PRISM® 3100 Genetic Analyzer and 3100-Avant Genetic Analyzer User Reference Guide* (PN 4335393).	

Table 3   User-supplied materials‡ *(continued)*

Item	Source
3130/3130*xl* Analyzer materials	
96-Well Plate Septa	4315933
Reservoir Septa	4315932
3100/3130*xl* Genetic Analyzer Capillary Array, 36-cm	4315931
POP-4™ Polymer for 3130/3130*xl* Genetic Analyzers	4352755
3130/3130*xl* Genetic Analyzer Autosampler Plate Kit, 96-well	4316471
GeneScan™ 500 LIZ® Size Standard	4322682
Running Buffer, 10✕	402824
DS-33 Matrix Standard Kit (Dye Set G5)	4345833
MicroAmp® Optical 96-Well Reaction Plate	N8010560
For a complete list of parts and accessories for the 3130*xl* instrument, refer to Appendix A of the *Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide* (PN 4352716).	
310 Analyzer materials	
310 DNA Analyzer Capillary Array, 47-cm	402839
0.5 mL Sample Tray	5572
96-Well Tray Adaptor (for 9700 thermal cycler trays)	4305051
GeneScan™ 500 LIZ® Size Standard	4322682
Running Buffer, 10✕	4335643
Genetic Analyzer Septa Retainer Clips for 96-Tube Sample Tray	402866
Genetic Analysis Sample Tubes (0.5-mL)	401957
Septa for 0.5-mL Sample Tubes	401956
DS-33 Matrix Standard Set (6-FAM™, 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).	

Table 3   User-supplied materials‡ *(continued)*

Item	Source
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-Cap Strip	N8010535
MicroAmp® 96-Well Tray/Retainer Set	403081
MicroAmp® 96-Well Base	N8010531
MicroAmp® Clear Adhesive Film	4306311
MicroAmp® Optical Adhesive Film	4311971
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
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

Work area setup and lab design

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

- If you are using the AmpF*l*STR® Identifiler® 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 (**http://nij.ncjrs.gov/publications/pubs_db.asp**).

- If you are using the AmpF*l*STR® Identifiler® 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 AmpF*l*STR® Identifiler® 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).

To prevent contamination by human DNA, be careful while handling and processing samples. 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: These laboratory design resources and guidances constitute only a sample of the precautions that need to be observed when using PCR technology. Refer to your laboratory's internal policies and procedures for additional information and references.

PCR-setup tools

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 tools

The following PCR systems should be placed in the amplified DNA work area.

- GeneAmp® PCR System 9700 with the Silver 96-Well Block
- GeneAmp® PCR System 9700 with the Gold-plated Silver 96-Well Block
- Veriti™ 96-Well Thermal Cycler

Required user-supplied reagents

In addition to the AmpF*l*STR® Identifiler® 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 procedure below or order it from Teknova (Cat # T0223).

To prepare low TE buffer:

1. 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

   Note: Adjust the volumes accordingly for specific needs.

2. Aliquot and autoclave the solutions.

3. Store at room temperature.

DNA quantification

Importance of quantification

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 AmpF*l*STR® Identifiler® Plus Kit is 1.0 ng in a maximum input volume of 10 µL for 28 PCR cycles and 0.5 ng in a maximum input volume of 10 µL for 29 PCR cycles.

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 of quantifying DNA	Applied Biosystems provides several kits for quantifying DNA in samples. See the reference cited in the following table for details about these kits.	

Product	Description	References
Quantifiler® Human DNA Quantification Kit (PN 4343895) *and* Quantifiler® Y Human Male DNA Quantification Kit (PN 4343906)	**Properties:** The Quantifiler® Human and Quantifiler® Y Human Male Kits are highly specific for human DNA, and they individually detect total human or male DNA, respectively. The kits detect single-stranded and degraded DNA. **How they work:** The Quantifiler® DNA Quantification Kits consist of target-specific and internal control 5' nuclease assays. The Quantifiler® Human and Quantifiler® Y Human Male Kits contain different target-specific assays (human DNA or human male DNA, respectively) that each consist of two locus-specific PCR primers and one TaqMan® MGB probe labeled with FAM™ dye for detecting the amplified sequence. The kits each contain a separate 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.	Quantifiler® Human DNA Quantification Kits User's Manual (PN 4344790)
Quantifiler® Duo DNA Quantification Kit (PN 4387746)	**Properties:** The Quantifiler® Duo Kit is highly specific for human DNA. This kit combines the detection of both total human and male DNA in one PCR reaction. The kit detects single-stranded and degraded DNA. **How it works:** The Quantifiler® Duo DNA Quantification Kit consists of target-specific and internal control 5' nuclease assays. The Quantifiler® Duo kit combines two human-specific assays in one PCR reaction (for total human DNA and human male DNA). The two human DNA specific assays each consist of two PCR primers and a TaqMan® probe. The TaqMan® probes for the human DNA and human male DNA assays are labeled with VIC® and FAM™ dyes, respectively. In addition, the kit contains an internal PCR control (IPC) assay similar in principle to that used in the other Quantifiler kits, but labeled with NED™ dye.	Quantifiler® Duo DNA Quantification Kit User's Manual (PN 4391294)

Prepare the amplification kit reactions

1. Calculate the volume of each component needed to prepare the reactions, using the table below.

DNA sample	Volume per reaction (μL)
AmpFℓSTR® Identifiler® Plus Master Mix	10.0
AmpFℓSTR® Identifiler® 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® Identifiler® Plus Kit Master Mix and the AmpFℓSTR® Identifiler® Plus Kit Primer Set, then vortex 3 seconds and centrifuge 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, they 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® tube.

6. Prepare the DNA samples:

DNA sample	To prepare...
Negative control	Add 10 μL of low TE buffer (10mM Tris, 0.1mM EDTA, pH 8.0).
Test sample	Dilute a portion of the test DNA sample with low TE buffer so that 1.0 ng of total DNA is in a final volume of 10 μL. Add 10 μL of the diluted sample to the reaction mix.
Positive control	Add 10 μL of 9947A control DNA (0.1 ng/μL).

The final reaction volume (sample or control plus reaction mix) is 25 µL.

7. Seal the MicroAmp® Optical 96-Well Reaction Plate with MicroAmp® Clear Adhesive Film or MicroAmp® Optical Adhesive Film, or cap the tubes.

8. Vortex the reaction mix for 3 seconds, then centrifuge the tubes at 3000 rpm for about 20 seconds in a tabletop centrifuge (with plate holders if using 96-well plates) to remove bubbles.

9. Amplify the samples in a GeneAmp® PCR System 9700 with the silver 96-well block, a GeneAmp® PCR System 9700 with the gold-plated silver 96-well block, or a Veriti® 96-Well Thermal Cycler.

Note: The AmpF*l*STR® Identifiler® Plus Kit is not validated for use with the GeneAmp PCR System 9700 with the aluminium 96-well block. Use of this thermal cycling platform may adversely affect performance of the AmpF*l*STR® Identifiler® Plus Kit.

Select the appropriate PCR cycle number

All AmpF*l*STR® kits are optimized for a specific number of amplification cycles to deliver well-balanced and high quality results. However, increases in the number of low-level DNA samples being submitted for analysis have prompted many laboratories to evaluate increasing the number of amplification cycles to increase the sensitivity of the assay. Before increasing the cycle number, perform a comprehensive validation study to establish new performance criteria for the higher cycle number.

Higher cycle numbers can cause the following to occur:

*   Exaggerated stochastic effects resulting from low DNA input amounts
*   Greater difference between the presence and absence of an allele
*   Greater heterozygote peak imbalance
*   Possible differences in expected stutter position and percentage
*   Possible increase in artifacts and/or background in the profile to accompany the increase in sample allele signal

The Identifiler® Plus Kit offers two PCR-cycle-number options:

*   **Standard 28-PCR-cycle protocol** – Provides high sensitivity to consistently generate full STR profiles with 125 pg of DNA input. Use with the optimum 1.0 ng DNA input amount in a maximum input volume of 10 μL.
*   **29-PCR-cycle protocol** – Adds the extra sensitivity when amplifying <125 pg DNA inputs. Recommended for use when the total DNA input amount is <0.5 ng.

The results of the developmental validation at both PCR cycle numbers is presented in .

Perform PCR

⚠ **WARNING! PHYSICAL INJURY HAZARD.** Thermal cycler.

1. Program the thermal cycling conditions:
   - When using the GeneAmp PCR System 9700 with either 96-well silver or gold-plated silver block, select the **9600 Emulation Mode**.
   - When using the Veriti® 96-Well Thermal Cycler, refer to the following document for instructions on how to configure the Veriti instrument to run in the 9600 Emulation Mode: *User Bulletin: Veriti® 96-Well Thermal Cycler AmpFℓSTR® Kit Validation* (PN 4440754).

Initial incubation step	Cycle (28 or 29 cycles‡)		Final extension	Final hold
	Denature	Anneal/Extend		
HOLD	CYCLE		HOLD	HOLD
95 °C 11 min	94 °C 20 sec	59 °C 3 min	60 °C 10 min	4 °C ∞

‡ Refer to the previous section for selecting the appropriate PCR cycle number

2. Load the plate into the thermal cycler and close the heated cover.

   IMPORTANT! If using adhesive clear film instead of caps to seal the plate wells, be sure to place a MicroAmp® compression pad (PN 4312639) on top of the plate to prevent evaporation during thermal cycling.

3. Start the run.

4. On completion of the run, store the amplified DNA and protect from light.

If you are storing the DNA...	Then place at...
< 2 weeks	2 to 8 °C
> 2 weeks	−15 to −25 °C

   IMPORTANT! Store the amplified products so that they are protected from light.

Amplification using bloodstained FTA® cards

 FTA® cards can be useful for collecting, storing, and processing biological samples. A small punch disc of the card containing the sample can be placed directly into an amplification tube, purified, and amplified, without transferring the disc. Applied Biosystems studies indicate that a 1.2-mm bloodstained disc contains approximately 5 to 20 ng DNA. An appropriate cycle number for this high quantity of DNA is 24 cycles as determined by Applied Biosystems validation studies. However, it is recommended that each laboratory determine the optimum cycle number based on internal validation studies.

In the example shown in Figure 4, a 1.2-mm disc of a bloodstained FTA card was purified using three washes with FTA Purification Reagent and two washes with 1✕ low TE buffer. The purified punch disc was then amplified in the MicroAmp® tube for 24 cycles.
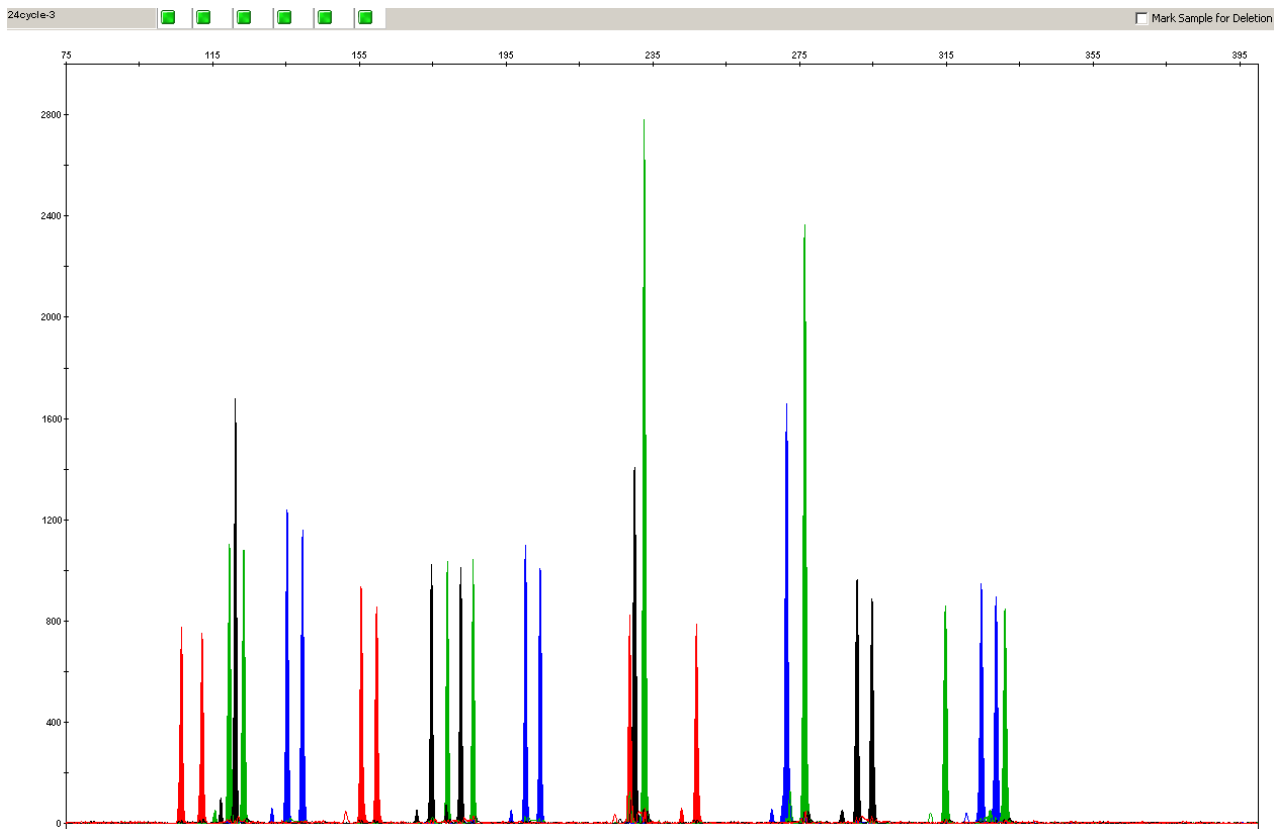

Figure 4   AmpF*l*STR® Identifiler® Plus PCR Amplification Kit results from a 1.2-mm FTA bloodstain disc (24-cycle amplification), analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer

Chapter 3

Electrophoresis

Electrophoresis

3

This chapter covers:

Allelic ladder requirements

To accurately genotype samples, you must run an allelic ladder sample along with the unknown samples. For samples run on the:

- ABI PRISM® 310 Genetic Analyzer: Run at least one allelic ladder for every 10 sample injections.
- ABI PRISM® 3100 and Applied Biosystems 3130 series Genetic Analyzers: Run at least one allelic ladder per every set of 16 samples.

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

- 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

When genotyping, it is critical to use an allelic ladder 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.
- Variation in laboratory temperature can affect migration speed (see IMPORTANT above).

Section 3.1   3100/3100-*Avant* and 3130/3130*xl* instruments

Set up the 3100/3100-*Avant* or 3130/3130*xl* instrument for electrophoresis

Reagents and parts

Table 3 on page 10 lists the required materials not supplied with the AmpF*l*STR® Identifiler® Plus Kit.

IMPORTANT! The fluorescent dyes attached to the primers are light-sensitive. Protect the AmpF*l*STR® Identifiler® Plus Primer Set from light when not in use. Amplified DNA, AmpF*l*STR® Identifiler® Plus Allelic Ladder, and GeneScan™ 500 LIZ® Size Standard should also be protected from light. Keep freeze-thaw cycles to a minimum.

3100/3100-*Avant* or 3130/3130*xl* instrument requirements

The following table lists Data Collection Software and the run modules that can be used to analyze Identifiler® Plus PCR products. For details on the procedures, refer to the documents listed in the table.

Operating system	Data Collection Software	Run modules and conditions	References
Windows XP	3.0‡ (3130/3130*xl* Analyzer)	• HIDFragmentAnalysis36_POP4_1 Injection conditions: – 3130 = 3 kV/5 sec – 3130*xl* = 3 kV/10 sec • Dye Set G5	*Applied Biosystems 3130/3130xl Genetic Analyzers Using Data Collection Software v3.0, Protocols for Processing AmpF*l*STR PCR Amplification Kit PCR Products User Bulletin* (PN 4363787)
Windows 2000	2.0 (3100 Analyzer)	• HIDFragmentAnalysis36_POP4_1 Injection condition: 3 kV/10 sec • Dye Set G5	*ABI PRISM® 3100/3100-Avant Genetic Analyzers Using Data Collection Software v2.0, Protocols for Processing AmpF*l*STR PCR Amplification Kit PCR Products User Bulletin* (PN 4350218)
Windows NT®	1.1 (3100 Analyzer)	• GeneScan36vb_DyeSetG5Module Injection condition: 3 kV/10 sec • GS500Analysis.gsp	*ABI PRISM® 3100/3100-Avant Genetic Analyzers Protocols for Processing AmpF*l*STR PCR Amplification Kit PCR Products User Bulletin* (PN 4332345)
	1.0 (3100-*Avant* Analyzer)	• GeneScan36Avb_DyeSetG5Module Injection condition: 3 kV/5 sec • GS500Analysis.gsp	*ABI PRISM® 3100/3100-Avant Genetic Analyzers Protocols for Processing AmpF*l*STR PCR Amplification Kit PCR Products User Bulletin* (PN 4332345)

‡ Applied Biosystems conducted validation studies for the Identifiler® Plus Kit using this configuration.

Prepare samples for electrophoresis on the 3100/3100-*Avant* or 3130/3130*xl* instrument

Prepare the samples

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

1. Calculate the volume of Hi-Di™ Formamide and GeneScan™ 500 LIZ® Size Standard needed to prepare the samples, using the table below.

Reagent	Volume per reaction (μL)
GeneScan 500 LIZ Size Standard	0.3
Hi-Di Formamide	8.7

   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 and experiments.

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

3. Vortex the tube, then centrifuge briefly.

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 collected at the bottom.

6. Heat the reaction plate in a thermal cycler for 3 minutes at 95 °C.

7. Immediately place the plate on ice for 3 minutes.

8. Prepare the plate assembly on the autosampler.

9. Start the electrophoresis run.

Section 3.2   310 instrument

Set up the 310 instrument for electrophoresis

Reagents and parts

Table 3 on page 10 lists the required materials not supplied with the AmpF*l*STR® Identifiler® Plus Kit.

IMPORTANT! The fluorescent dyes attached to the primers are light-sensitive. Protect the AmpF*l*STR® Identifiler® Plus Primer Set from light when not in use. Amplified DNA, AmpF*l*STR® Identifiler® Plus Allelic Ladder, and GeneScan™ 500 LIZ® Size Standard should also be protected from light. Keep freeze-thaw cycles to a minimum.

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310 instrument requirements

The following table lists Data Collection Software and the run modules that can be used to analyze Identifiler® Plus PCR products. For details on the procedures, refer to the documents listed in the table.

Operating system	Data Collection Software	Run modules and conditions	References
Windows XP *or* Windows NT® and Windows 2000	3.1‡ *or* 3.0	GS STR POP4 (1mL) G5 v2.md5 Injection condition: 15 kV/5 sec	ABI PRISM® *310 Genetic Analyzer User's Manual (Windows)* (PN 4317588) ABI PRISM® *310 Protocols for Processing AmpF*l*STR PCR Amplification Kit Products with Microsoft Windows NT Operating System: User Bulletin* (PN 4341742)

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

ABI PRISM® 310 Genetic Analyzer

Prepare samples for electrophoresis on the 310 instrument

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

1. Calculate the volume of Hi-Di™ Formamide and GeneScan™ 500 LIZ® Internal Size Standard needed to prepare the samples, using the table below.

Reagent	Volume per reaction (μL)
GeneScan™ 500 LIZ® Size Standard	0.5
Hi-Di™ Formamide	24.5

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 and experiments.

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

3. Vortex the tube, then centrifuge briefly.

4. Into a 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 the appropriate septa, then briefly vortex and centrifuge the tubes to ensure that the contents of each tubes are mixed and collected at the bottom.

6. Heat the tubes in a thermal cycler for 3 minutes at 95 °C.

7. Immediately place the tubes on ice for 3 minutes.

8. Place the sample tray on the autosampler.

9. Start the electrophoresis run.

Chapter 4

Data Analysis

Data Analysis

4

This chapter covers:

Section 4.1   GeneMapper® *ID* Software

Overview of GeneMapper® *ID* Software

GeneMapper® *ID* Software is an automated genotyping software for forensic casework, databasing, and paternity data analysis.

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

Instruments   Refer to for a list of compatible instruments.

Before you start   When using GeneMapper® *ID* Software v3.2.1 to perform human identification (HID) analysis with AmpFℓSTR® kits, be aware that:

- HID analysis requires at least one allelic ladder sample per run folder. Your laboratory can use multiple ladder samples in an analysis, provided individual laboratories conduct 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 must be labeled as "Allelic Ladder" in the Sample Type column in a project. Failure to apply this setting for ladder samples results in failed analysis.

- 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.

- Alleles that are not in the AmpFℓSTR® Allelic Ladders do exist. Off-ladder (OL) 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.

If you are using GeneMapper w Software to perform Human Identification (HID) analysis with AmpF*l*STR kits, go to "Set up GeneMapper® ID-X Software for data analysis" on page 52 or refer to the *GeneMapper® ID-X Software Version 1.0 Human Identification Analysis Getting Started Guide* (PN 4375574).

Set up GeneMapper® *ID* Software for data analysis

Workflow

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

- Import panels and bins into the Panel Manager, as explained in "Import panels and bins" on page 39.
- Import an analysis method, as explained in "Import an HID analysis method" on page 42.
- Import a size standard, as explained in "Import an HID size standard" on page 47.
- Define custom views of analysis tables.
- Define custom views of plots.

For more info

For details about GeneMapper® *ID* Software workflow and features, refer to:

- *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)
- *Installation Procedures and New Features for GeneMapper® ID* Software *v3.2 User Bulletin* (PN 4352543)

GeneMapper *ID* Software

Import panels and bins

To import the AmpF*l*STR® Identifiler® Plus Kit panel and bin set from the Applied Biosystems web site into the GeneMapper® *ID* Software v3.2.1 database:

1. Download and open the file containing panels and bins:

   a. From the Support menu of **www.appliedbiosystems.com**, select **Software Downloads, Patches & Updates**. Select **GeneMapper® ID Software v3.2** from the drop-down menu. Select **Updaters & Patches**, and download the file **Identifiler Plus Analysis Files GMID**.

   b. Unzip the file.

2. Start the GeneMapper® *ID* Software, then log in with the appropriate user name and password.


   IMPORTANT! If you need logon instructions, refer to page 2-7 of the *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide* (PN 4338775).

   ---

3. Select **Tools ▸ Panel Manager**.

4. Find, then open the folder containing the panels and bins:
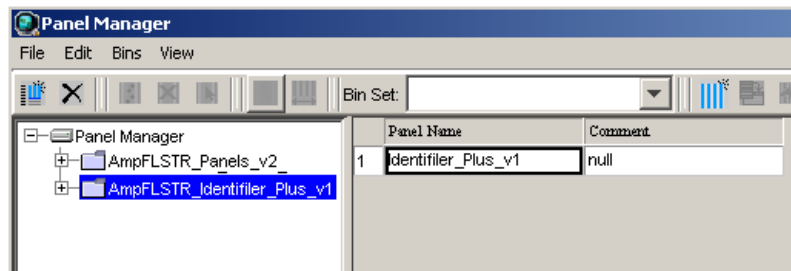
   a. Select **Panel Manager** in the navigation pane.
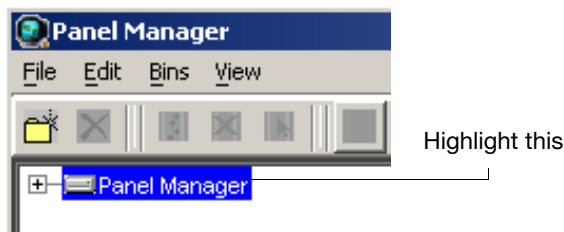
   Highlight this

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

   c. Navigate to, then open the **Identifiler Plus Analysis Files GMID** folder that you unzipped in step 1.

5. Select Identifiler_Plus_Panels_v1, then click **Import**.

   Note: Importing this file creates a new folder in the navigation pane of the Panel Manager, AmpFLSTR_Identifiler_Plus_v1. This folder contains the panel and associated markers.

   ---
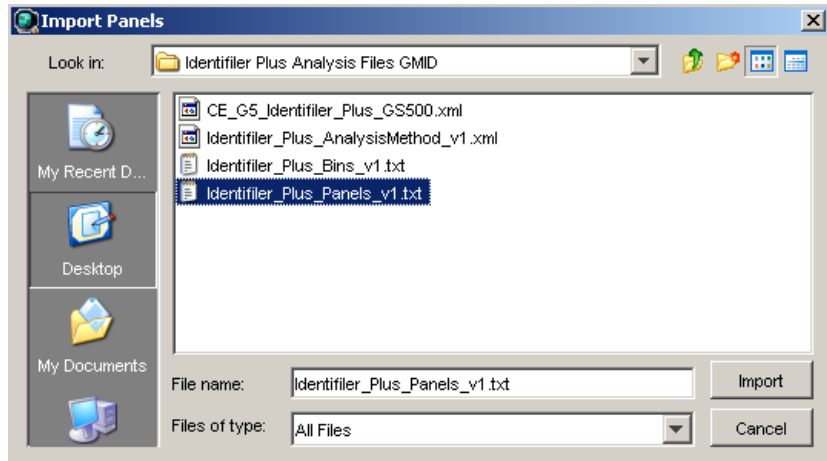
6. Import Identifiler_Plus_Bins_v1:

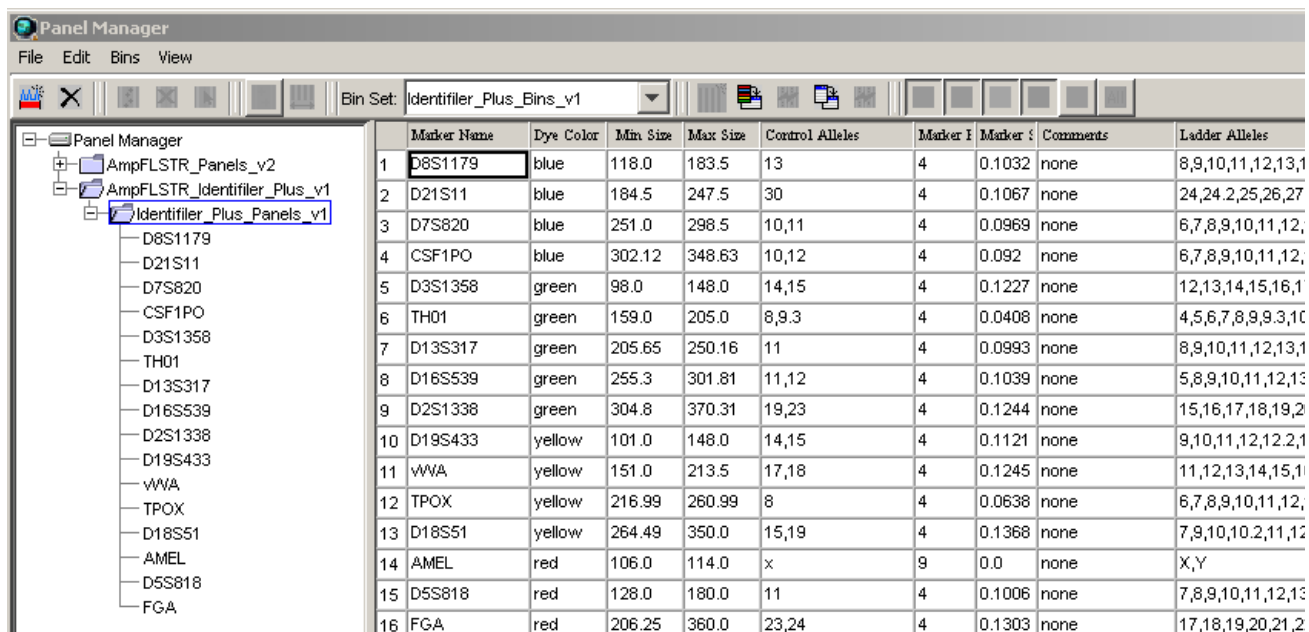   a. Select the **AmpFLSTR_Identifiler_Plus_v1** folder in the navigation pane.

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

   c. Navigate to, then open the **Identifiler Plus Analysis Files GMID** folder.

   d. Select **Identifiler_Plus_Bins_v1**, then click **Import**.
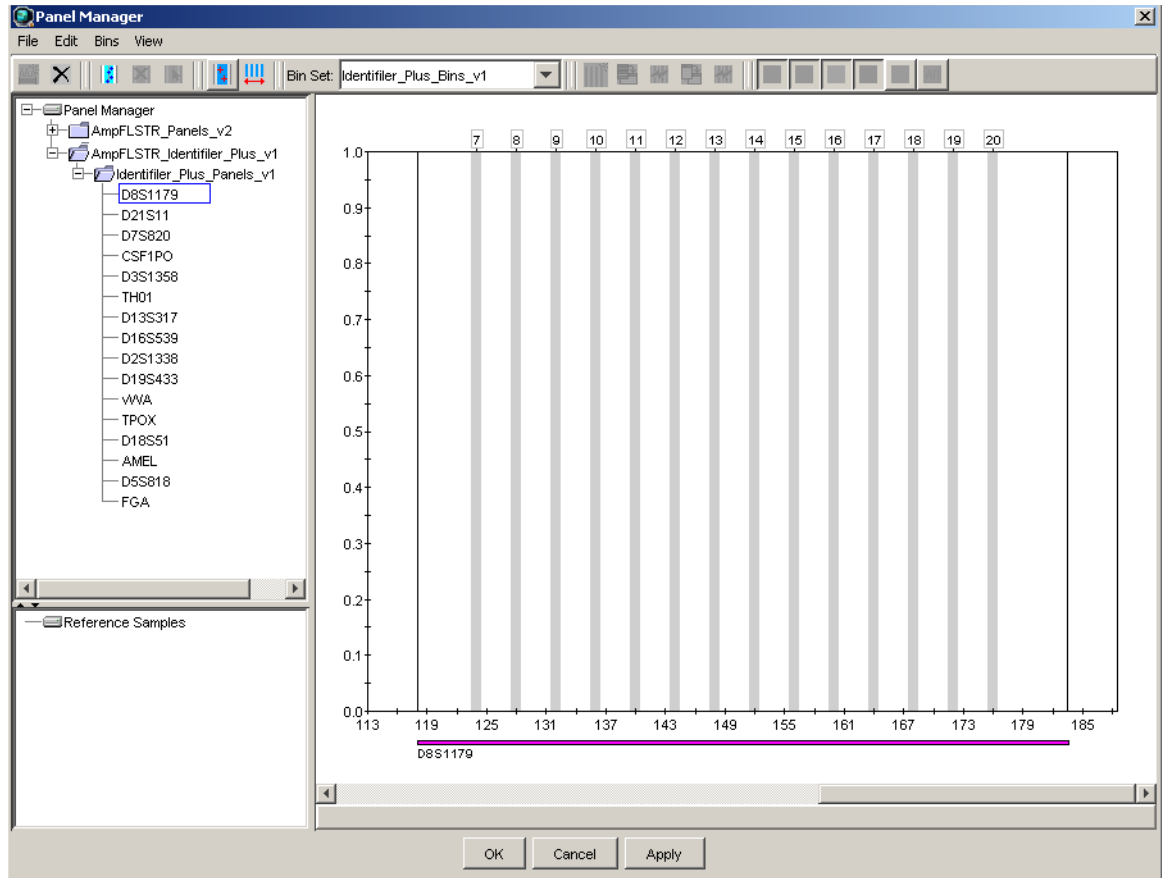
   Note: Importing this file associates the bin set with the panels in the Identifiler_Plus_Panels_v1 folder.
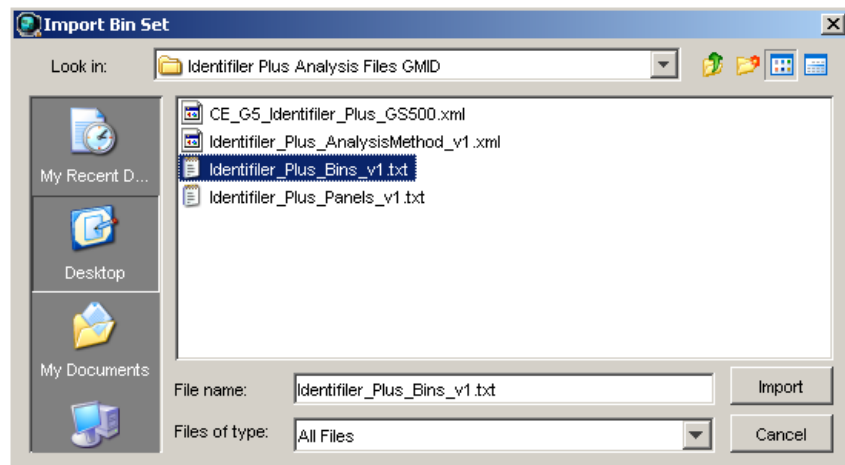
7. View the imported panels in the navigation pane:

a. Double-click the **AmpFLSTR_Identifiler_Plus_v1** folder to view the Identifiler_Plus_Panels_v1 folder.

b. Double-click the **Identifiler_Plus_Panels_v1** folder to display the panel information in the right pane.

	Marker Name	Dye Color	Min Size	Max Size	Control Alleles	Marker F	Marker S	Comments	Ladder Alleles
1	D8S1179	blue	118.0	183.5	13	4	0.1032	none	8,9,10,11,12,13,1
2	D21S11	blue	184.5	247.5	30	4	0.1067	none	24,24.2,25,26,27
3	D7S820	blue	251.0	298.5	10,11	4	0.0969	none	6,7,8,9,10,11,12,
4	CSF1PO	blue	302.12	348.63	10,12	4	0.092	none	6,7,8,9,10,11,12,
5	D3S1358	green	98.0	148.0	14,15	4	0.1227	none	12,13,14,15,16,1
6	TH01	green	159.0	205.0	8,9.3	4	0.0408	none	4,5,6,7,8,9,9.3,10
7	D13S317	green	205.65	250.16	11	4	0.0993	none	8,9,10,11,12,13,1
8	D16S539	green	255.3	301.81	11,12	4	0.1039	none	5,8,9,10,11,12,13
9	D2S1338	green	304.8	370.31	19,23	4	0.1244	none	15,16,17,18,19,2
10	D19S433	yellow	101.0	148.0	14,15	4	0.1121	none	9,10,11,12,12.2,1
11	VWA	yellow	151.0	213.5	17,18	4	0.1245	none	11,12,13,14,15,1
12	TPOX	yellow	216.99	260.99	8	4	0.0638	none	6,7,8,9,10,11,12,
13	D18S51	yellow	264.49	350.0	15,19	4	0.1368	none	7,9,10,10.2,11,12
14	AMEL	red	106.0	114.0	x	9	0.0	none	X,Y
15	D5S818	red	128.0	180.0	11	4	0.1006	none	7,8,9,10,11,12,13
16	FGA	red	206.25	360.0	23,24	4	0.1303	none	17,18,19,20,21,2

8. Select **D8S1179** to display the Bin view for the marker in the right pane.

9. Click **Apply**, then **OK** to add the AmpF*l*STR® Identifiler® Plus Kit panel and bin set to the GeneMapper® *ID* Software database.

IMPORTANT! If you close the Panel Manager without clicking OK, the panels and bins are not imported into the GeneMapper® *ID* Software database.
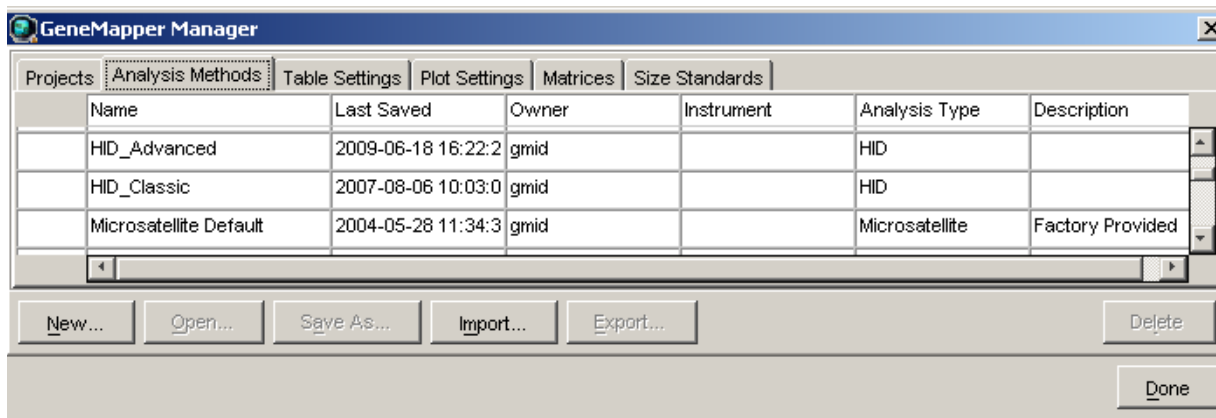

Import an HID analysis method

The HID Advanced analysis method for the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit uses the Identifiler_Plus_Bins_v1 file described in step 6 on page 40.

Use the following procedure to import the analysis method from the folder that you downloaded from the Applied Biosystems web site, into the GeneMapper® *ID* Software database. Refer to step 1 on page 39 for downloading instructions.

Note: The Identifiler_Plus_AnalysisMethod_v1 has been provided to assist you in getting started with Identifiler Plus kit data analysis. Analysis parameters should be established by each individual laboratory based on the laboratory's internal validation studies.

---

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 **Identifiler Plus Analysis Files GMID** folder.

   c. Select **Identifiler_Plus_AnalysisMethod_v1**, then click **Import** to import the Identifiler_Plus_AnalysisMethod_v1 into the GeneMapper® *ID* Software database.
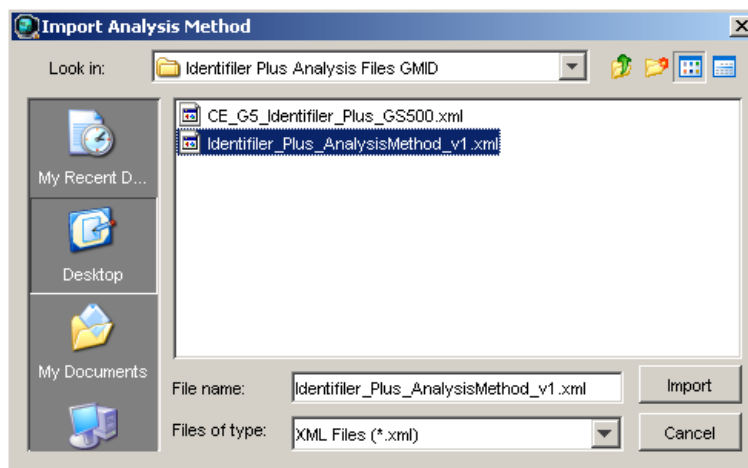
To view the settings for Identifiler_Plus_AnalysisMethod_v1, select the **Analysis Methods** tab, then select **Identifiler_Plus_AnalysisMethod** in the Name column and click **Open**.
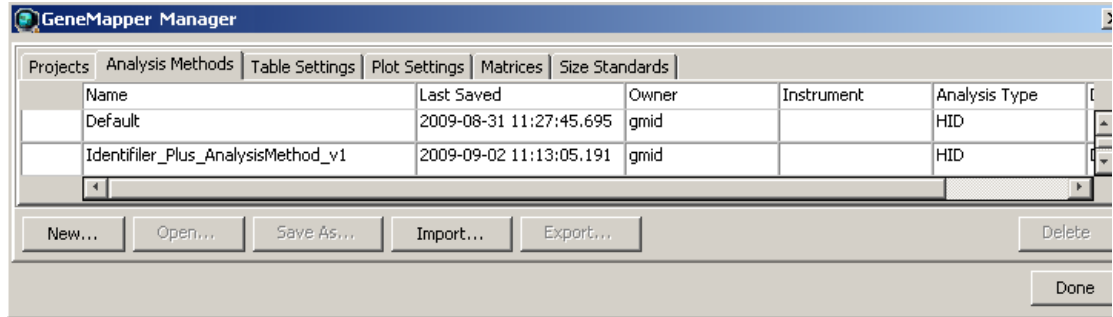
Figure 5   Analysis Method Editor - HID: General tab settings

Figures 6 through 9 below show the settings for each tab of the Analysis Method Editor - HID.
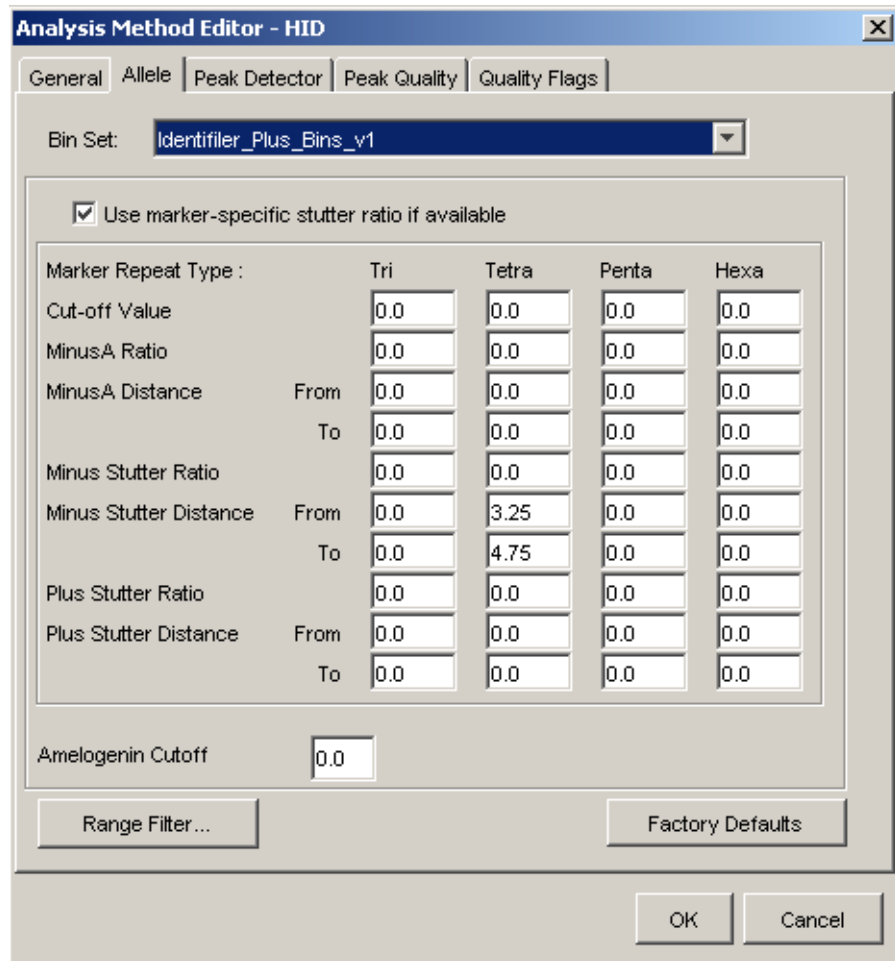
Figure 6   Analysis Method Editor - HID: Allele tab settings

- 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 Identifiler_Plus_Panels_v1 file.

For more information about allele filters, refer to:

- *GeneMapper® ID* Software *Version 3.1 Human Identification Analysis User Guide*, Chapter 3 (PN 4338775)
- *GeneMapper® ID* Software *Versions 3.1 and 3.2 Human Identification Analysis Tutorial* (PN 4335523)
- *Installation Procedures and New Features for GeneMapper® ID* Software *v3.2 User Bulletin* (PN 4352543)
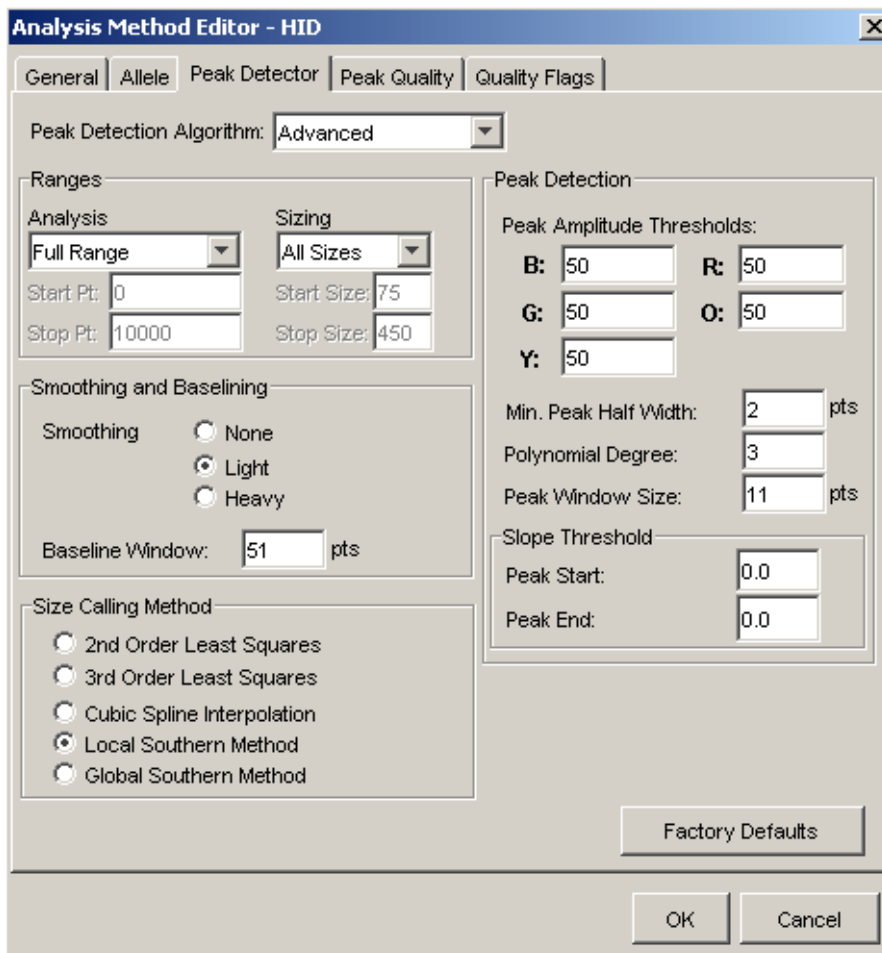
Figure 7   Analysis Method Editor - HID: Peak Detector tab settings

IMPORTANT! Laboratories need to perform the appropriate internal validation studies to determine the peak amplitude thresholds that allow for reliable interpretation of AmpFℓSTR® Identifiler® Plus PCR Amplification Kit data.

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

Note: The analysis range is set by the user based on the locations of the primer peaks and size standard peaks.

For more information about peak detection algorithms, refer to:

- *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, Appendix A* (PN 4335523)
- *Installation Procedures and New Features for GeneMapper® ID* Software *v3.2 User Bulletin* (PN 4352543)
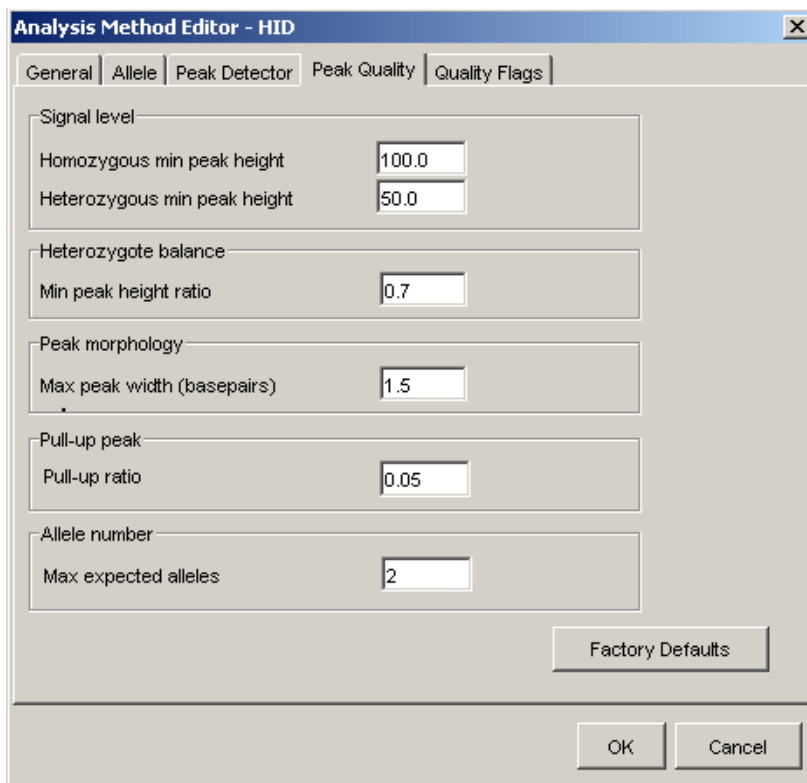

Figure 8   Analysis Method Editor - HID: Peak Quality tab settings

IMPORTANT! Laboratories need to perform the appropriate internal validation studies to determine the minimum heterozygous and homozygous minimum peak height thresholds and the minimum peak height ratio threshold that allow for reliable interpretation of AmpF*ℓ*STR® Identifiler® Plus PCR Amplification Kit data.
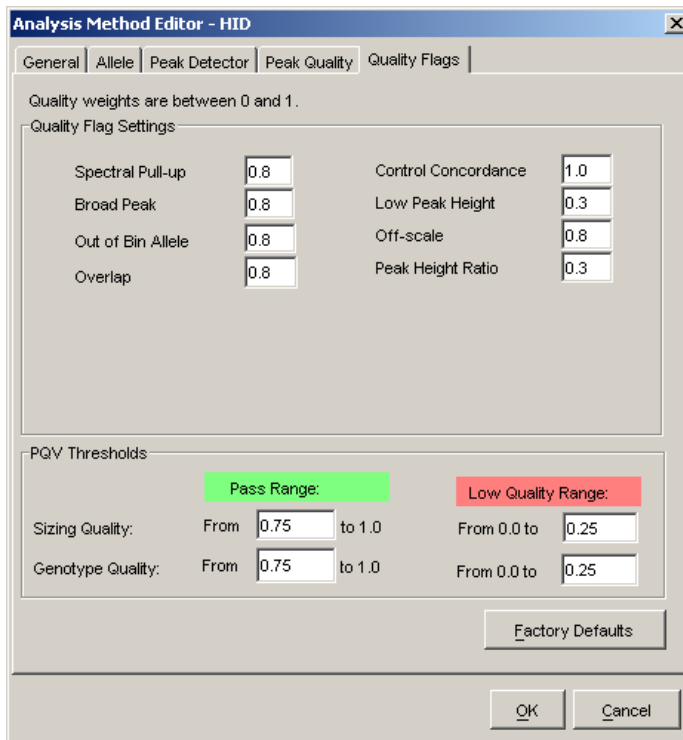
Figure 9   Analysis Method Editor - HID: Quality Flags tab settings

Import an HID size standard

The size standard for the Identifiler® Plus Kit uses the following GS500 peaks in its sizing algorithm: 75, 100, 139, 150, 160, 200, 300, 350, 400, and 450.
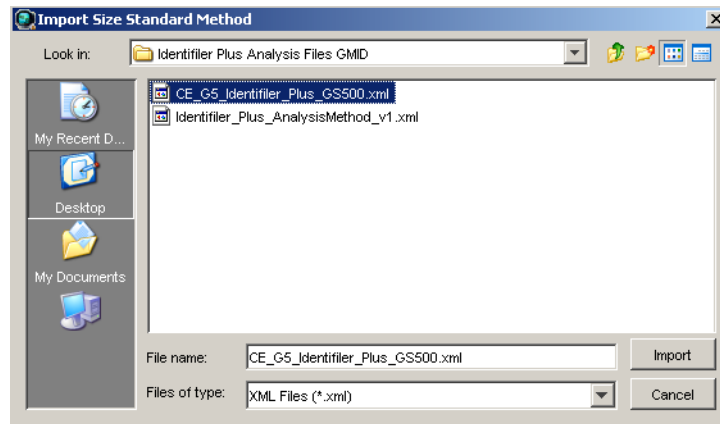
Use the following procedure to import the size standard for the AmpF*l*STR® Identifier® Plus PCR Amplification Kit from the folder that you downloaded from the Applied Biosystems web site, into the GeneMapper® *ID* Software database. Refer to for downloading instructions.

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

2. Import a size standard:

   a. Select the **Size Standards** tab, then click **Import**.
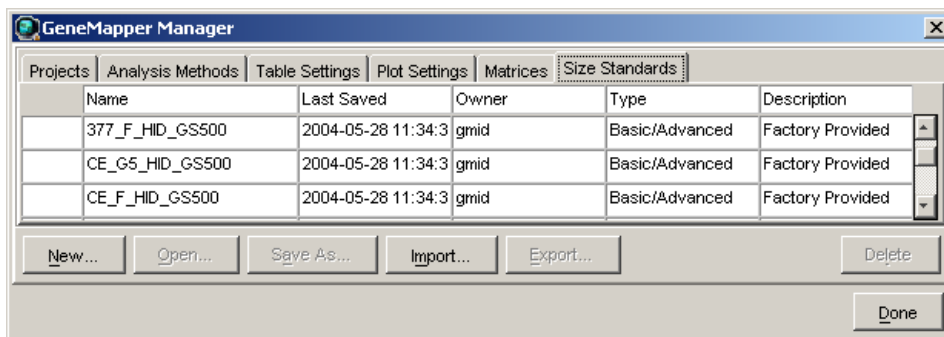
   b. Navigate to, then open the **Identifiler Plus Analysis Files GMID** folder.

c. Select **CE_G5_Identifiler_Plus_GS500**, then click **Import** to import the **CE_G5_Identifiler_Plus_GS500** size standard into the GeneMapper® *ID* Software database.

Analyze and edit sample files with GeneMapper® *ID* Software

Analyze 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	Settings
Sample Type	Select the sample type.
Analysis Method	Identifiler_Plus_AnalysisMethod_v1
Panel	Identifiler_Plus_Panels_v1
Size Standard‡	CE_G5_Identifiler_Plus_GS500§#

‡ 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_Identifiler_Plus_GS500 size standard provided with the AmpF*l*STR® Identifiler® Plus Kit: 75, 100, 139, 150, 160, 200, 300, 350, 400, and 450. For additional information about size standards, refer to the *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide* (PN 4338775), Appendix D.

Neither the 250-nt nor the 340-nt peak are included in the size standard definition. These peaks can be used as an indicator of precision within a run.

3. Click ▶ (Analyze), enter a name for the project (in the Save Project dialog box), then click **OK** to start analysis.

    • The status bar displays the progress of analysis:

        – As a completion bar extending to the right with the percentage completed indicated

        – With text messages on the left

- The Samples table (figure below) 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.

	Status	Sample File	Sample Name	Sample Type	Analysis Method	Panel	Size Standard	Instrument Type
1		Cerebus_3130XL	IB_0329	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
2		Cerebus_3130XL	IB_0330	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
3		Cerebus_3130XL	IB_0331	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
4		Cerebus_3130XL	IB_0332	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
5		Cerebus_3130XL	IB_0333	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
6		Cerebus_3130XL	IB_0334	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
7		Cerebus_3130XL	IB_0335	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
8		Cerebus_3130XL	IB_0336	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
9		Cerebus_3130XL	IB_0337	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
10		Cerebus_3130XL	IB_0338	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
11		Cerebus_3130XL	IB_0339	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
12		Cerebus_3130XL	IB_0340	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
13		Cerebus_3130XL	IB_0341	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
14		Cerebus_3130XL	IB_0342	Sample	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130
15		Cerebus_3130XL	ladder3	Allelic Ladder	Identifiler_Plus_AnalysisMethod_v1	Identifiler_Plus_Panels_v1	CE_G5_Identifiler_Plus_GS500	ABI3130

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

Examine and edit 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:

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

Section 4.2   GeneMapper® *ID-X* Software

Overview of GeneMapper® *ID-X* Software

GeneMapper® *ID-X* Software is an automated genotyping software for forensic casework, databasing, and paternity data analysis.

After electrophoresis, the Data Collection Software stores information for each sample in a .fsa file. Using GeneMapper® *ID-X* Software v1.0.1, v1.1, or v1.1.1, you can then analyze and interpret the data from the .fsa files.

Instruments   Refer to for a list of compatible instruments.

Before you start   When using GeneMapper® *ID-X* Software v1.0.1, v1.1, or v1.1.1 to perform human identification (HID) analysis with AmpF*l*STR® kits, be aware that:

- HID analysis requires at least one allelic ladder sample per run folder. Your laboratory can use multiple ladder samples in an analysis, provided individual laboratories conduct the appropriate validation studies.

    For multiple ladder samples, the GeneMapper® *ID-X* 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 must be labeled as "Allelic Ladder" in the Sample Type column in a project. Failure to apply this setting for ladder samples results in failed analysis.

- 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.

- Alleles that are not in the AmpF*l*STR® Allelic Ladders do exist. Off-ladder (OL) 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.

Set up GeneMapper® *ID-X* Software for data analysis

Workflow
Before you can analyze sample (.fsa) files using GeneMapper® *ID-X* Software v1.0.1, v1.1, or v1.1.1 for the first time:

- Import panels, bins, and marker stutter into the Panel Manager, as explained in "Import panels, bins, and marker stutter" on page 52.
- Import an analysis method, as explained in "Import an analysis method" on page 57.
- Import a size standard, as explained in "Import a HID size standard" on page 62.
- Define custom views of analysis tables.
- Define custom views of plots.

For more info
For quick set up instructions, refer to the GeneMapper® *ID-X* Software *Version 1.0 Getting Started Guide* (PN 4375574).

For details about the GeneMapper® *ID-X* Software workflow and features, refer to:

- GeneMapper® *ID-X* Software *Version 1.0 Getting Started Guide* (PN 4375574)
- GeneMapper® *ID-X* Software *Version 1.0 Quick Reference Guide* (PN 4375670)
- GeneMapper® *ID-X* Software *Version 1.0 Reference Guide* (PN 4375671)

Import panels, bins, and marker stutter
To import the AmpF*l*STR® Identifiler® Plus Kit panels, bin sets, and marker stutter from the Applied Biosystems web site into the GeneMapper® *ID-X* Software v1.0.1, v1.1, or v1.1.1 database:

1. Download and open the file containing panels, bins, and marker stutter:

   a. From the Support menu of **www.appliedbiosystems.com**, select **Software Downloads, Patches & Updates**. Select **GeneMapper® ID-X Software** from the drop-down menu. Select **Updaters & Patches**, and download the file **Identifiler Plus Analysis Files GMIDX**.

   b. Unzip the file.

2. Start the GeneMapper® *ID-X* Software, then log in with the appropriate user name and password.

   IMPORTANT! If you need logon instructions, refer to the *GeneMapper® ID-X Software Version 1.0 Getting Started Guide* (PN 4375574).

3. Select **Tools ▸ Panel Manager**.

4. Find, then open the folder containing the panels, bins, and marker stutter:

   a. Select **Panel Manager** in the navigation pane.
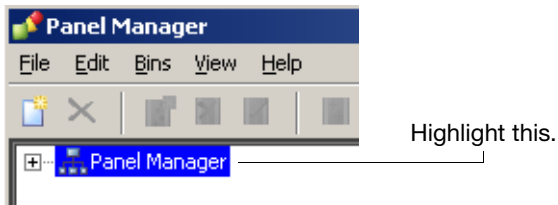
GeneMapper® ID-X Software

Highlight this.

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

c. Navigate to, then open the Identifiler Plus Analysis Files GMIDX folder that you unzipped in step 1 on page 52.

5. Select **Identifiler_Plus_Panels_v1X**, then click **Import**.

Note: Importing this file creates a new folder in the navigation pane of the Panel Manager "AmpFLSTR_Identifiler_Plus_v1X." This folder contains the panel and associated markers.
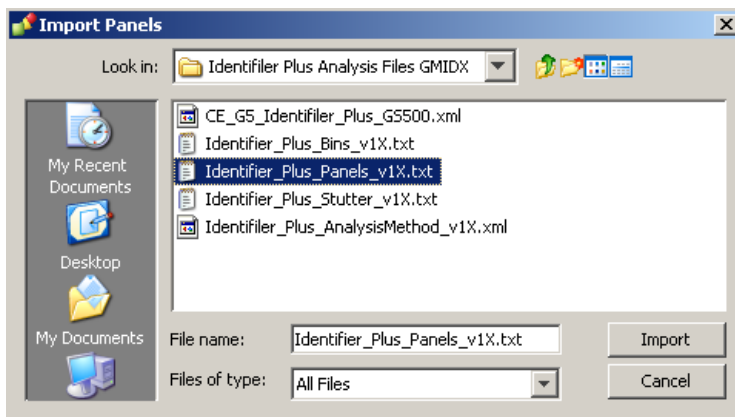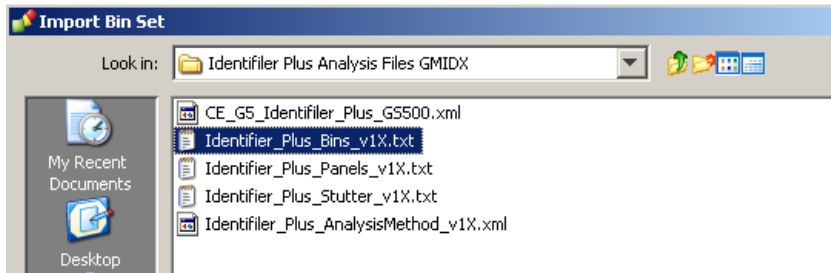

6. Import Identifiler_Plus_Bins_v1X:

a. Select the **AmpFLSTR_Identifiler_Plus_v1X** folder in the navigation pane.
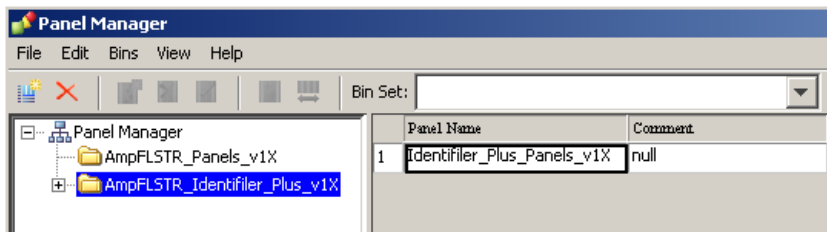
b. Select **File ▸ Import Bin Set** to open the Import Bin Set dialog box.

c. Navigate to, then open the **AmpFLSTR_Identifiler_Plus_v1X** folder.

d. Select **Identifiler_Plus_Bins_v1X**, then click **Import**.
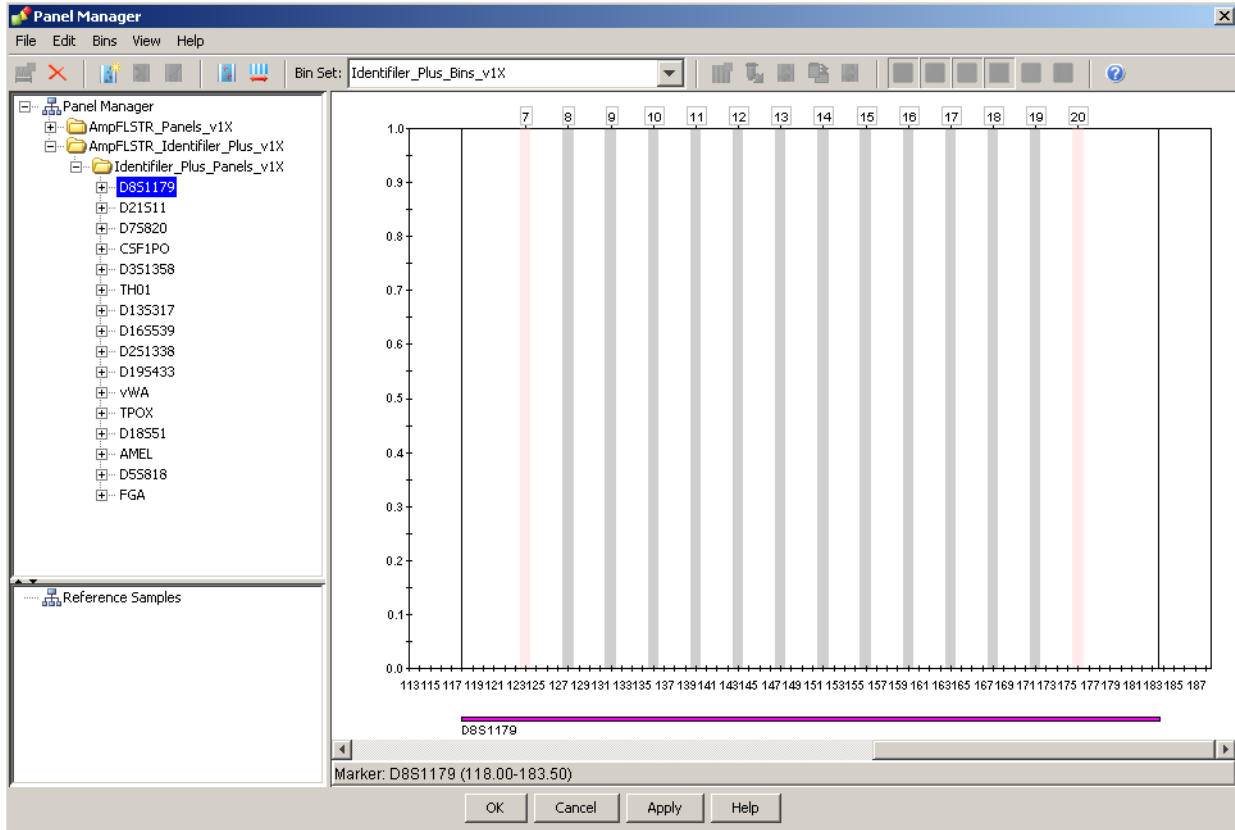
> **Note:** Importing this file associates the bin set with the panels in the Identifiler_Plus_Panels_v1X folder.

7. View the imported panels in the navigation pane:

   a. Double-click the **AmpFLSTR_Identifiler_Plus_v1X** folder to view the Identifiler_Plus_Panel_v1X folder.

   b. Double-click the **Identifiler_Plus_Panel_v1X** folder to display the panel information in the right pane and the markers below it.
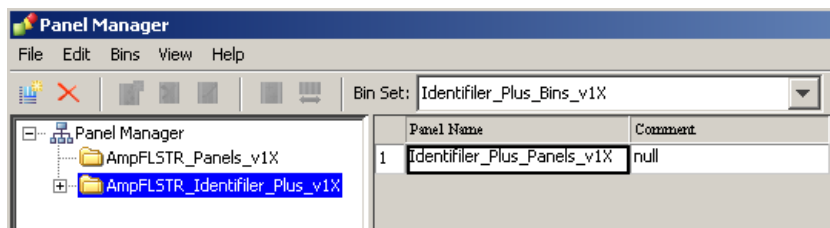

8. Select **D2S1388** to display the Bin view for the marker in the right pane.

9. Import Identifiler_Plus_Stutter_v1X:

   a. Select the **AmpFLSTR_Identifiler_Plus_v1X** folder in the navigation panel.

   b. Select File ▸ **Import Marker Stutter** to open the Import Marker Stutter dialog box.

   c. Navigate to, then open the **Identifiler Plus Analysis Files GMIDX** folder.

   d. Select **Identifiler_Plus_Stutter_v1X**, then click **Import**.

   Note: Importing this file associates the marker stutter ratio with the bin set in the Identifiler_Plus_Bins_v1X folder.
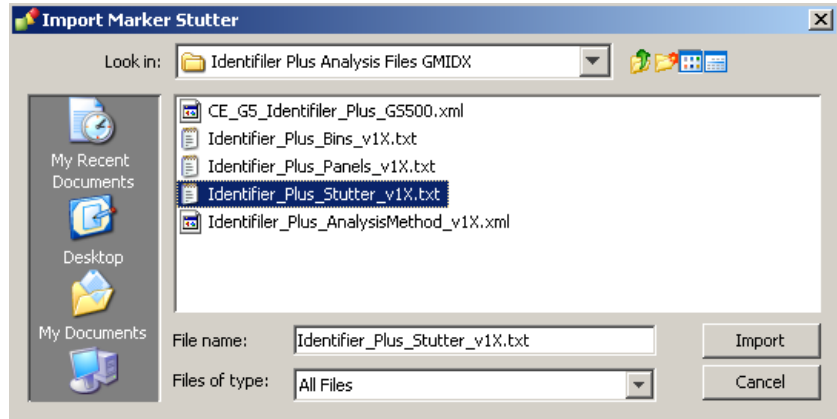
10. View the imported marker stutters in the navigation pane:

    a. Select the **Identifiler_Plus_Panel_v1X** folder to display its list of markers in the right pane.

    b. Double-click the **Identifiler_Plus_Panel_v1X** folder to display its list of markers below it.

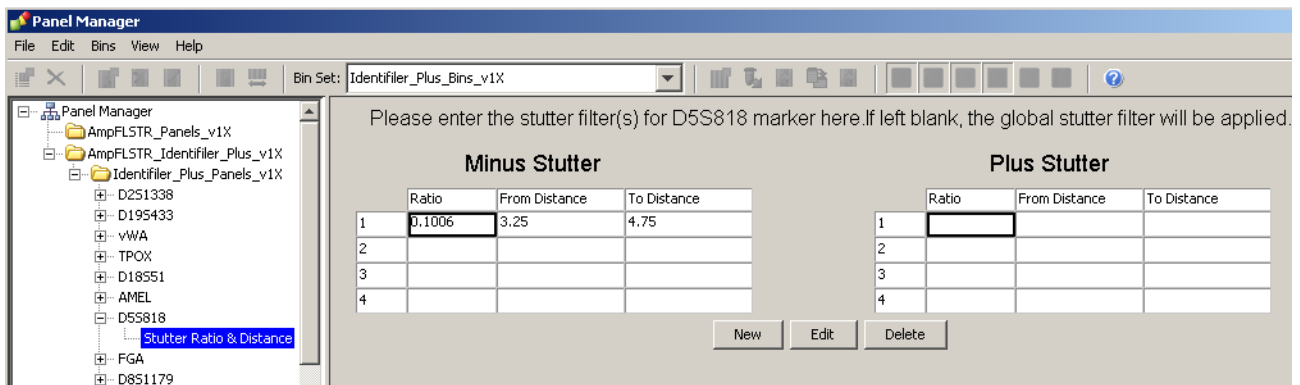   c. Double-click **D5S818** to display the Stutter Ratio & Distance view for the marker in the right pane.

11. Click **Apply**, then **OK** to add the AmpFℓSTR® Identifiler® Plus Kit panels, bin sets, and marker stutter to the GeneMapper® *ID-X* Software database.

> **IMPORTANT!** If you close the Panel Manager without clicking **Apply**, the panels, bin sets, and marker stutter will not be imported into the GeneMapper® *ID-X* Software database.

GeneMapper® ID-X Software

Import an analysis method

Use the following procedure to import the analysis method for the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit from the folder that you downloaded from the Applied Biosystems web site, into the GeneMapper® *ID-X* Software database. Refer to for downloading instructions.

Note: The Identifiler_Plus_AnalysisMethod_v1x has been provided to assist you in getting started with Identifiler Plus kit data analysis. Analysis parameters should be established by each individual laboratory based on the laboratory's internal validation studies.

1. Select **Tools ▸ GeneMapper® ID-X Manager** to open the GeneMapper *ID-X* Manager.

2. Import an analysis method:

   a. Select the **Analysis Methods** tab, then click **Import**.
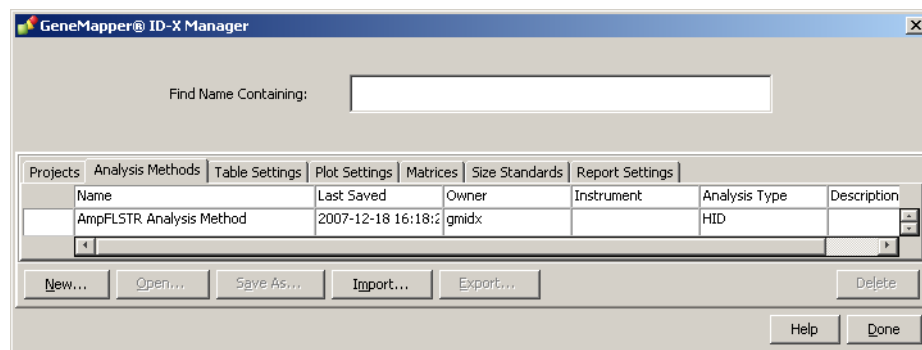
   b. Navigate to, then open the **Identifiler Plus Analysis Files GMIDX** folder.

   c. Select **Identifiler_Plus_AnalysisMethod_v1X**, then click **Import** to import Identifiler_Plus_AnalysisMethod_v1X into the GeneMapper® *ID-X* Software database.
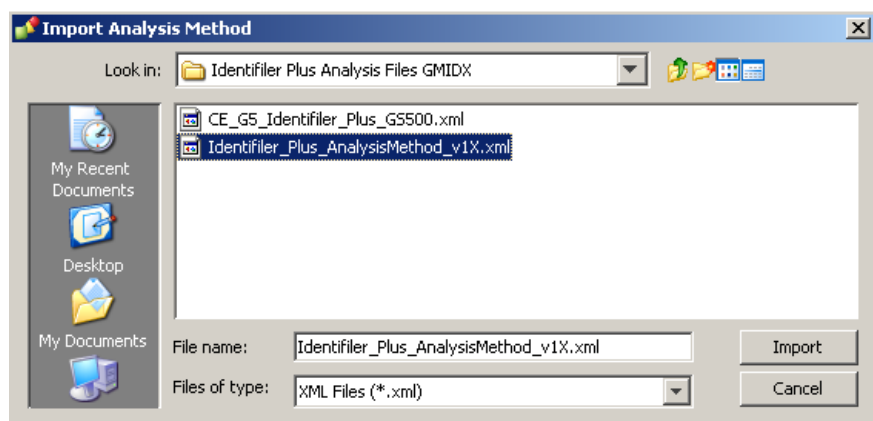
3. To view the settings for Identifiler_Plus_AnalysisMethod_v1X, select the **Analysis Methods** tab, then select **Identifiler_Plus_AnalysisMethod_v1X** in the Name column and click **Open**.
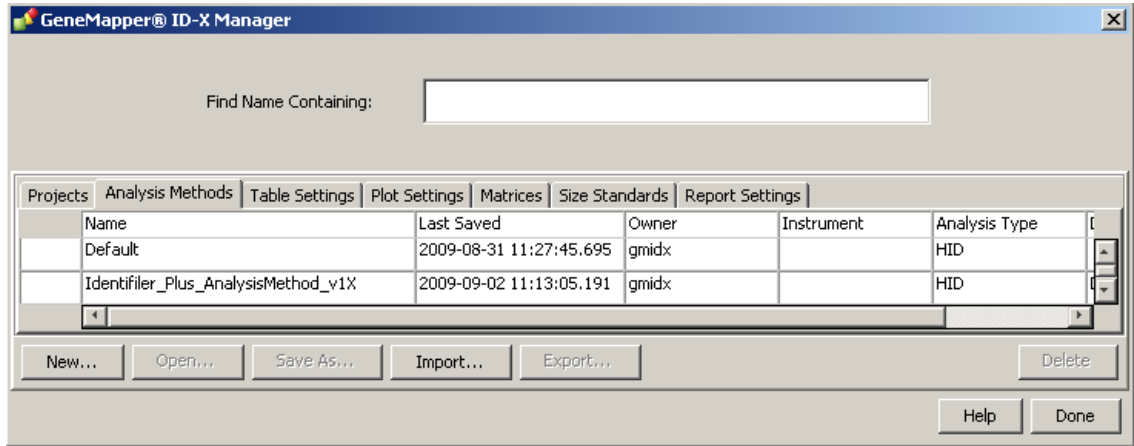
Figure 10   Analysis Method Editor: General tab settings

through below show the settings for each tab of the Analysis Method Editor.

GeneMapper® ID-X Software
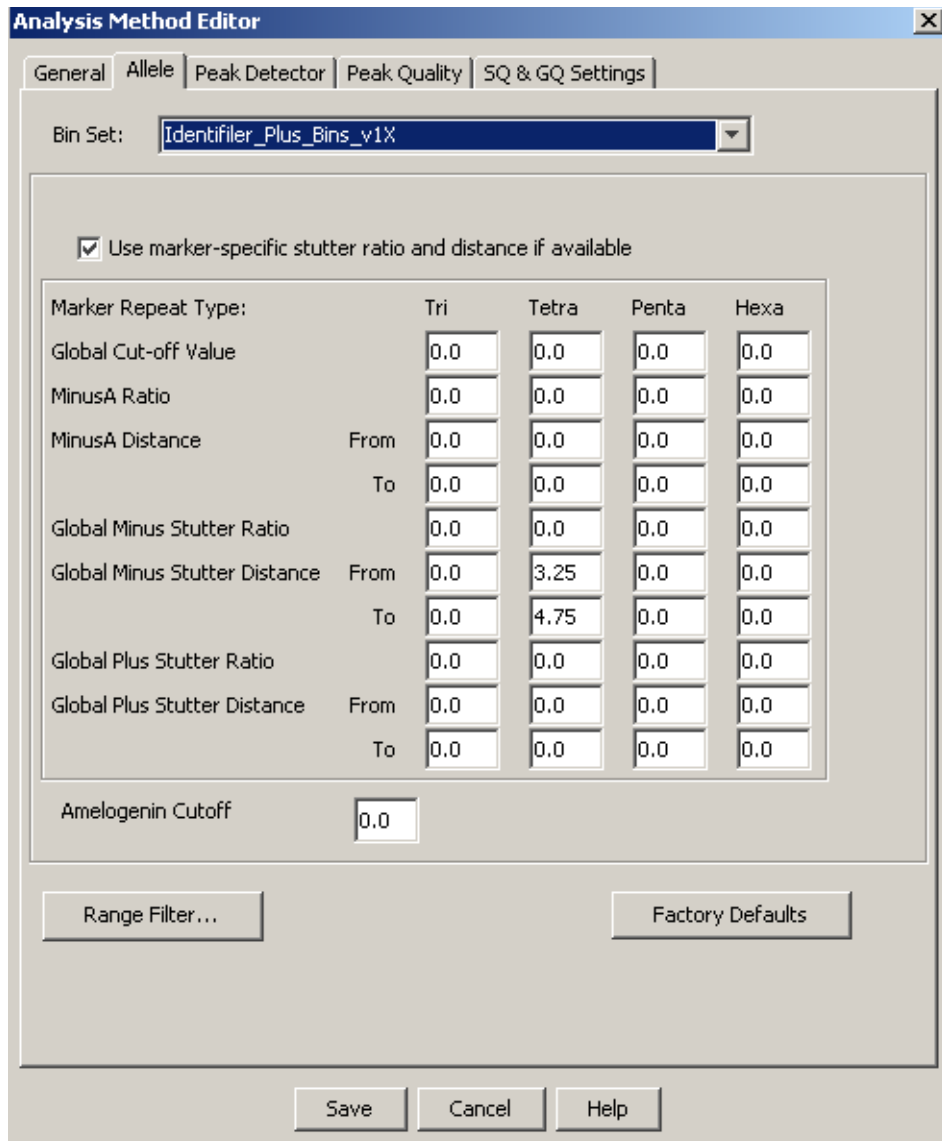
Figure 11   Analysis Method Editor: Allele tab settings

- GeneMapper® *ID-X* Software 1.0.1, 1.1, or 1.1.1 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 Identifiler_Plus_Stutter_v1X file.

For more information about allele filters, refer to:

- GeneMapper® *ID-X* Software *Version 1.0 Getting Started Guide* (PN 4375574)
- GeneMapper® *ID-X* Software *Version 1.0 Quick Reference Guide* (PN 4375670)
- GeneMapper® *ID-X* Software *Version 1.0 Reference Guide* (PN 4375671)
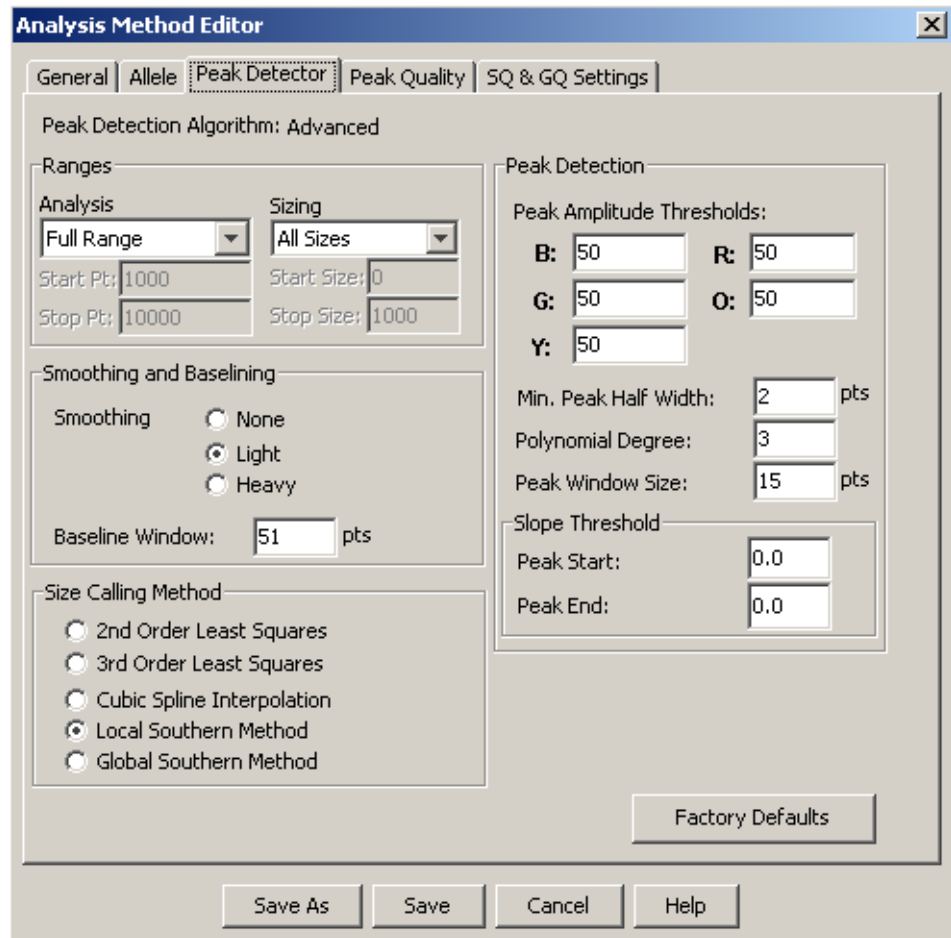
Figure 12   Analysis Method Editor: Peak Detector tab settings

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

IMPORTANT! Laboratories need to perform the appropriate internal validation studies to determine the peak amplitude thresholds that allow for reliable interpretation of AmpFℓSTR® Identifiler® Plus PCR Amplification Kit data.

For more information about peak detection algorithms, refer to:

- GeneMapper® *ID-X* Software *Version 1.0 Getting Started Guide* (PN 4375574)
- GeneMapper® *ID-X* Software *Version 1.0 Quick Reference Guide* (PN 4375670)
- GeneMapper® *ID-X* Software *Version 1.0 Reference Guide* (PN 4375671)

Figure 13   Analysis Method Editor: Peak Quality tab settings

IMPORTANT! Laboratories need to perform the appropriate internal validation studies to determine the minimum heterozygous and homozygous minimum peak height thresholds and the minimum peak height ratio threshold that allow for reliable interpretation of AmpF*l*STR® Identifiler® Plus PCR Amplification Kit data.
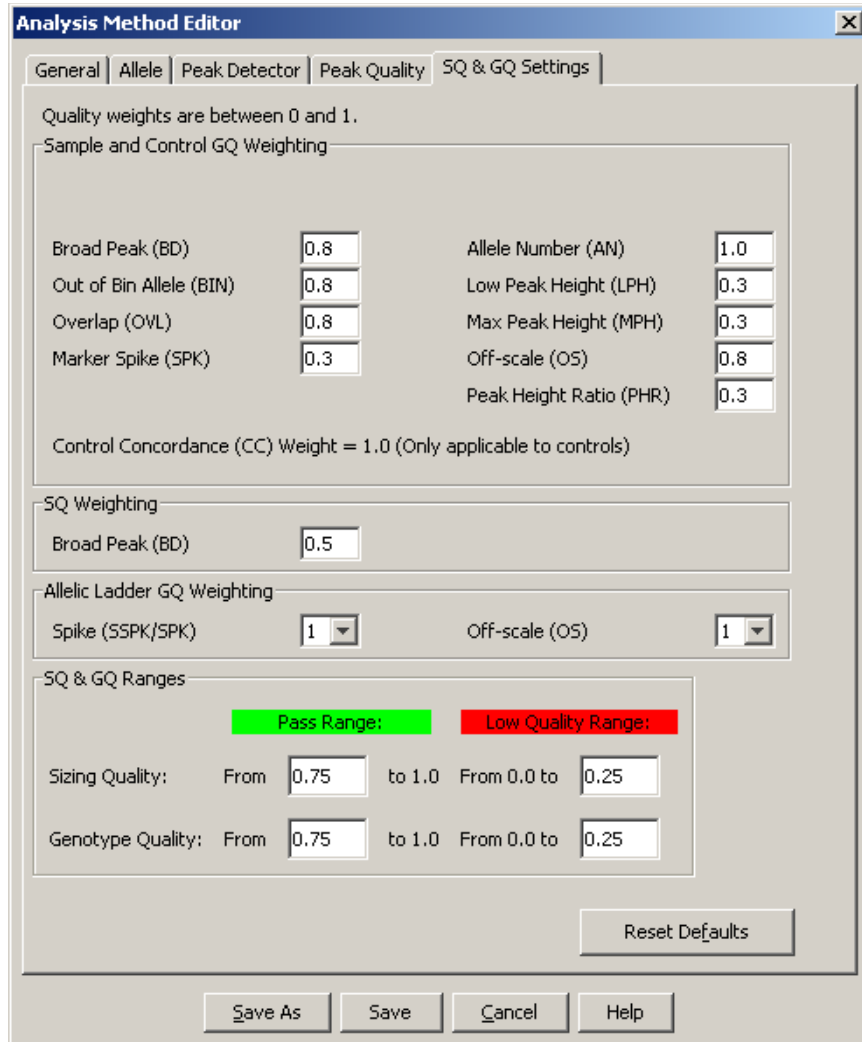
Figure 14   Analysis Method Editor: SQ and GQ tab settings

Import a HID size standard

The size standard for the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit uses the following GeneScan 500 LIZ size standard peaks in its sizing algorithm: 75, 100, 139, 150, 160, 200, 300, 350, 400, and 450.

Use the following procedure to import the size standard for the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit from the folder that you downloaded from the Applied Biosystems web site, into the GeneMapper® *ID-X* Software database. Refer to step 1 on page 52 for downloading instructions.

1.   Select **Tools ▸ GeneMapper® ID-X Manager** to open the GeneMapper® *ID-X* Manager.

2. Import a size standard:

    a. Select the **Size Standards** tab, then click **Import**.
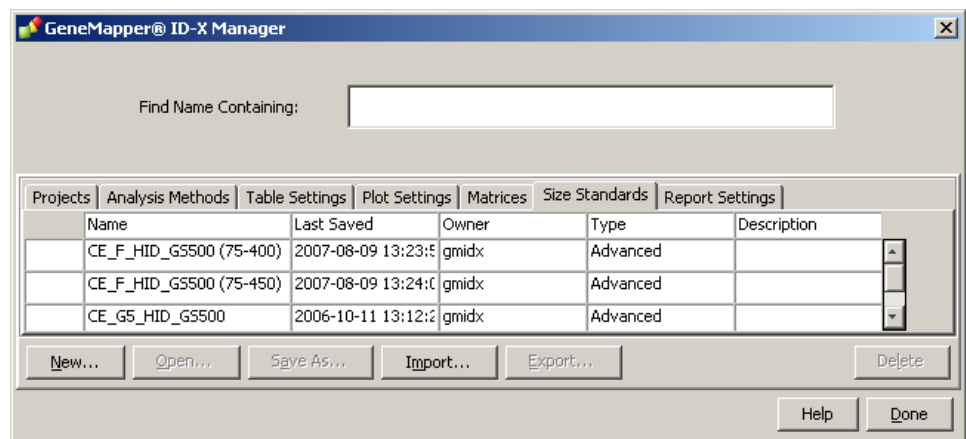
    b. Navigate to, then open the **Identifiler Plus Analysis Files GMIDX** folder.

    c. Select **CE_G5_Identifiler_Plus_GS500**, then click **Import** to import the CE_G5_Identifiler_Plus_GS500 analysis method into the GeneMapper® *ID-X* Software database.
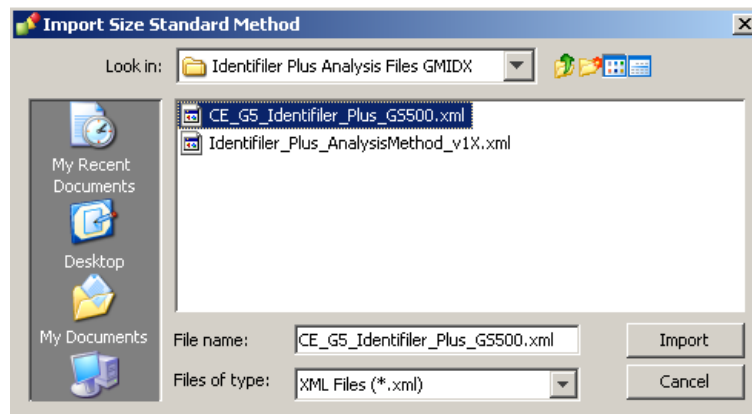
Analyze and edit sample files with GeneMapper® *ID-X* Software

Analyze 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	Settings
Sample Type	Select the sample type.
Analysis Method	Identifiler_Plus_AnalysisMethod_v1X
Panel	Identifiler_Plus_Panel_v1X
Size Standard‡	CE_G5_Identifiler_Plus_GS500§#

‡ 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_Identifiler_Plus_GS500 size standard provided with the AmpF*l*STR® Identifiler® Plus Kit: 75, 100, 139, 150, 160, 200, 300, 350, 400, and 450. For additional information about size standards, refer to the *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide* (PN 4338775), Appendix D.

Neither the 250-nt nor the 340-nt peak are included in the size standard definition. These peaks can be used as an indicator of precision within a run.

3. Click ▶ (Analyze), enter a name for the project (in the Save Project dialog box), then click **OK** to start analysis.

   - The status bar displays the progress of analysis as a completion bar extending to the right with the percentage completed indicated.

   - The table displays the row of the sample currently being analyzed in green (or red if analysis failed for the sample).

   - The Analysis Summary tab (see figure on next page) is displayed upon completion of the analysis.

GeneMapper® ID-X Software

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

- GeneMapper® *ID-X* Software *Version 1.0 Getting Started Guide* (PN 4375574)
- GeneMapper® *ID-X* Software *Version 1.0 Quick Reference Guide* (PN 4375670)
- GeneMapper® *ID-X* Software *Version 1.0 Reference Guide* (PN 4375671)

Examine and edit 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 Analysis Summary tab of the Project window (assuming the analysis is complete).

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

- *GeneMapper® ID-X Software Version 1.0 Getting Started Guide* (PN 4375574)
- *GeneMapper® ID-X Software Version 1.0 Quick Reference Guide* (PN 4375670)
- *GeneMapper® ID-X Software Version 1.0 Reference Guide* (PN 4375671)

Experiments and Results

5

This chapter covers:

Overview

Experiments using the AmpF*l*STR® Identifiler® Plus Kit

This chapter provides results of the developmental validation experiments performed by Applied Biosystems using the AmpF*l*STR® Identifiler® Plus PCR Amplification 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 which 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).

Experiment conditions

Experiments to evaluate the performance of the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit were performed at Applied Biosystems. The 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 conducted 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 optimum PCR product yield and that met reproducible performance standards.While these experiments are not exhaustive, they are appropriate for a manufacturer of STR kits intended for forensic and/or parentage testing use.

IMPORTANT! Each laboratory using the AmpF*l*STR® Identifiler® 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 thermal cycling 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 AmpF*ℓ*STR® Identifiler® 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 1.0 ng of the Control DNA 9947A for 28-cycles is shown in Figure 15. Applied Biosystems observed that the performance of the multiplex is robust within a ±30% window of the optimum magnesium chloride concentration.
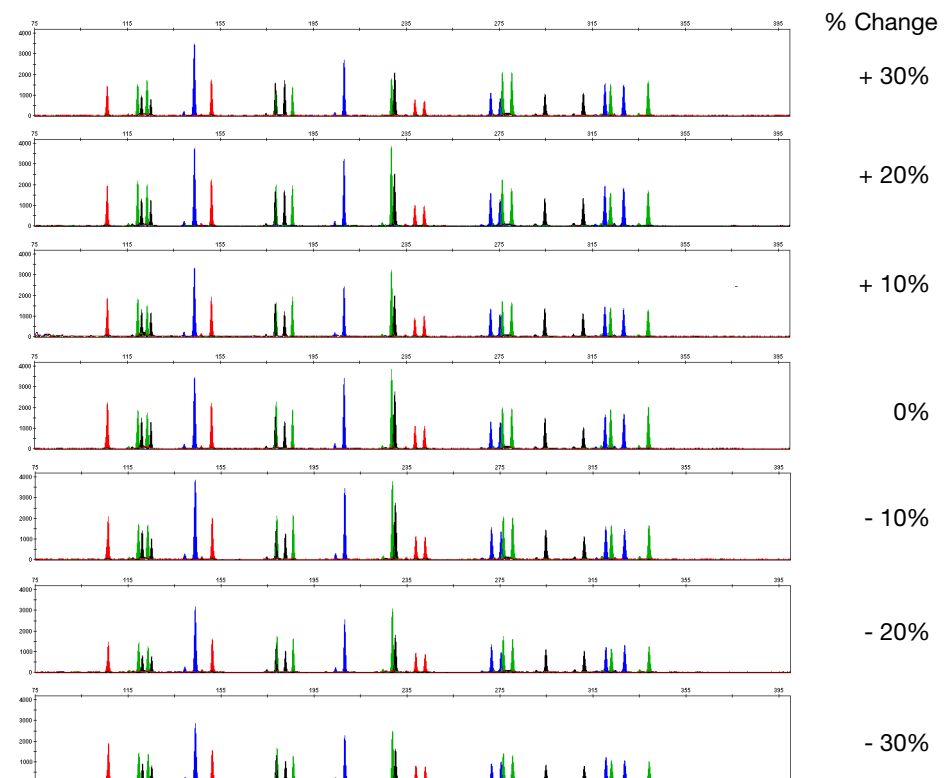
Figure 15   1.0 ng of Control DNA 9947A amplified with the AmpF*ℓ*STR® Identifiler® Plus Kit for 28-cycles in the presence of varying concentrations of magnesium chloride and analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer

Thermal cycler parameters

Thermal cycling parameters were established for amplification of the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit. Thermal cycling times and temperatures of GeneAmp® PCR systems were verified. Varying annealing/extension and denaturation temperature windows were tested to verify that a specific PCR product with the desired sensitivity of at least 1.0 ng of AmpF*l*STR Control DNA 9947A was produced.

For example, annealing/extension temperatures were tested at 55, 57, 59, 61, and 63 °C for 3-minute hold times in the Silver 96-Well GeneAmp® PCR System 9700 (Figure 16). The PCR products were analyzed using the Applied Biosystems 3130*xl* Genetic Analyzer.

Of the tested annealing/extension 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/extension temperature of 59 °C.
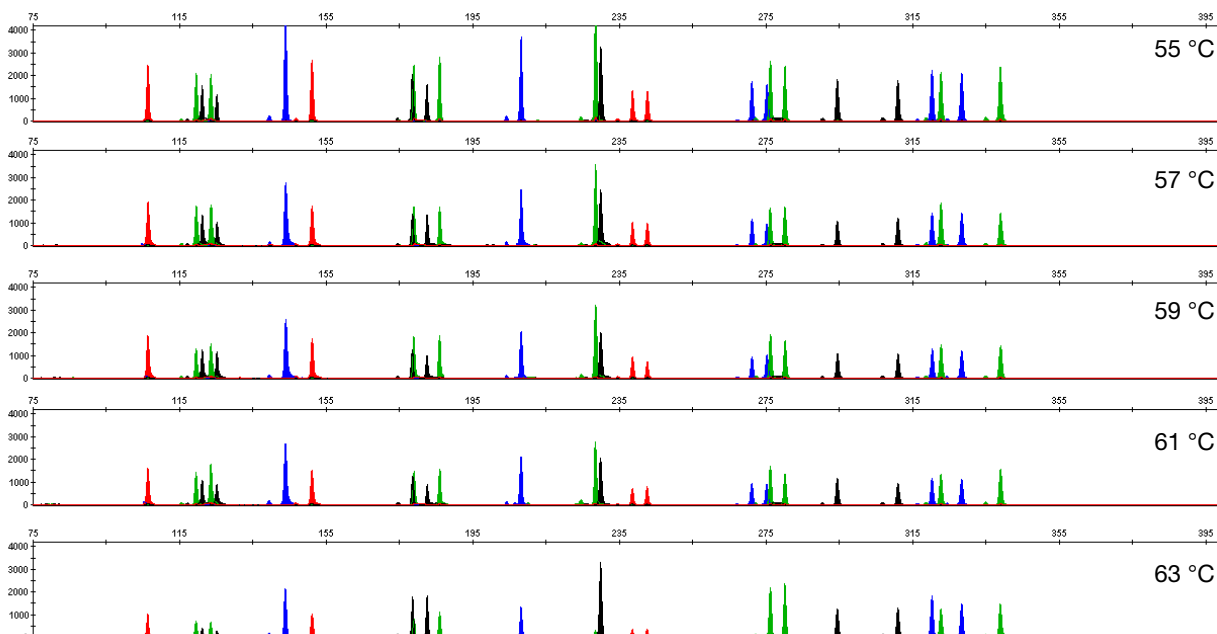
Figure 16 Electropherograms obtained from amplification of 1.0 ng of Control DNA 9947A at annealing/extension temperatures of 55 °C, 57 °C, 59 °C, 61 °C, and 63 °C, analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer, Y-axis scale (0 to 4,000 RFUs)

PCR cycle number

AmpF*l*STR® Identifiler® Plus PCR Amplification Kit reactions were amplified for 26, 27, 28, 29, and 30 cycles on the Silver 96-Well GeneAmp® PCR System 9700 using 1.0 ng from three DNA samples. As expected, the amount of PCR product increased with the number of cycles. A full profile was generated at 26 cycles and off-scale data were collected for several allele peaks at 30 cycles (Figure 17).

Although none of the cycle numbers tested produced nonspecific peaks, 28 cycles was found to give optimal peak heights with 1 ng of DNA input when the amplified products were examined on Applied Biosystems 3130*xl* Genetic Analyzers.
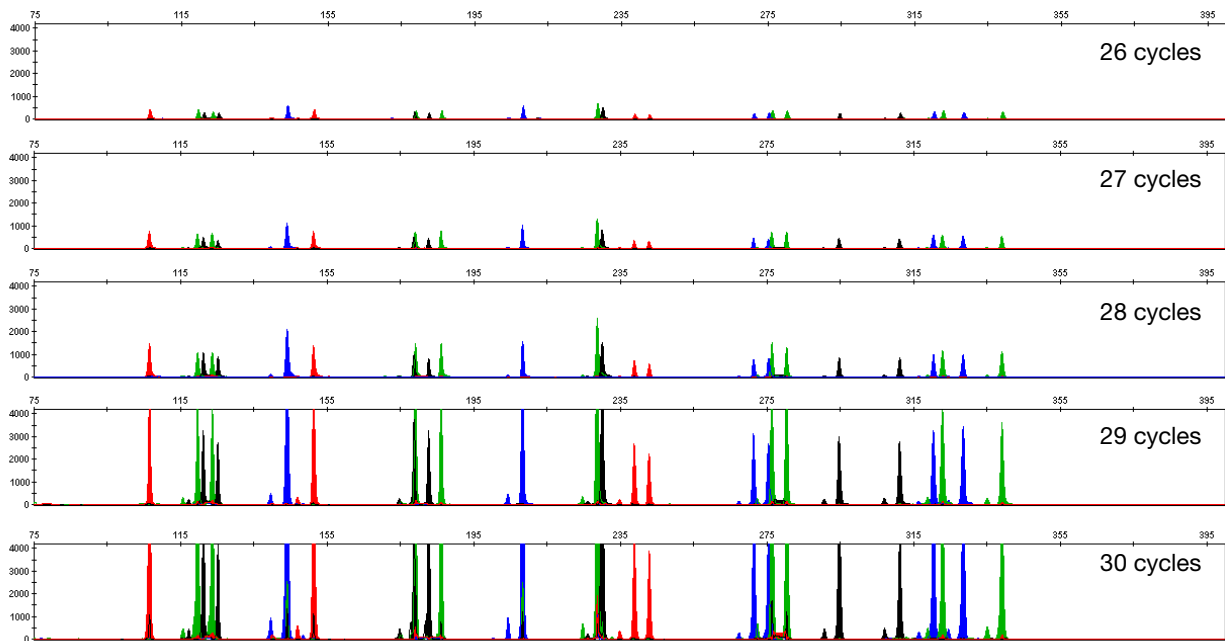
Figure 17   Representative AmpF*l*STR® Identifiler® Plus Kit profiles obtained from amplification of 1.0 ng DNA template using 26, 27, 28, 29, and 30 cycles, analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer, Y-axis scale, (0 to 4,000 RFUs)

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 AmpF*l*STR Identifiler® Plus profiles have been determined from various sample types. Figure 18 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 18 represents the nominal nucleotide sizes for the AmpF*l*STR Identifiler® 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.
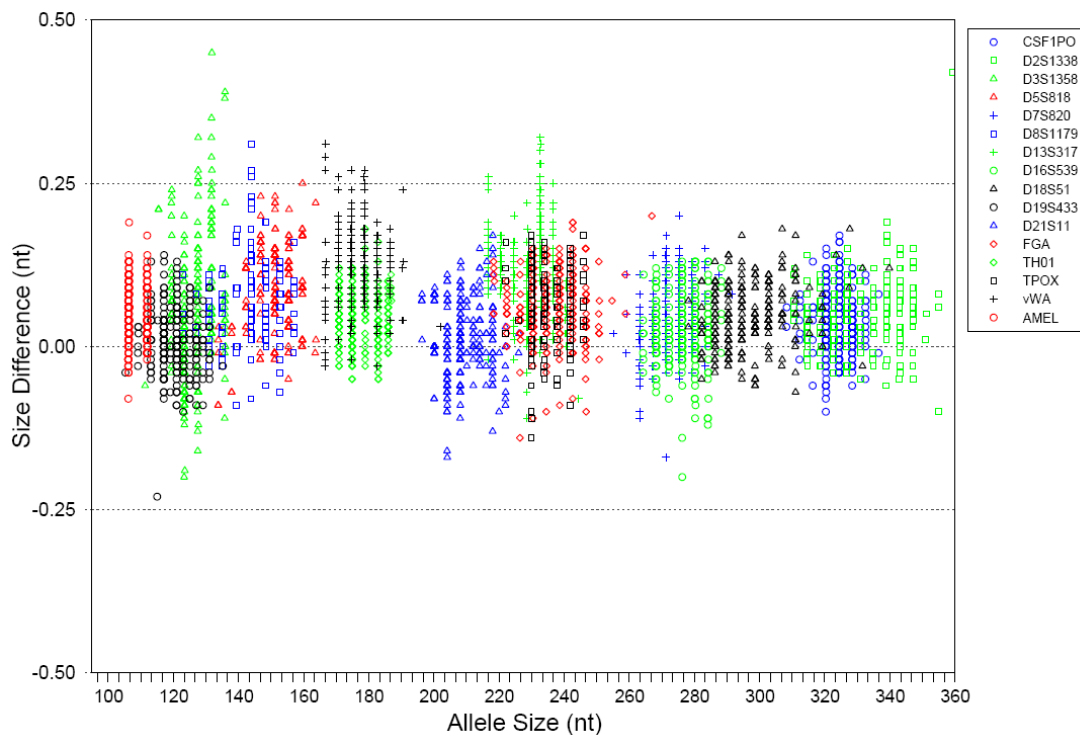
Figure 18   Size deviation of 200 samples analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer. For each sample, 1.0 ng of DNA was amplified for 28 PCR cycles.

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 AmpF*l*STR Identifiler® 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 AmpF*l*STR Identifiler® 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 4 on page 75 shows typical precision results obtained from five runs (16 capillaries/run) of the AmpF*l*STR® Identifiler® 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™ 500 LIZ® Size Standard. The results were obtained within a set of injections on a single capillary array.

Sample alleles may occasionally size outside of 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 18 on page 73 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 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.

GeneMapper® *ID* Software and GeneMapper® *ID-X* Software automatically flag sample alleles that do not size within the prescribed window around an allelic ladder allele by labelling the allele as OL (off-ladder).

Maximum precision is obtained with a set of capillary injections on each of the supported platforms however the determined allele sizes will vary between the different platforms. Cross-platform sizing differences occur from a number of factors including type and concentration of polymer, run temperature, and electrophoresis conditions. Variations in sizing can also occur between runs on the same instrument and between runs on different instruments of the same platform type because of these factors.

Applied Biosystems strongly recommends that the allele sizes be compared to the sizes obtained for known alleles in the AmpF*l*STR® Identifiler® Plus Allelic Ladder from the same run and then be converted to genotypes (as described in "Before you start" on pages 37 and 51). See Table 4 for the results of five runs of the AmpF*l*STR® Identifiler® Plus Allelic Ladder. For more information on precision and genotyping, see Lazaruk *et al.,* 1998 and Mansfield *et al.*, 1998.

In Table 4, the mean sizes for all the alleles in each run (16 capillaries) were calculated. The mean range shown in the table represents the lowest- and highest-mean size values obtained across all 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 4 represents the lowest and highest standard deviation values obtained across all five runs.

Table 4   Precision results of five runs (16 capillaries/run) of the AmpF*l*STR® Identifiler® Plus Allelic Ladder

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
Amelogenin		
X	106.03–106.13	0.033–0.045
Y	111.69–111.8	0.03–0.042
CSF1PO		
6	303.99–304.12	0.041–0.063
7	308.04–308.17	0.037–0.058
8	312.1–312.2	0.039–0.065
9	316.13–316.25	0.035–0.045
10	320.18–320.3	0.034–0.055
11	324.24–324.34	0.03–0.046
12	328.3–328.39	0.025–0.047
13	332.36–332.44	0.032–0.037
14	336.39–336.49	0.024–0.039
15	340.42–340.53	0.038–0.05
D13S317		
8	216.36–216.48	0.031–0.064
9	220.34–220.48	0.035–0.051
10	224.32–224.45	0.034–0.059
11	228.31–228.45	0.031–0.065
12	282.42–32.55	0.031–0.063
13	236.3–236.43	0.038–0.066
14	240.24–240.37	0.043–0.058
15	244.23–244.37	0.037–0.066

Table 4   Precision results of five runs (16 capillaries/run) of the AmpF*l*STR® Identifiler® Plus Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
D16S539		
5	252.01–252.15	0.05–0.06
8	264–264.15	0.05–0.061
9	268–268.14	0.05–0.063
10	272–272.15	0.045–0.059
11	276.02–276.17	0.04–0.064
12	280.03–280.18	0.039–0.067
13	284.05–284.22	0.045–0.06
14	288.08–288.23	0.044–0.054
15	292.12–292.26	0.038–0.059
D18S51		
7	261.8–261.9	0.037–0.049
9	269.94–270.03	0.037–0.051
10	274.02–274.12	0.043–0.051
10.2	276.03–276.13	0.037–0.048
11	278.11–278.22	0.042–0.059
12	282.2–282.29	0.037–0.046
13	286.29–286.39	0.039–0.051
13.2	288.29–288.4	0.034–0.052
14	290.38–290.49	0.032–0.051
14.2	292.39–292.5	0.043–0.057
15	294.48–294.59	0.029–0.049
16	298.57–298.69	0.035–0.053
17	302.69–302.81	0.039–0.056
18	306.83–306.95	0.039–0.056
19	310.96–311.07	0.034–0.049
20	315.08–315.18	0.036–0.045
21	319.2–319.31	0.036–0.045
22	323.39–323.5	0.03–0.044
23	327.46–327.53	0.025–0.052
24	331.59–331.65	0.026–0.043
25	335.69–335.76	0.032–0.043
26	339.8–339.88	0.036–0.047
27	343.87–343.99	0.031–0.048

Table 4   Precision results of five runs (16 capillaries/run) of the
AmpF*l*STR® Identifiler® Plus Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
D19S433		
9	101.25–101.34	0.022–0.034
10	105.16–105.25	0.028–0.037
11	109.09–109.17	0.021–0.033
12	113.04–113.12	0.024–0.036
12.2	115.06–115.13	0.027–0.035
13	117.02–117.09	0.026–0.036
13.2	119.03–119.1	0.027–0.038
14	121.02–121.07	0.025–0.038
14.2	123.05–123.1	0.028–0.037
15	125.03–125.09	0.03–0.041
15.2	127.08–127.13	0.027–0.04
16	129.08–129.13	0.031–0.039
16.2	131.13–131.19	0.023–0.042
17	133.16–133.21	0.034–0.046
17.2	135.23–135.28	0.034–0.041
D21S11		
24	184.41–184.46	0.024–0.042
24.2	186.39–186.45	0.025–0.04
25	188.35–188.4	0.024–0.038
26	192.27–192.34	0.027–0.035
27	196.21–196.28	0.024–0.039
28	200.06–200.13	0.026–0.039
28.2	202.03–202.1	0.026–0.036
29	204.02–204.09	0.025–0.044
29.2	206.08–206.14	0.027–0.041
30	208.06–208.11	0.028–0.04
30.2	210.03–210.09	0.031–0.037
31	212.04–212.12	0.031–0.037
31.2	214.03–214.1	0.023–0.04
32	216.04–216.11	0.028–0.042
32.2	218.03–218.09	0.023–0.0351
33	220.05–220.1	0.031–0.043
33.2	221.98–222.05	0.033–0.038
34	224.12–224.18	0.024–0.033
34.2	226.03–226.09	0.029–0.041

Table 4   Precision results of five runs (16 capillaries/run) of the
AmpF*l*STR® Identifiler® Plus Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
35	228.1–228.17	0.03–0.048
35.2	230.03–230.09	0.03–0.037
36	232.02–232.09	0.03–0.047
37	236.08–236.17	0.026–0.041
38	240.04–240.1	0.033–0.045
D2S1338		
15	306.27–306.39	0.033–0.058
16	310.35–310.47	0.031–0.055
17	314.39–314.53	0.029–0.042
18	318.45–318.58	0.029–0.046
19	322.52–322.63	0.025–0.046
20	326.58–326.67	0.029–0.039
21	330.66–330.74	0.034–0.045
22	334.71–334.8	0.031–0.043
23	338.74–338.85	0.026–0.045
24	342.75–342.89	0.026–0.05
25	346.78–346.92	0.026–0.051
26	350.77–350.89	0.028–0.049
27	354.69–354.81	0.026–0.045
28	358.87–359.01	0.028–0.045
D3S1358		
12	111.12–111.22	0.024–0.047
13	115.23–115.32	0.03–0.046
14	119.2–119.31	0.03–0.044
15	123.14–123.22	0.031–0.045
16	127.32–127.41	0.032–0.042
17	131.54–131.62	0.027–0.039
18	135.64–135.71	0.021–0.042
19	139.72–139.81	0.024–0.045
D5S818		
7	133.69–133.75	0.029–0.039
8	137.8–137.86	0.031–0.037
9	142.17–142.24	0.022–0.035
10	146.64–146.71	0.025–0.039
11	151.05–151.12	0.031–0.043
12	155.32–155.39	0.028–0.041

Table 4   Precision results of five runs (16 capillaries/run) of the AmpF*l*STR® Identifiler® Plus Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
13	159.55–159.61	0.027–0.051
14	163.63–163.7	0.032–0.038
15	167.68–167.74	0.026–0.051
16	171.7–171.76	0.035–0.04
D7S820		
6	255.08–255.19	0.029–0.058
7	259.13–259.22	0.04–0.056
8	263.16–263.25	0.037–0.053
9	267.19–267.29	0.046–0.053
10	271.25–271.34	0.039–0.051
11	275.28–275.4	0.037–0.06
12	279.34–279.45	0.034–0.05
13	283.38–283.49	0.039–0.049
14	287.44–287.54	0.039–0.051
15	291.51–291.62	0.043–0.052
D8S1179		
8	122.49–122.61	0.03–0.044
9	126.56–126.68	0.037–0.045
10	130.66–130.76	0.026–0.044
11	134.8–134.89	0.031–0.041
12	138.98–139.09	0.019–0.043
13	143.58–143.68	0.028–0.042
14	148.03–148.14	0.03–0.046
15	152.43–152.54	0.025–0.043
16	156.73–156.83	0.026–0.039
17	160.93–161.04	0.031–0.042
18	165.03–165.12	0.024–0.046
19	169.1–169.2	0.035–0.044
FGA		
17	214.11–214.23	0.041–0.05
18	218.14–218.26	0.043–0.052
19	222.17–222.3	0.039–0.054
20	226.21–226.35	0.044–0.057
21	230.26–230.38	0.045–0.055
22	234.29–234.42	0.05–0.058
23	238.33–238.47	0.038–0.057'

Table 4   Precision results of five runs (16 capillaries/run) of the
AmpF*l*STR® Identifiler® Plus Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
24	242.37–242.52	0.044–0.067
25	246.42–246.57	0.044–0.056
26	250.48–250.62	0.038–0.069
26.2	252.49–252.64	0.046–0.066
27	254.5–254.65	0.047–0.057
28	258.55–258.71	0.045–0.064
29	262.63–262.78	0.049–0.062
30	266.72–266.88	0.052–0.069
30.2	268.53–268.7	0.049–0.065
31.2	272.62–272.78	0.036–0.062
32.2	276.71–276.86	0.05–0.068
33.2	280.77–280.94	0.043–0.069
42.2	317.89–318.06	0.045–0.062
43.2	322.01–322.16	0.038–0.055
44.2	326.14–326.27	0.034–0.05
45.2	330.28–330.39	0.039–0.048
46.2	334.28–334.4	0.044–0.05
47.2	338.37–338.49	0.039–0.055
48.2	342.51–342.66	0.034–0.055
50.2	350.59–350.76	0.041–0.061
51.2	354.54–354.7	0.039–0.063
TH01		
4	162.72–162.77	0.025–0.04
5	166.78–166.84	0.027–0.035
6	170.82–170.87	0.03–0.046
7	174.83–174.9	0.029–0.045
8	178.84–178.9	0.02–0.046
9	182.82–182.89	0.027–0.034
9.3	185.84–185.9	0.022–0.042
10	186.77–186.83	0.026–0.036
11	190.71–190.77	0.027–0.034
12	201.48–201.55	0.026–0.037
TPOX		
6	221.82–221.91	0.029–0.05
7	225.8–225.88	0.029–0.053
8	229.79–229.86	0.034–0.048

Table 4   Precision results of five runs (16 capillaries/run) of the
AmpF*l*STR® Identifiler® Plus Allelic Ladder *(continued)*

Applied Biosystems 3130*xl* Genetic Analyzer		
Allele	**Mean**	**Standard Deviation**
9	233.77–233.86	0.039–0.048
10	237.76–237.83	0.025–0.053
11	241.75–241.83	0.028–0.04
12	245.78–245.85	0.04–0.049
13	249.76–249.85	0.036–0.048
vWA		
11	154.07–154.14	0.028–0.042
12	158.26–158.34	0.028–0.04
13	162.42–162.49	0.031–0.043
14	166.66–166.73	0.031–0.05
15	170.59–170.66	0.029–0.044
16	174.62–174.68	0.03–0.04
17	178.61–178.67	0.028–0.051
18	182.54–182.61	0.021–0.037
19	186.5–186.56	0.024–0.043
20	190.43–190.49	0.028–0.04
21	194.29–194.36	0.024–0.044
22	198.17–198.24	0.023–0.036
23	202.01–202.09	0.028–0.041
24	206.36–206.42	0.034–0.041

Extra peaks in the electropherogram

Causes of extra peaks

Peaks other than the target alleles may be detected on the electropherogram. Causes for the appearance of 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 one repeat unit smaller (or less frequently, one repeat larger) than the major STR product (Butler, 2005 and 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 samples (n = 500) amplified using the 28-cycle-protocol (DNA input ~1ng) at the loci used in the Identifiler® Plus Kit. All data were generated on the Applied Biosystems 3130*x*l Genetic Analyzer.

Some conclusions from these measurements and observations are:

- For each AmpF*ℓ*STR® Identifiler® Plus Kit locus, the percent stutter generally increases with allele length, as shown in Figure 19 to Figure 22 on pages 83 through 85.

- 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 with other alleles in the locus.

- The stutter value for each locus shown in Table 5 on page 85 was determined by taking the mean plus three times the standard deviation. These values are the stutter filter percentages in the Identifiler® Plus stutter file; they will be used during the filtering step in the GeneMapper® ID Software v3.2.1 or GeneMapper® ID-X Software v1.0.1, v1.1, or v1.1.1. Peaks in the stutter position that are above the stutter filter percentage will not be 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 30 on page 99.

- The percent stutter cannot be accurately measured for allele peaks that are off-scale and may appear unusually high relative to the main peak.
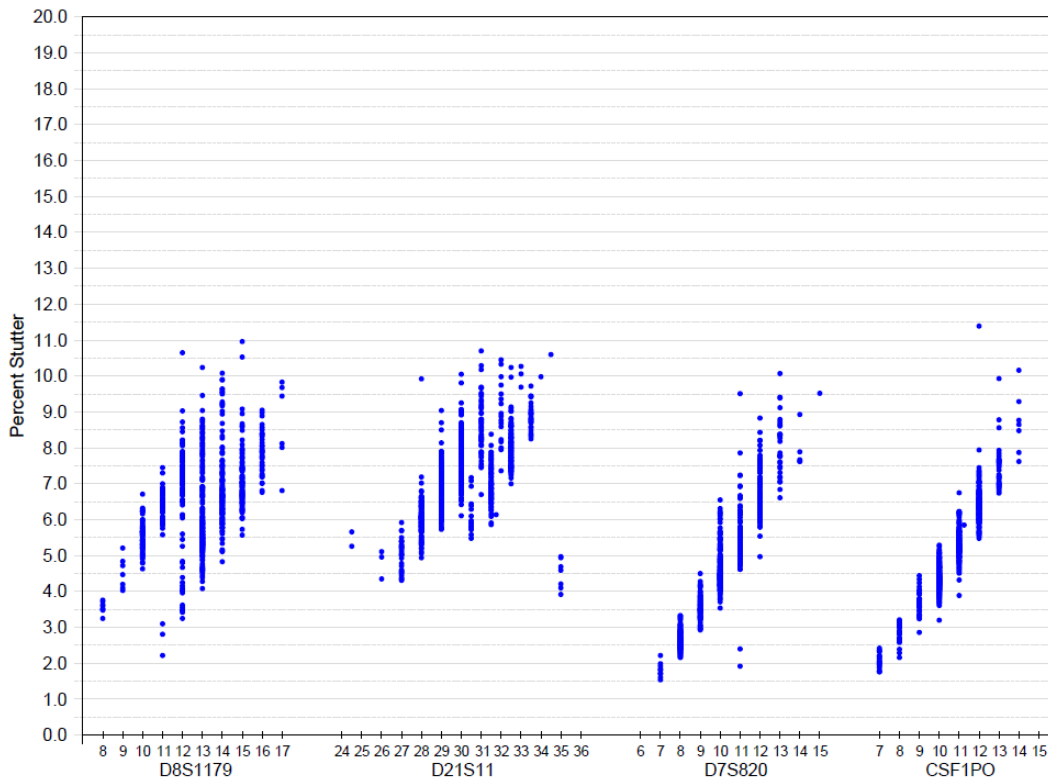
Figure 19   Stutter percentages for the D8S1179, D21S11, D7S820, and CSF1PO loci
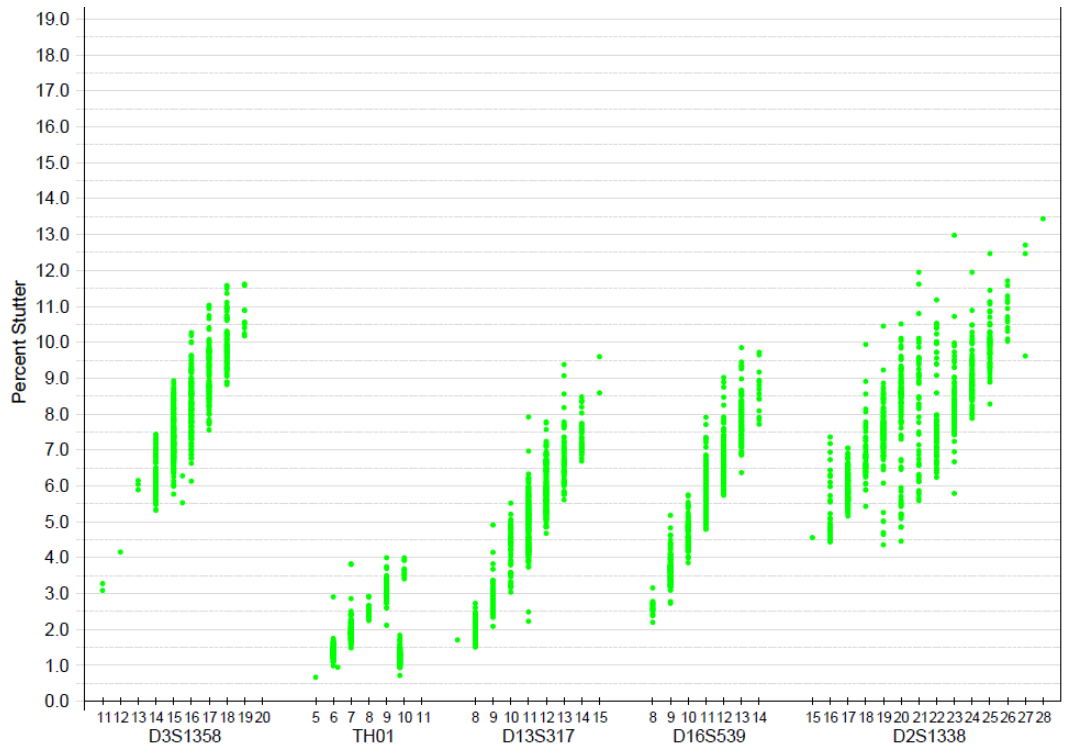
Figure 20   Stutter percentages for the D3S1358, TH01, D13S317, D16S539, and D2S1338 loci
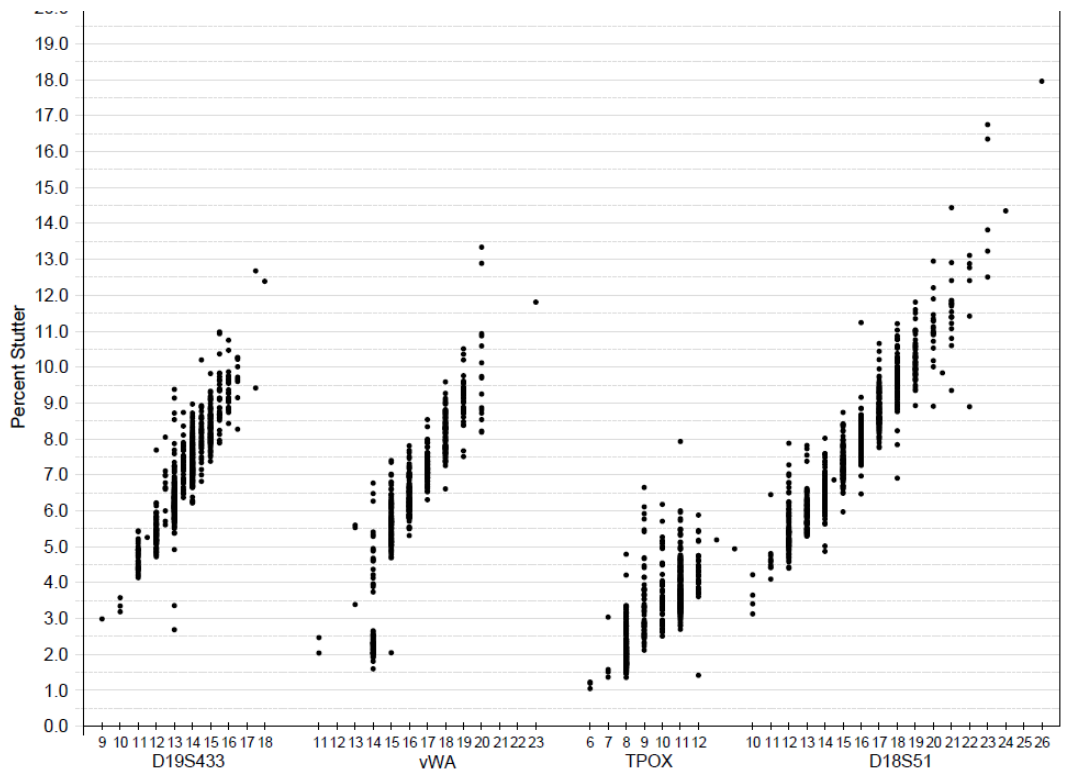
Figure 21   Stutter percentages for the D19S443, vWA, TPOX, and D18S51 loci
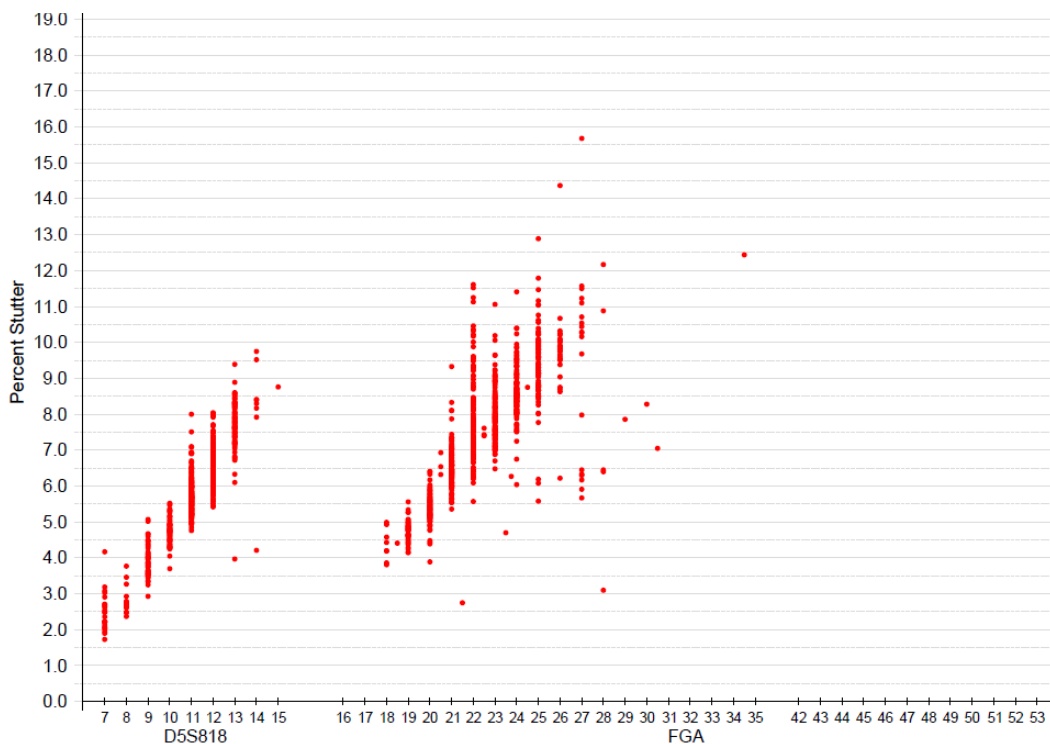
Figure 22   Stutter percentages for the D5S818 and FGA loci

**Table 5   Marker-specific stutter filter percentages for AmpF*ℓ*STR®
Identifiler® Plus PCR Amplification Kit loci‡**

Locus	% Stutter
CSF1PO	9.2041
D13S317	9.9348
D16S539	10.3945
D18S51	13.6799
D19S433	11.2096
D21S11	10.6714
D2S1338	12.4409
D3S1358	12.2719
D5S818	10.0599
D7S820	9.6926
D8S1179	10.3155
FGA	13.028
TH01	4.0813

Table 5   Marker-specific stutter filter percentages for AmpF*ℓ*STR®
Identifiler® Plus PCR Amplification Kit loci‡ *(continued)*

Locus	% Stutter
TPOX	6.3832
vWA	12.446

‡ These percentages are used as stutter filters in used in GeneMapper *ID* v3.2.1
   Identifiler_Plus_Panels_v1 and GeneMapper® *ID-X* software v1.0.1, v1.1, or v1.1.1
   Identifiler_Plus_Panels_v1x.

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 and 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 Identifiler® Plus Kit includes two main 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 10 min.

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."
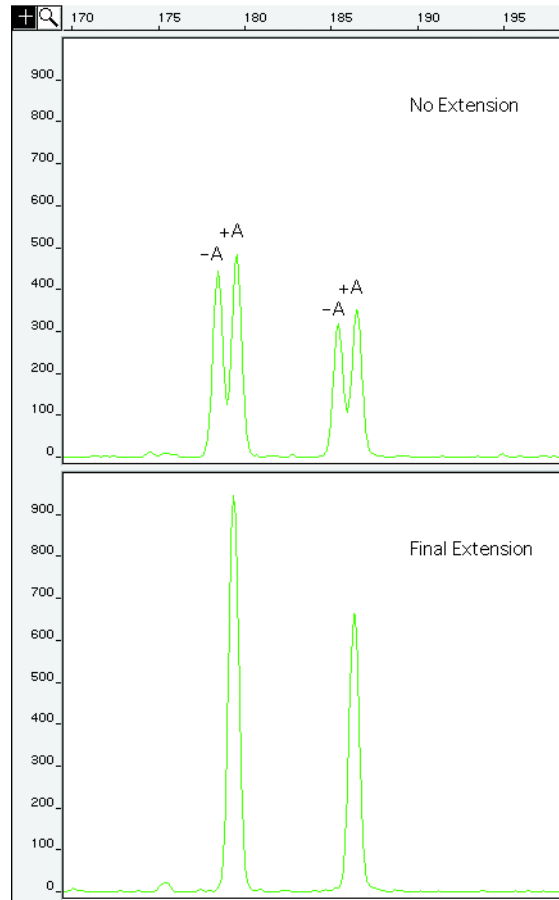
Figure 23   Omitting the final extension step results in split peaks due to incomplete A nucleotide addition. Data are from an ABI PRISM® 310 Genetic Analyzer using another AmpFℓSTR kit.

Lack of complete +A nucleotide addition may be observed in AmpFℓSTR® Identifiler® Plus PCR Amplification 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 while anomalies are non-reproducible, intermittent occurrences that are not observed consistently in a system (for example, spikes and baseline noise). Reproducible artifacts have not been seen in data produced, with either 28 or 29 cycles of amplification, on the genetic analyzers used during developmental validation of the Identifiler® Plus Kit.

However, you should consider possible noise and artifacts when interpreting data from the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit on the Applied Biosystems 3130/3130*xl*, ABI PRISM® 3100/3100-*Avant*, and ABI PRISM® 310 Genetic Analyzers.

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 15 loci and the sex-determining marker, Amelogenin, which are amplified with the AmpF*l*STR® Identifiler® Plus Kit. These loci have been extensively characterized by other laboratories.

Nature of the polymorphisms

The primers for the Amelogenin locus flank a 6-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 AmpF*l*STR® Identifiler® Plus Kit loci 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.

All the alleles in the AmpF*l*STR® Identifiler® Plus Allelic Ladder, including microvariants, have been subjected to sequencing at Applied Biosystems. In addition, other groups in the scientific community have sequenced alleles at some of these loci (Nakahori *et al.,* 1991; Puers *et al.,* 1993; Möller *et al.,* 1994; Barber *et al.,* 1995; Brinkmann and Möller, 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 AmpF*l*STR® Identifiler® 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).

Three CEPH family DNA sets were examined. One nanogram of DNA from each sample was amplified using the Identifiler® Plus Kit, followed by analysis using an Applied Biosystems 3130*xl* Genetic Analyzer. The families examined included #1333 (9 offspring), #1340 (7 offspring), and #1345 (7 offspring), representing 23 meiotic divisions.

In family #1340, we observed two parent/offspring pairs with mutations at locus D8S1179. The genotypes differed by one repeat unit between the two generations. Calculation of a mutation rate based on these data would be inaccurate due to the small sample size. The other parent-offspring allele transfers were in accordance with Mendelian rules.

Mapping
: The Identifiler® Plus Kit loci Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX, and vWA have been mapped, and the chromosomal locations have been published (Nakahori *et al.,* 1991; Edwards *et al.,* 1992; Kimpton *et al.,* 1992; Kong et al., 2004; 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® Identifiler® 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 AmpFℓSTR® Identifiler® Plus PCR Amplification Kit experiments on nonhuman DNA sources are shown in Figure 24 on page 90.

Figure 24 shows amplification for: Control DNA 9947A (1.0 ng, panel 1), chimpanzee (1.0 ng, panel 2), dog (10 ng. panel 3), cat (10 ng, panel 4), horse (10 ng, panel 5), microbial DNA pool (equivalent to 105 copies of *Candida albicans*, *Staphylococcus aureus*, *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 AmpFℓSTR® Identifiler® Plus PCR Amplification Kit and analyzed using the Applied Biosystems 3130*xl* Genetic Analyzer.

- Primates: gorilla, chimpanzee, orangutan, and macaque (1.0 ng each)
- Non-primates: mouse, dog, sheep, rabbit, cat, horse, hamster, rat, chicken, and cow (10 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 350 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 PET® dye.
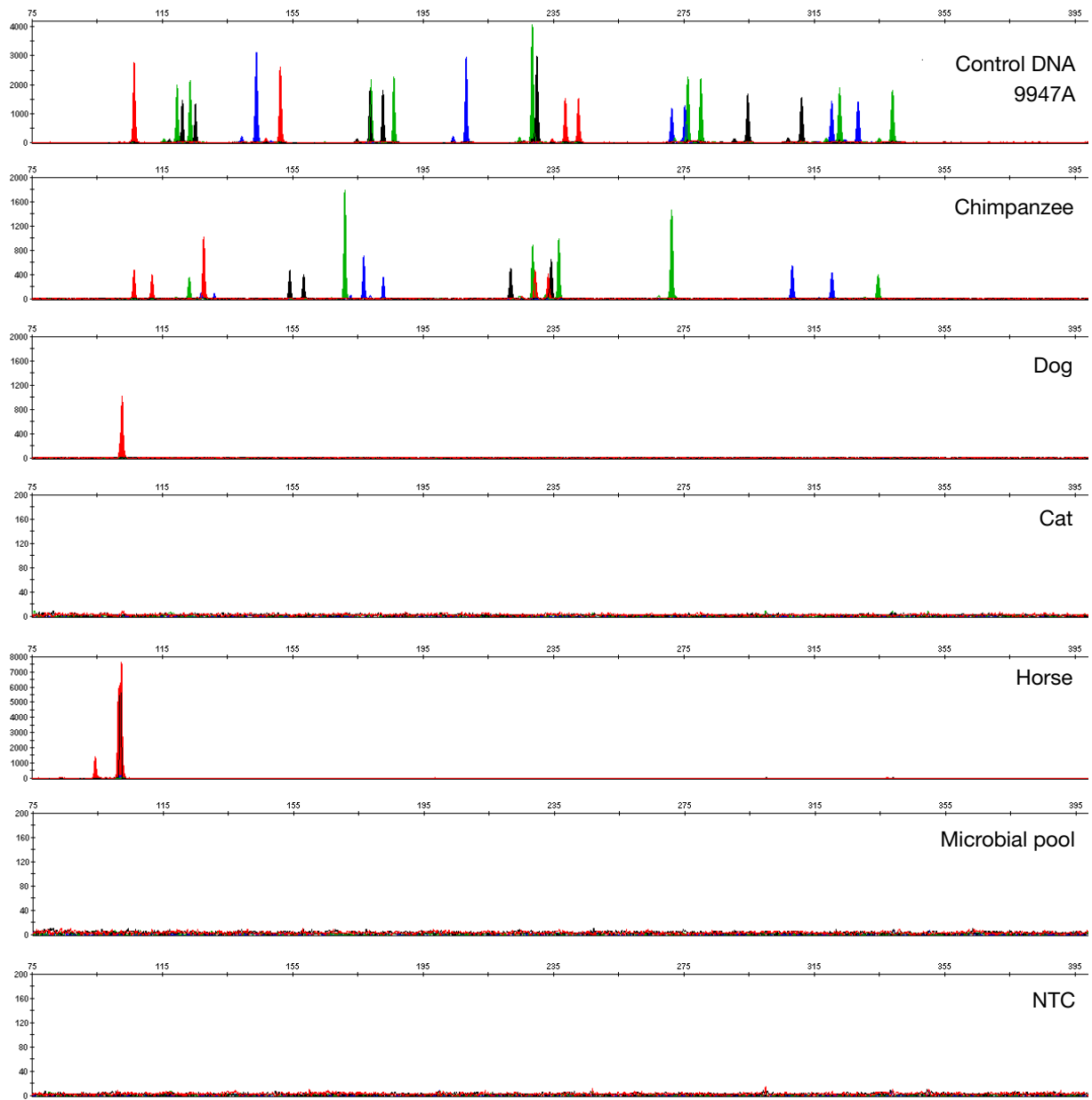
Figure 24   Representative electropherograms from a species specificity study including positive and non-template controls (NTC) run for 28 PCR cycles

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 AmpF*l*STR® Identifiler® Plus PCR Amplification Kit should be between 0.75 and 1.0 ng for 28-cycle amplification. 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 0.075 to 0.1 ng/µL so that 0.75 to 1.0 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 Figures 25 and 26, the control DNA 9947A was serially diluted from 1 ng to 0.031 ng. With the 28-PCR-cycle protocol, full profiles (26 alleles) 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. With the 29-PCR-cycle protocol, full profiles (26 alleles) were consistently obtained at 0.062 ng, but occasional partial profiles that are missing anywhere from 1 to 5 alleles were observed at 0.031 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 because of 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. For information on selecting the appropriate cycle number for your DNA input amount, see "Select the appropriate PCR cycle number" on page 20.

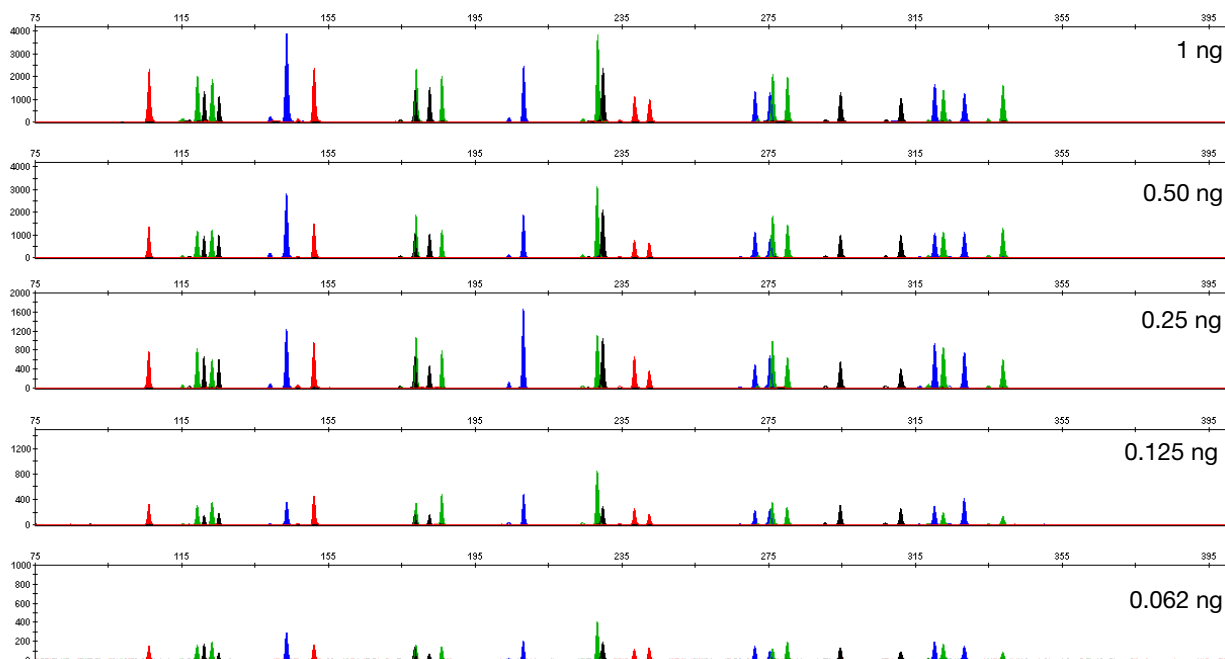**Figure 25   Effect of amplifying 1 ng, 0.50 ng, 0.25 ng, 0.125 ng, and 0.062 ng of Control DNA 9947A using the 28-PCR-cycle protocol**
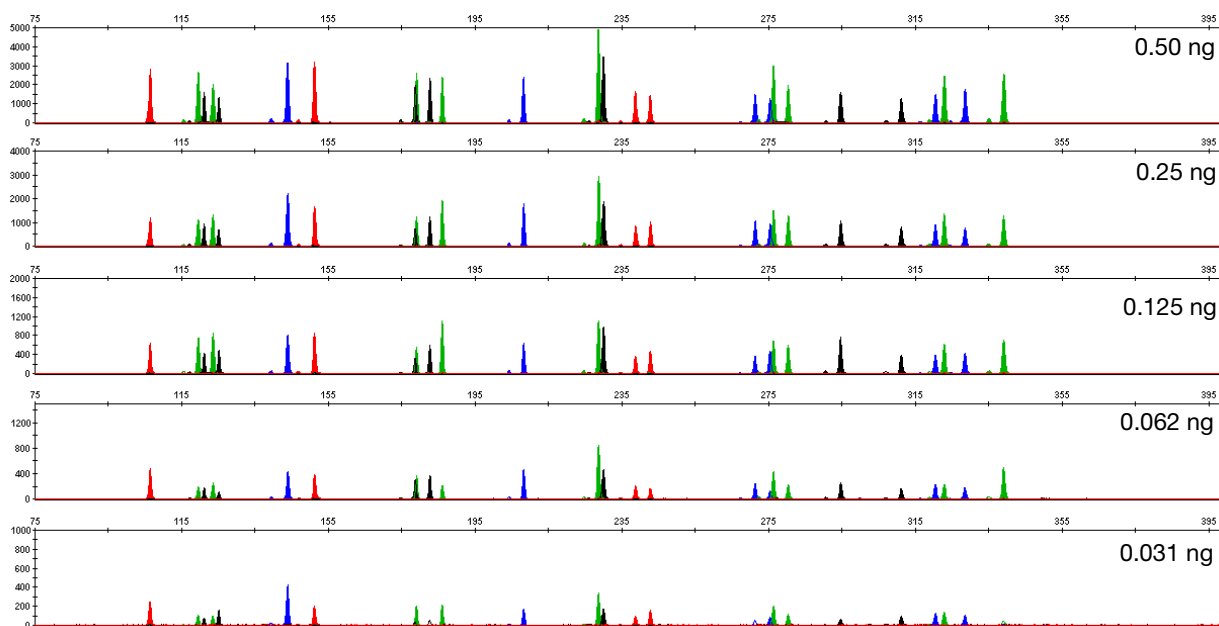

Figure 26   Effect of amplifying 0.5 ng, 0.25 ng, 0.125 ng, 0.062 ng, and 0.031 ng of Control DNA 9947A using the 29-PCR-cycle protocol

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 reduction 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 AmpF*l*STR® Identifiler® 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.
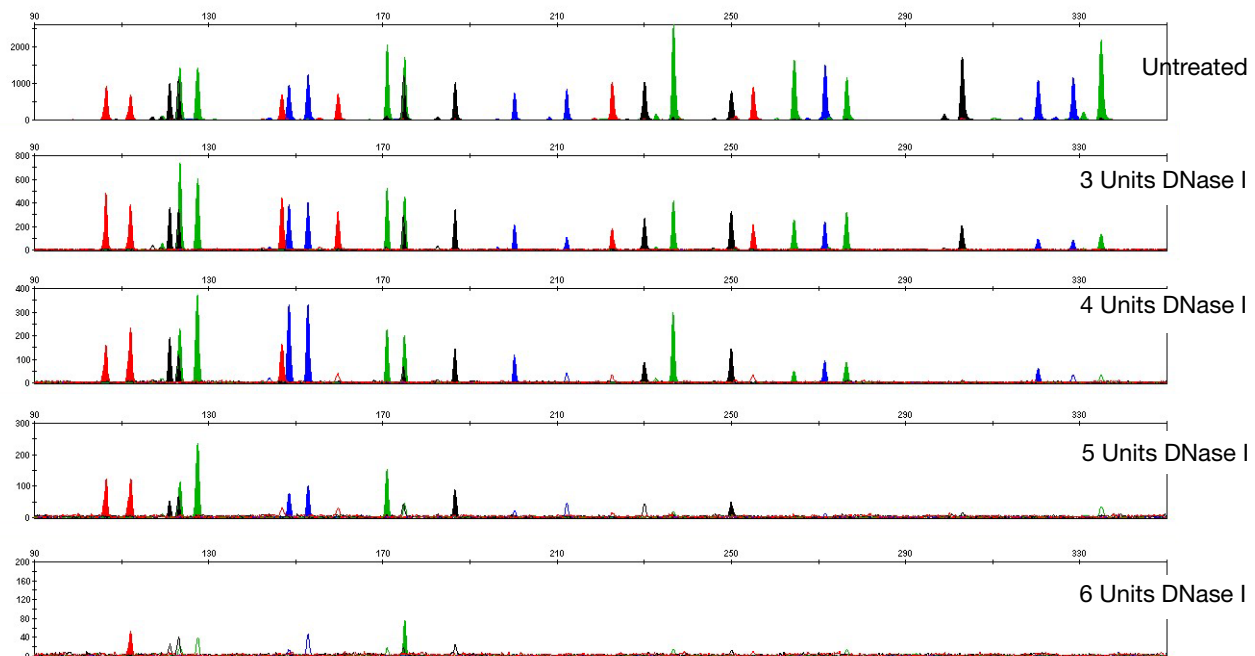
Figure 27   28-PCR-cycle amplification of Raji DNA samples sonicated and incubated with increasing doses of DNase I. Panels 1, 2, 3, 4, and 5 correspond to 0, 3, 4, 5, and 6 units of DNase I.

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 that it subsequently interferes with PCR by inhibiting polymerase activity.

To examine the effects of hematin on the amplification results obtained by the Identifiler® Plus Kit, Control DNA 9947A (1 ng input DNA) was amplified with increasing concentrations of hematin for 28 cycles of amplification (Figure 28 on page 94). The concentrations of hematin used were 0 μM, 100 μM, 200 μM, and 300 μM (see Table 6).
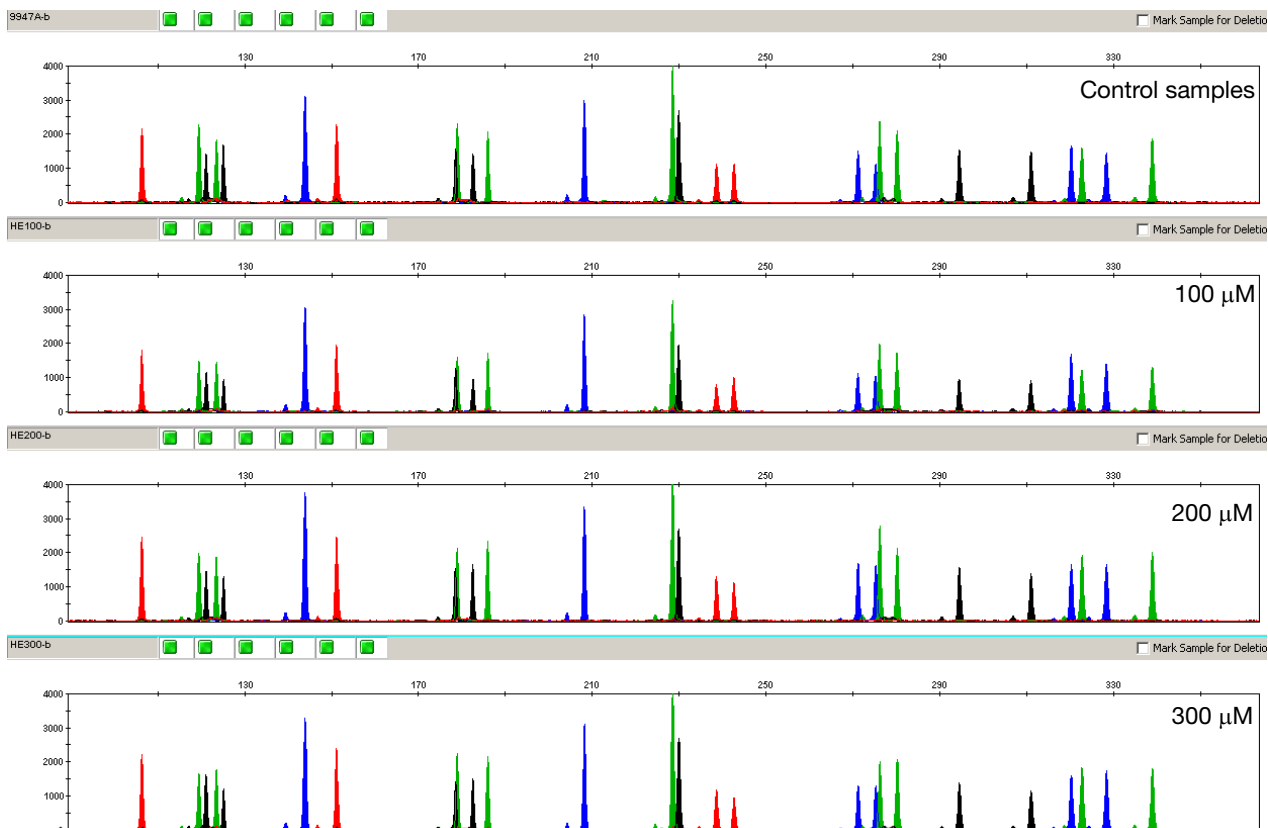
Figure 28   Amplification with the AmpF*l*STR® Identifiler® Plus Kit in the presence and absence of hematin. Panel 1 corresponds to control samples; panels 2–4 correspond to samples amplified in the presence of 100, 200, and 300 μM of hematin.

Table 6   Performance in simulated model of hematin inhibition (n = 3) ‡

Hematin (µM)	Identifiler® Plus Kit Alleles detected/Total alleles
0	26/26, 26/26, 26/26
100	26/26, 26/26, 26/26

Table 6   Performance in simulated model of hematin inhibition
(n = 3) *(continued)*[‡]

Hematin (μM)	Identifiler® Plus Kit Alleles detected/Total alleles
200	26/26, 26/26, 26/26
300	26/26, 26/26, 26/26

‡ Only those peaks >50 RFUs were counted. A complete profile with Control 9947A DNA
yields 26 peaks using the AmpF*l*STR® Identifiler® Plus Kit

**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 9947A with the Identifiler® Plus
Kit for 28 cycles of amplification (see Figure 29). The concentrations of humic acid
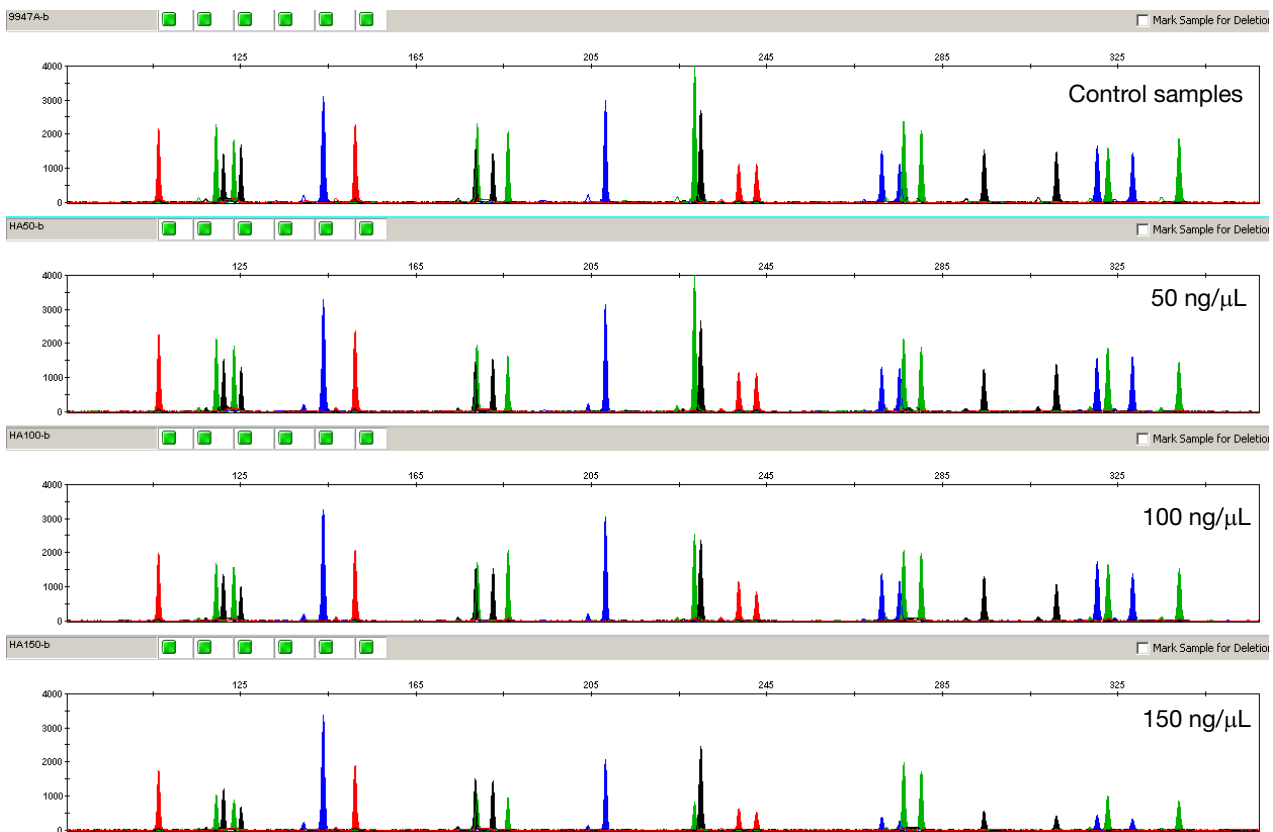tested were 0, 50, 100, and 150 ng/μL (see Table 7).

Figure 29   Amplification with the AmpF*l*STR® Identifiler® Plus PCR Amplification
Kit in the presence and absence of humic acid. Panel 1 corresponds to control
samples; panels 2–4 correspond to samples amplified in the presence of 50, 100,
and 150 ng/μL humic acid.

Table 7 Performance in simulated model of humic acid inhibition (n = 3)[‡]

Humic Acid (ng/μL)	Identifiler® Plus Kit
0	26/26, 26/26, 26/26
50	26/26, 26/26, 26/26
100	26/26, 26/26, 26/26
150	26/26, 26/26, 26/26

‡ Only those peaks >50 RFUs were counted. A complete profile with Control 9947A DNA yields 26 peaks using the AmpFℓSTR® Identifiler® Plus Kit.

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® Identifiler® Plus PCR Amplification Kit loci in unmixed population database samples are shown in Table 8.

Table 8   Peak height ratios for ~1.0 ng[‡] of input DNA amplified for 28 PCR cycles

Locus	Number of Observations (n)	Mean	Median	Minimum	Maximum
Amel	320	90.23	91.29	46.21	99.89
CSF1PO	378	90.37	91.56	67.19	99.96
D13S317	375	90.61	92.09	68.28	99.94
D16S539	389	90.03	91.15	68.11	99.87
D18S51	432	90.06	91.24	62.26	99.94
D19S433	399	90.2	91.2	57.29	99.96
D21S11	428	90.3	91.12	69.05	100
D2S1388	436	90.36	91.56	63.86	100
D3S1358	356	91.39	92.66	62.82	99.94
D5S818	357	91.15	92.21	66.07	100
D7S820	395	90.49	92.07	46.61	99.94
D8S1179	396	91.22	92.7	67.42	100
FGA	429	89.83	91.07	60.38	99.87
TH01	362	91.62	93.04	70.09	100
TPOX	333	91.17	92.18	70.65	100
vWA	414	91.16	92.33	65.22	100

‡ Actual DNA input amounts: 0.7 ng to 1.3 ng

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 comprise (at a single locus) 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.

Limit of detection of the minor component

Mixtures of two genomic DNA samples were examined at various ratios (0:1, 1:1, 3:1, 7:1, 10:1, 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 Figures 30 (28 PCR cycles) and 31 (29 PCR cycles) on page 99 where samples A and B were mixed according to the ratios provided. Using either the 28- or 29-PCR-cycle protocol, the minor component allele calls at non-overlapping loci are highlighted. The amplification of the minor contributor at 3:1, 7:1 (0.875:0.125 ng), and 10:1 (0.9:0.09 ng) mixture ratios was readily typeable. 15:1 (0.9375:0.0625 ng) mixture ratios resulted in full or partial profiles for the minor component. The profiles of these samples are described in Table 9.
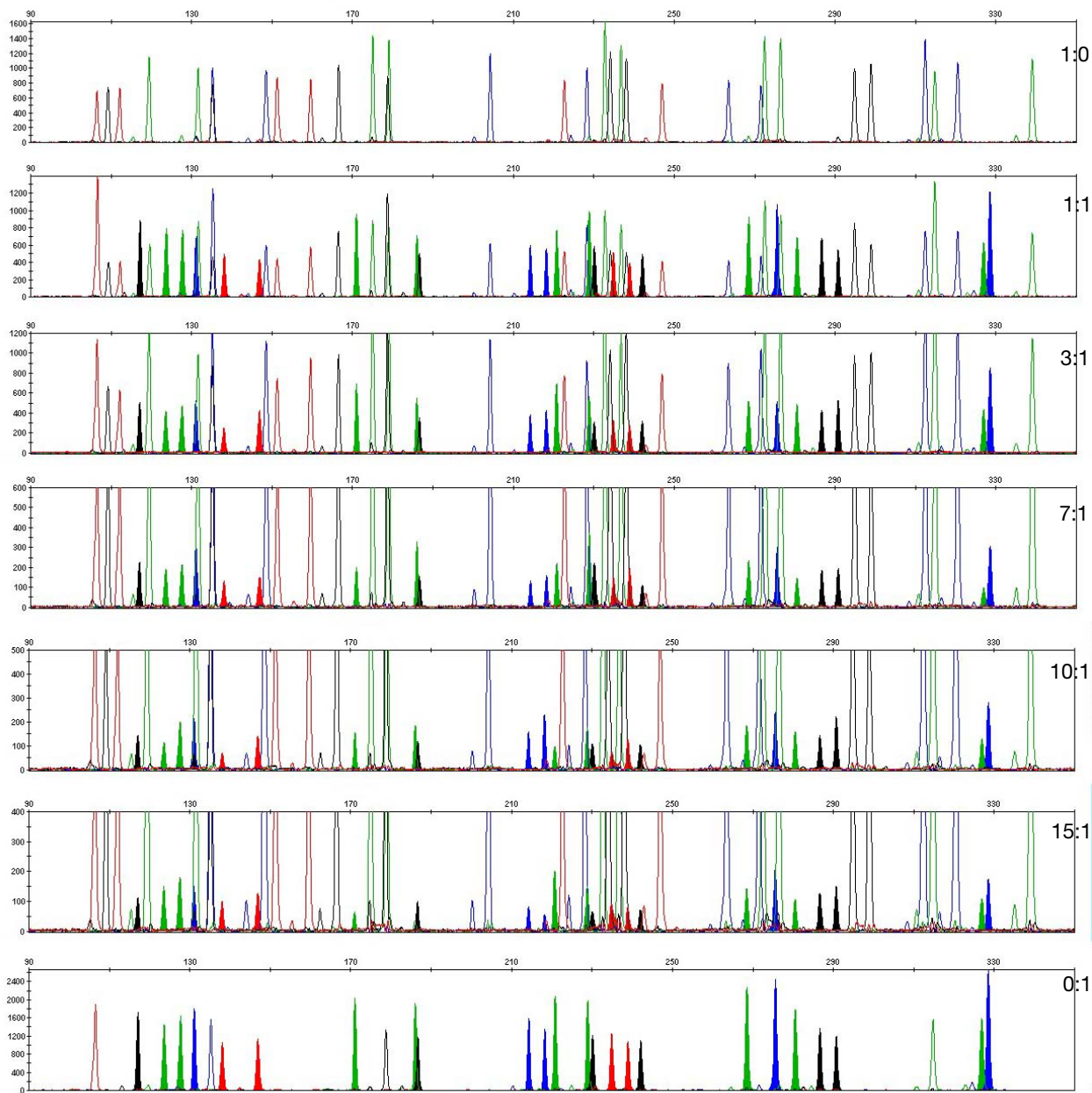
Figure 30   Amplification of DNA mixtures at various ratios using the 28-PCR-cycle protocol. Minor allele peaks that do not overlap with the major contributor peaks are highlighted.
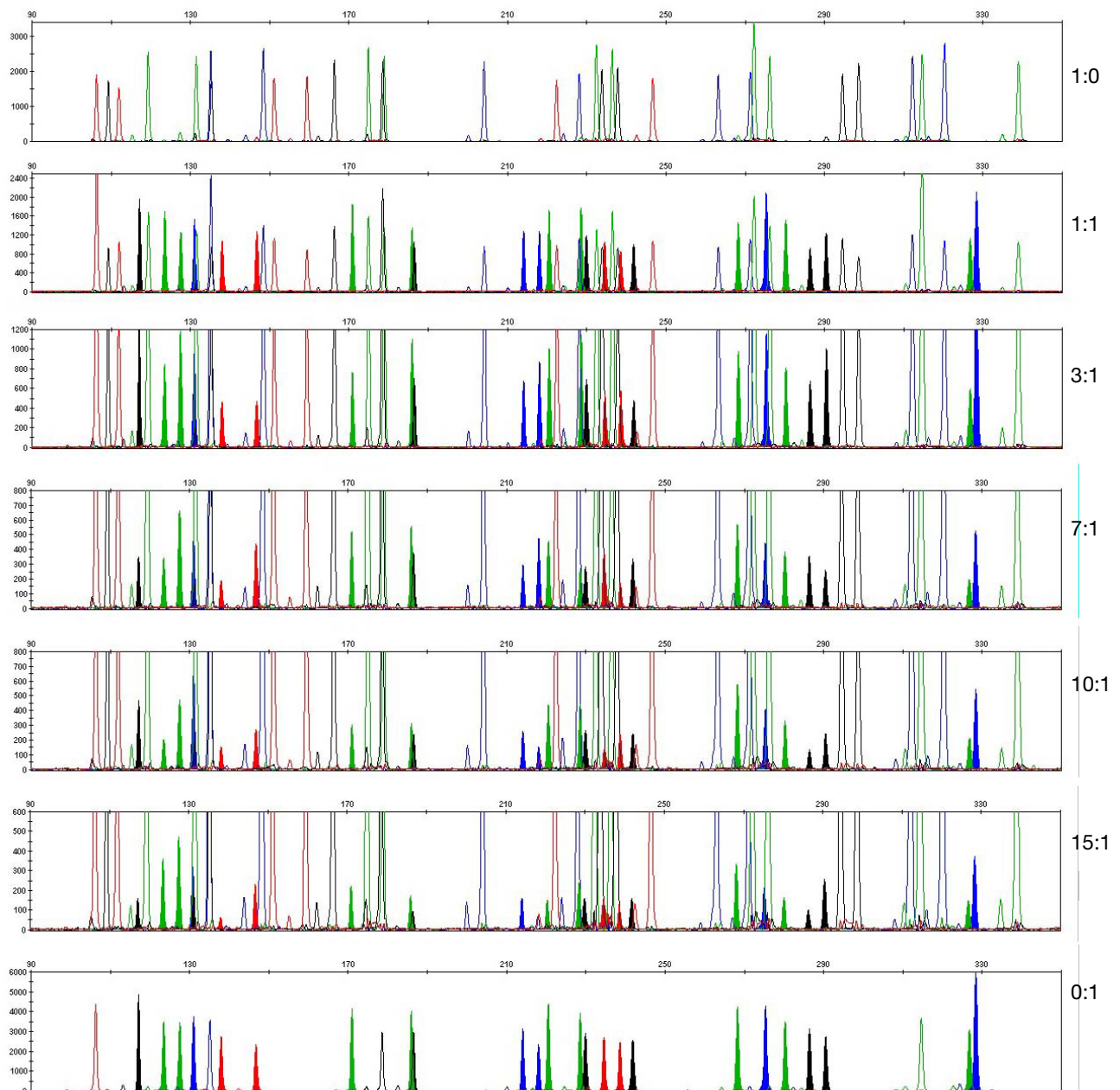
Figure 31   Amplification of DNA mixtures at various ratios using the 29-PCR-cycle protocol. Minor allele peaks that do not overlap with the major contributor peaks are highlighted.

Table 9   Genotypes of mixed DNA samples

Locus	Profile Sample A	Profile Sample B
D8S1179	11, 14	10, 11
D21S11	29, 35	31.2, 32.2

Table 9   Genotypes of mixed DNA samples *(continued)*

Locus	Profile Sample A	Profile Sample B
D7S820	8, 10	11
CSF1PO	8, 10	12
D3S1358	14, 17	15, 16
TH01	7, 8	6, 9.3
D13S317	12, 13	9, 11
D16S539	10, 11	9, 12
D2S1338	17, 23	17, 20
D19S433	11, 17.2	13
vWA	14, 17	17, 19
TPOX	9, 10	8, 11
D18S51	15, 16	13, 14
AMEL	X, Y	X
D5S818	11, 13	8, 10
FGA	19, 25	22, 23

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, you must 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 a suspects reference sample, then the suspect is excluded as the donor of the biological evidence that was 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).

Population samples used in these studies
The AmpF*l*STR® Identifiler® PCR Amplification Kit, prior to the addition of the D8S1179 degenerate primer, was used to generate the population data provided in this section. Samples were collected from individuals throughout the United States with no geographical preference.

African-American

357 samples were provided by the Kentucky State Police and the Federal Bureau of Investigation.

U.S. Caucasian

349 samples were provided by the Kentucky State Police and the Federal Bureau of Investigation.

U.S. Hispanic

290 samples were provided by the Minnesota Bureau of Criminal Apprehension/Memorial Blood Center of Minneapolis and the Federal Bureau of Investigation.

Native American

191 samples were provided by the Minnesota Bureau of Criminal Apprehension/Memorial Blood Center of Minneapolis.

In addition to the alleles that were observed and recorded in the Applied Biosystems databases, other alleles have been published or reported to Applied Biosystems by other laboratories (see the STRBase at **www.cstl.nist.gov/div831/strbase).**

AmpF*l*STR®
Identifiler® Plus
Kit allele
frequencies

Table 10 shows the AmpF*l*STR® Identifiler® Plus Kit allele frequencies in four populations, listed as percentages.

Table 10   AmpF*l*STR Identifiler Plus Kit allele frequencies

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
CSF1PO				
6	‡	‡	‡	‡
7	4.62	0.14‡	0.34‡	‡
8	7.56	0.29‡	0.17‡	0.52‡
9	3.78	1.72	0.86‡	8.38
10	27.87	24.21	23.10	30.89
11	20.59	31.91	28.28	21.99
11.3	0.14‡	‡	‡	‡
12	29.13	32.81	39.66	32.72
13	5.32	7.31	6.38	4.71
14	0.98	1.43	0.86‡	0.79‡
15	‡	0.29‡	‡	‡
D2S1338				
15	0.14‡	‡	‡	‡
16	5.32	4.73	2.41	2.62
17	10.78	17.34	21.21	9.95
18	5.60	6.30	4.14	7.07
19	14.15	13.75	22.76	29.58
20	6.02	14.61	13.79	9.69
21	14.01	2.58	2.59	2.38
22	13.17	4.01	7.41	15.18
23	10.78	11.46	11.36	11.78
24	9.80	11.75	8.45	7.85
25	8.12	10.60	5.17	3.14
26	1.96	2.72	0.69‡	0.79‡
27	0.14‡	0.14‡	‡	‡
28	‡	‡	‡	‡
D3S1358				
<11	0.42‡	0.14‡	‡	‡
11	‡	‡	‡	0.26‡
12	0.56‡	‡	0.17‡	‡
13	0.70‡	0.29‡	0.17‡	‡
14	12.04	15.76	7.41	6.81
15	30.53	25.36	39.14	40.84
15.2	0.14‡	‡	‡	‡

Table 10 AmpF*l*STR Identifiler Plus Kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
16	28.57	22.78	26.72	32.98
17	19.47	18.19	16.03	9.95
18	6.72	16.48	8.97	8.38
19	0.84	1.00	1.03	0.79‡
20	‡	‡	0.34‡	‡
D5S818				
7	0.14‡	‡	6.72	15.71
8	5.46	‡	0.69‡	‡
9	1.68	4.15	5.17	6.02
10	6.72	5.44	5.17	4.19
11	25.49	39.26	39.14	41.10
12	36.41	35.24	29.31	23.30
13	21.57	15.47	12.59	9.42
14	2.38	0.14‡	0.69‡	0.26‡
15	‡	0.29‡	0.18‡	‡
16	‡	‡	0.17‡	‡
17	0.14‡	‡	0.17‡	‡
D7S820				
6	‡	0.14‡	0.17‡	‡
7	0.42‡	1.29	1.72	0.52‡
8	18.77	16.48	11.72	13.09
9	13.73	17.62	6.21	8.12
10	34.45	27.22	27.41	21.99
11	19.89	18.05	28.79	28.80
12	10.78	14.76	20.17	24.08
13	1.54	3.72	3.45	3.40
14	0.42‡	0.72	0.34‡	‡
15	‡	‡	‡	‡
D8S1179				
8	0.42‡	2.29	0.34‡	0.52‡
9	0.42‡	1.15	0.34‡	0.26‡
10	2.38	9.74	8.45	4.71
11	3.92	6.02	5.86	3.40
12	13.31	14.04	12.07	11.52
13	23.25	32.52	32.93	37.43
14	30.11	21.35	26.21	30.63
15	20.17	9.89	10.86	9.42

Table 10   AmpF*l*STR Identifiler Plus Kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
16	4.62	2.72	2.41	1.57
17	1.12‡	0.29‡	0.52‡	0.52‡
18	0.28‡	‡	‡	‡
19	‡	‡	‡	‡
D13S317				
8	3.08	12.18	9.66	4.97
9	2.52	7.74	21.72	17.80
10	3.78	4.44	9.14	13.61
11	24.51	29.80	23.10	24.35
12	46.22	30.80	20.86	23.04
13	15.41	11.17	10.17	7.85
14	4.34	3.72	5.34	8.12
15	0.14‡	0.14‡	‡	0.26‡
D16S539				
5	‡	‡	‡	‡
8	3.22	1.72	1.72	0.79‡
9	19.05	10.46	9.31	12.30
10	10.92	5.59	15.69	15.45
11	31.51	31.95	30.17	30.89
12	18.77	30.23	29.48	27.75
13	14.85	16.76	11.55	10.73
14	1.54	3.01	2.07	2.09
15	0.14‡	0.29‡	‡	‡
D18S51				
7	‡	‡	‡	‡
9	0.14‡	‡	‡	‡
10	0.28‡	0.86	0.52‡	0.79‡
10.2	0.14‡	‡	‡	‡
11	0.28‡	1.15	1.21‡	‡
12	7.00	13.90	10.34	14.92
13	4.34	12.18	14.48	9.16
13.2	0.42‡	‡	‡	‡
14	6.86	16.76	15.52	26.96
14.2	0.28‡	‡	‡	‡
15	19.47	13.61	16.55	12.04
16	16.53	13.61	11.72	10.73
17	18.21	12.32	14.14	14.66

Table 10 AmpF*l*STR Identifiler Plus Kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
18	11.90	7.74	6.72	2.62
19	6.02	4.44	4.14	3.93
20	4.90	1.72	2.24	1.83
21	2.10	1.00	1.03	1.31
22	0.70‡	0.43‡	0.52‡	0.79‡
23	0.42‡	0.14‡	0.52‡	0.26‡
24	‡	0.14‡	0.17‡	‡
25	‡	‡	0.17‡	‡
26	‡	‡	‡	‡
27	‡	‡	‡	‡
D19S433				
9	‡	0.14‡	0.17‡	‡
10	1.54	‡	‡	‡
11	7.14	0.72	0.52‡	0.52‡
11.2	0.14‡	‡	0.17‡	‡
12	10.78	7.74	6.21	3.14
12.2	6.30	0.57‡	1.90	‡
13	29.83	28.94	16.03	17.80
14	21.01	34.10	31.72	24.87
14.2	4.20	0.86	5.00	3.66
15	4.76	15.76	13.45	13.35
15.2	3.36	2.72	8.79	10.73
16	2.38	4.15	4.31	3.93
16.2	2.38	1.72	2.93	1.83
17	‡	0.29‡	0.17‡	0.79‡
17.2	0.28‡	0.29‡	‡	2.88
18.2	0.14‡	0.29‡	‡	1.05‡
D21S11				
24	‡	‡	‡	‡
24.2	0.14‡	0.43‡	0.17‡	‡
24.3	‡	‡	‡	‡
25	‡	‡	‡	‡
25.2	‡	0.14‡	0.17‡	‡
26	0.14‡	0.14‡	0.17‡	‡
27	5.04	4.58	1.21	0.52‡
28	22.97	16.76	9.14	6.28
28.2	‡	‡	‡	‡

Table 10   AmpF*l*STR Identifiler Plus Kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
29	19.33	20.49	21.21	16.75
29.2	0.14‡	‡	0.52‡	0.26‡
29.3	0.14‡	‡	‡	‡
30	17.23	25.21	29.31	34.29
30.2	1.40	3.30	2.93	1.83
31	7.98	7.16	6.72	5.76
31.2	7.98	9.46	8.62	18.85
32	1.12	1.43	1.55	0.79‡
32.2	5.88	7.16	12.93	9.69
33	0.56‡	‡	‡	0.52‡
33.2	3.78	3.30	4.14	3.66
34	1.26	‡	‡	‡
34.1	0.14‡	‡	‡	‡
34.2	0.14‡	0.29‡	0.86‡	0.79‡
35	2.94	‡	0.34‡	‡
35.1	0.14‡	‡	‡	‡
35.2	‡	0.14‡	‡	‡
36	0.84	‡	‡	‡
37	0.28‡	‡	‡	‡
38	0.14‡	‡	‡	‡
FGA				
16	‡	0.14‡	‡	‡
16.1	0.14‡	‡	‡	‡
17	‡	0.29‡	0.17‡	‡
17.2	0.14‡	‡	‡	‡
18	0.70‡	2.72	0.52‡	1.31
18.2	1.40	‡	‡	‡
19	6.72	6.16	7.07	10.21
19.2	0.28‡	‡	‡	‡
20	7.00	13.90	7.41	12.30
20.2	‡	0.14‡	‡	‡
21	12.89	16.91	14.66	12.83
22	21.57	16.91	17.24	10.47
22.2	0.28‡	1.29	0.34‡	0.26‡
22.3	0.14‡	0.14‡	‡	‡
23	14.99	15.19	11.90	15.97
23.2	0.14‡	0.14‡	0.86‡	0.26‡

Table 10   AmpF*l*STR Identifiler Plus Kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
24	17.51	13.75	15.34	15.71
24.2	‡	0.14‡	0.17‡	‡
25	7.98	8.60	14.14	14.14
26	3.50	2.72	6.90	4.45
26.2	‡	‡	‡	0.52
29	0.56‡	‡	‡	‡
30	‡	‡	‡	‡
30.2	0.14‡	‡	‡	‡
31.2	‡	‡	‡	‡
32.2	‡	‡	‡	‡
31.2	‡	‡	‡	‡
33.2	‡	‡	‡	‡
34.2	0.14‡	‡	‡	‡
42.2	‡	‡	‡	‡
43.2	‡	‡	‡	‡
44.2	0.28‡	‡	‡	‡
45.2	‡	‡	‡	0.26‡
46.2	0.14‡	‡	‡	‡
47.2	‡	‡	‡	‡
48.2	0.14‡	‡	‡	‡
50.2	‡	‡	‡	‡
51.2	‡	‡	‡	‡
TH01				
4	‡	‡	‡	‡
5	0.28‡	0.43‡	0.17‡	‡
6	11.06	20.49	22.76	20.68
7	42.86	21.78	33.62	43.98
8	20.73	11.46	8.45	5.24
8.3	‡	0.14‡	‡	‡
9	12.32	16.19	14.14	6.28
9.3	11.62	29.08	20.34	23.56
10	0.98	0.43‡	0.52‡	0.26‡
11	‡	‡	‡	‡
13.3	0.14‡	‡	‡	‡
TPOX				
6	6.72	0.14‡	0.34‡	‡
7	2.24	‡	0.34‡	0.26‡

Table 10   AmpF*l*STR Identifiler Plus Kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
8	36.13	53.30	49.66	37.96
9	21.15	11.60	7.24	4.19
10	9.24	4.30	4.66	3.40
11	21.43	25.93	27.24	39.27
12	3.08	4.73	10.52	14.92
13	‡	‡	‡	‡
vWA				
11	0.28‡	‡	0.17‡	‡
12	‡	‡	‡	0.26‡
13	1.26	0.43‡	‡	0.26‡
14	7.14	8.31	6.90	4.45
15	20.03	11.32	10.00	7.07
16	26.75	23.35	34.31	32.98
17	20.59	24.50	21.55	33.51
18	14.71	22.49	18.45	15.45
19	6.72	8.31	7.07	4.71
20	1.96	1.15	1.38	1.05‡
21	0.28‡	‡	0.17‡	0.26‡
22	0.28‡	‡	‡	‡
23	‡	‡	‡	‡
24	‡	0.14‡	‡	‡

‡ A minimum allele frequency (0.7% for the African-American database, 0.7% for the U.S. Caucasian database, 0.9% for the U.S. Hispanic database, and 1.3% for the Native American database) is suggested by the National Research Council in forensic calculations.

Low-frequency alleles

Some alleles of the AmpF*l*STR® Identifiler® Plus Kit loci occur at a low frequency. For these alleles, a minimum frequency (5 divided by 2n, where n equals the number of individuals in the database) was assigned for the AmpF*l*STR Identifiler Plus Kit African-American, Native-American, U.S. Caucasian, and U.S. Hispanic databases, as suggested in the 1996 report of the Committee on DNA Forensic Science (National Research Council, 1996). These databases are summarized in Table 13 on page 116. The minimum reportable genotype frequency at each locus is: 1.19×10^{-4} for the African-American database; 1.19×10^{-4} for the U.S. Caucasian database; 1.70×10^{-4} for the U.S. Hispanic database; and 2.97×10^{-4} for the Native American database [p2 + p(1–p) θ, where θ = 0.01].

Evaluation of Hardy-Weinberg equilibrium

Estimates of expected heterozygosity (HExp) were computed as described by Nei, M. (1973) using the program PopGene 1.32. Possible divergence from Hardy-Weinberg expectations (HWE) was tested using various methods:

- By calculating the unbiased estimate of the expected homozygote/heterozygote frequencies (Nei, M., 1978).
- Using chi-square (HW X^2p) and likelihood ratio (HW G^2p) tests (as implemented in the program PopGene 1.32).
- With an exact test (HW Exact p), which is a Markov chain method, based on 1000 shuffling experiments, to estimate without bias the exact P-value of the Hardy-Weinberg test with multiple alleles (Guo and Thompson, 1992), as implemented in the program GenePop 3.4.
- An inter-class correlation test analysis (Burrows' composite measure of linkage disequilibria between pairs of loci and X^2 tests for significance [Weir, 1996]) was performed separately in each population to detect any correlations between alleles at any of the pair-wise comparisons of the 15 loci, using the program PopGene 1.32.

Observed heterozygosity (Ho), expected heterozygosity, information content, and tests for detecting departures from Hardy-Weinberg equilibrium are shown for each population in Table 11. While a number of the chi-square tests gave seemingly significant p-values (putatively indicating departures from Hardy-Weinberg equilibrium), chi-squared tests are very sensitive to small expected values (as in the case of multiple rare alleles where the expected number of certain genotypes is 1 or fewer, such as with some of these markers), and can greatly inflate the test statistic in this situation (Weir, 1990). With the exact test, the number of tests with p-value < 0.05 were 0 in the African American and U.S. Caucasian populations, 1 in the U.S. Hispanic population (D8S1179; p=0.0304) and 2 in the Native Americans (D21S11, p=0.0118; D5S818, p=0.0205). These are no more than would be expected by chance. No more alleles were observed to be in linkage disequilibrium than would be expected by chance alone. The average observed heterozygosity across the 15 STR loci was 0.804 in the African American population, 0.792 in the U.S. Caucasian sample population, 0.793 in the Hispanic sample population, and 0.757 in the Native Americans. The most heterozygous locus was FGA (mean observed heterozygosity across all populations of 0.875), and the least heterozygous STR locus was TPOX (mean observed heterozygosity across all populations of 0.677).

Table 11   Heterozygosity and p-values for Hardy-Weinberg tests of the 15 Identifiler® Plus Kit STR loci in four U.S. populations ‡

	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
CSF1P0				
HW X^2 p	0.13649	0.926431	0.951476	0.839278
HW G^2 p	0.08902	0.894972	0.918038	0.728023
HW Exact p	0.0762	0.2688	0.5456	0.6148
HExp	0.7829	0.7267	0.7051	0.7398
Ho	0.7703	0.7421	0.7138	0.7958

Table 11   Heterozygosity and p-values for Hardy-Weinberg tests of the
15 Identifiler® Plus Kit STR loci in four U.S. populations *(continued)*‡

	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
D2S1338				
HW X^2 p	0.409878	0.537758	0.975972	0.722543
HW G^2 p	0.962501	0.407932	0.973054	0.760953
HW Exact p	0.7838	0.3488	0.9794	0.5825
HExp	0.8936	0.8823	0.8529	0.8428
*H*o	0.8768	0.8653	0.8379	0.801
D3S1358				
HW X^2 p	0.947371	0.670787	0.681659	0.087223
HW G^2 p	0.907905	0.654776	0.852278	0.175807
HW Exact p	0.2967	0.2814	0.4684	0.0614
HExp	0.7681	0.7986	0.7361	0.7028
*H*o	0.7955	0.8166	0.7414	0.7382
D5S818				
HW X^2 p	0.993751	0.859805	0.944725	0.073002
HW G^2 p	0.989776	0.520417	0.979044	0.08025
HW Exact p	0.958	0.462	0.4662	0.0205
HExp	0.7476	0.6931	0.7351	0.7378
*H*o	0.7479	0.7077	0.7586	0.6806
D7S820				
HW X^2 p	0.987668	0.571989	0.336834	0.324754
HW G^2 p	0.969887	0.44694	0.687948	0.289733
HW Exact p	0.9818	0.2286	0.4028	0.1276
HExp	0.7758	0.8117	0.7822	0.7858
*H*o	0.7955	0.7908	0.7862	0.7487
D8S1179				
HW X^2 p	0.067164	0.545414	0.047783	0.446248
HW G^2 p	0.568837	0.275218	0.302937	0.760077
HW Exact p	0.2176	0.3264	0.0304	0.1656
HExp	0.7925	0.8047	0.7853	0.7403
*H*o	0.7899	0.8424	0.8	0.6806
D13S317				
HW X^2 p	0.014379	0.711127	0.353995	0.813948
HW G^2 p	0.609389	0.871173	0.190736	0.814681
HW Exact p	0.3818	0.667	0.2415	0.6851
HExp	0.6977	0.7797	0.8251	0.8222
*H*o	0.6695	0.7364	0.8207	0.8168

Table 11 Heterozygosity and p-values for Hardy-Weinberg tests of the 15 Identifiler® Plus Kit STR loci in four U.S. populations *(continued)*‡

	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
D16S539				
HW X^2 p	0.433216	0.67702	0.058631	0.996396
HW G^2 p	0.482435	0.594871	0.37601	0.981384
HW Exact p	0.3753	0.4328	0.3068	0.9986
HExp	0.7939	0.7632	0.7747	0.7766
*H*o	0.8263	0.7822	0.7828	0.7853
D18S51				
HW X^2 p	0.999844	0.628334	0.999203	0.343027
HW G^2 p	1	0.872113	0.999492	0.798859
HW Exact p	0.978	0.0982	0.9152	0.2265
HExp	0.8694	0.8769	0.8761	0.8463
*H*o	0.8824	0.8682	0.8862	0.8377
D19S433				
HW X^2 p	0.91703	0.806717	0.731222	0.810711
HW G^2 p	0.83419	0.999765	0.975476	0.898389
HW Exact p	0.4517	0.69	0.3475	0.4301
HExp	0.8364	0.7659	0.8310	0.8430
*H*o	0.8011	0.7622	0.8414	0.822
D21S11				
HW X^2 p	0.985687	0.936146	0	0
HW G^2 p	1	0.999757	0.999794	0.712937
HW Exact p	0.7627	0.7861	0.6476	0.0118
HExp	0.8585	0.8427	0.8290	0.8003
*H*o	0.8711	0.8567	0.7931	0.801
FGA				
HW X^2 p	0	0.904953	0.263223	0.999686
HW G^2 p	1	0.999812	0.960137	0.999946
HW Exact p	0.9761	0.4459	0.0891	0.9161
HExp	0.8659	0.8686	0.8751	0.8746
*H*o	0.8824	0.8854	0.8724	0.8482
TH01				
HW X^2 p	0.961911	0.997905	0.649467	0.329461
HW G^2 p	0.940414	0.99169	0.617212	0.318591
HW Exact p	0.8286	0.9716	0.4495	0.1377
HExp	0.7323	0.7866	0.7666	0.7016
*H*o	0.7395	0.7822	0.8103	0.6492

Table 11   Heterozygosity and p-values for Hardy-Weinberg tests of the
15 Identifiler® Plus Kit STR loci in four U.S. populations *(continued)*[‡]

	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
TPOX				
HW X^2 p	0.765163	0.801518	0.875348	0.333914
HW G^2 p	0.611014	0.757735	0.913091	0.229017
HW Exact p	0.7247	0.5775	0.8356	0.0647
HExp	0.7643	0.6311	0.6607	0.6765
*H*o	0.7563	0.6304	0.6759	0.6178
vWA				
HW X^2 p	0.925176	0.005048	0.641684	0.994248
HW G^2 p	0.964308	0.218817	0.934427	0.997184
HW Exact p	0.7033	0.0564	0.7066	0.8845
HExp	0.8141	0.8081	0.7818	0.7457
*H*o	0.8571	0.8138	0.7759	0.7277

‡   HW X^2 p, probability value of X^2 test for Hardy-Weinberg equilibrium; HW G^2 p, probability value of the G-statistic of the Likelihood Ratio test for multinomial proportions; HW Exact p; A Markov chain unbiased exact test to estimate the P-value of the Hardy-Weinberg test with multiple alleles; Hexp, Expected heterozygosity; *H*o, observed heterozygosity

Concordance studies

Applied Biosystems analyzed >500 samples by comparing allele calls between the AmpF*l*STR Identifiler and Identifiler® Plus Kits. The genotype data from all the analyzed samples showed 100% concordance between the Identifiler and Identifiler® Plus Kits.

Mutation Rate

Estimation of spontaneous or induced germline mutation at genetic loci can be achieved by comparing 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 ten STR loci that were 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; mutations were not detected at any of the other nine STR loci. The D18S11 mutation was represented by an increase of one 4-nt repeat unit, allele 17 was inherited as allele 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® Identifiler® Plus Kit loci.
- Methods for modifications of these mutation rates (to infer mutation rates indirectly for those loci where the rates are not large enough to be measured directly and/or to account for those events undetectable as Mendelian errors).

Probability of Identity

Table 12 shows the Probability of Identity (PI) values of the AmpF*l*STR® Identifiler® Plus Kit loci individually and combined.

Table 12 Probability of Identity values for the AmpF*l*STR Identifiler Plus Kit STR loci

Locus	African-American	U.S. Caucasian	U.S. Hispanic	Native American
CSF1PO	0.079	0.132	0.141	0.123
D2S1338	0.023	0.027	0.038	0.043
D3S1358	0.097	0.076	0.112	0.158
D5S818	0.104	0.147	0.115	0.110
D7S820	0.085	0.063	0.083	0.081
D8S1179	0.074	0.064	0.089	0.104
D13S317	0.132	0.079	0.056	0.056
D16S539	0.077	0.097	0.090	0.082
D18S51	0.033	0.031	0.031	0.046
D19S433	0.042	0.087	0.049	0.044
D21S11	0.037	0.044	0.047	0.074
FGA	0.034	0.035	0.032	0.031
TH01	0.109	0.079	0.097	0.134
TPOX	0.089	0.188	0.168	0.159
vWA	0.066	0.066	0.080	0.103
Combined	1.31×10^{-18}	5.01×10^{-18}	7.65×10^{-18}	3.62×10^{-17}

The P_I value is the probability that two individuals selected at random will have an identical AmpF*l*STR Identifiler Plus Kit genotype (Sensabaugh, 1982). The P_I values for the populations described in this section are then approximately $1/7.64 \times 10^{17}$ (African-American), $1/2.00 \times 10^{17}$ (U.S. Caucasian), $1/1.31 \times 10^{17}$ (U.S. Hispanic), and $1/2.76 \times 10^{16}$ (Native American).

Probability of Paternity Exclusion

Table 13 shows the Probability of Paternity Exclusion (P_E) values of the AmpFℓSTR®
Identifiler® Plus Kit STR loci individually and combined.

**Table 13   Probability of Paternity Exclusion values for the AmpFℓSTR
Identifiler Plus Kit loci**

Locus	African-American	U.S. Caucasian	U.S. Hispanic	Native American
CSF1PO	0.545	0.496	0.450	0.409
D2S1338	0.748	0.725	0.671	0.399
D3S1358	0.591	0.630	0.495	0.510
D5S818	0.506	0.440	0.525	0.601
D7S820	0.591	0.582	0.574	0.492
D8S1179	0.580	0.680	0.599	0.601
D13S317	0.383	0.487	0.638	0.370
D16S539	0.649	0.566	0.567	0.428
D18S51	0.760	0.731	0.767	0.329
D19S433	0.601	0.531	0.678	0.360
D21S11	0.737	0.708	0.586	0.399
FGA	0.760	0.766	0.739	0.309
TH01	0.492	0.566	0.618	0.646
TPOX	0.521	0.329	0.392	0.687
vWA	0.709	0.625	0.555	0.528
Combined	0.9999996	0.9999992	0.9999990	0.9999527

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

Troubleshooting

A

Follow the actions recommended in Table 14 to troubleshoot problems that occur during analysis.

Table 14   Troubleshooting

Observation	Possible causes	Recommended actions
Faint or no signal from both the 9947A and the DNA test samples at all loci	Incorrect volume or absence of either AmpF*l*STR Identifiler® Plus Master Mix or AmpF*l*STR Identifiler® 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.
	AmpF*l*STR Identifiler® 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® PCR System 9700.
	MicroAmp® Base used with tray/retainer set and tubes in GeneAmp® PCR System 9700	Remove MicroAmp Base from tray/retainer set and repeat test.
	Insufficient PCR product electrokinetically injected	**For ABI PRISM® 3100-*Avant* or Applied Biosystems 3100/3130*xl* runs:** Mix 1.0 µL of PCR product and 9 µL of Hi-Di™ Formamide/ GeneScan™ 500 LIZ® solution. **For ABI PRISM® 310 instrument runs:** Mix 1.5 µL of PCR product and 25 µL of Hi-Di™ Formamide/GeneScan™ 500 LIZ® solution.
	Degraded formamide	Check the storage of formamide; do not thaw and refreeze multiple times. Try Hi-Di™ Formamide.

Table 14   Troubleshooting *(continued)*

Observation	Possible causes	Recommended actions
Positive signal from AmpF*l*STR Control DNA 9947A but partial or no signal from DNA test samples	Quantity of test DNA sample is below assay sensitivity	Quantitate DNA and add 1.0 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 or use the AmpF*l*STR® MiniFiler™Kit.
	Dilution of test sample DNA in water or wrong buffer (for example, TE formula with incorrect EDTA concentration)	Redilute DNA using low 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.
	Amplification of stutter product	See "Stutter products" on page 81.
	Mixed sample	
	Incomplete 3´ A base addition (n-1 nt position)	See "Addition of 3´ A nucleotide" on page 86. Be sure to include the final extension step of 60 °C for 10 min in the PCR.
	Signal exceeds dynamic range of instrument (off-scale data)	Ensure cycle number is optimized according to instructions on page 21. Repeat PCR amplification using fewer PCR cycles or use your laboratory's SOP to analyze off-scale data.
	Poor spectral separation (bad matrix)	Follow the steps for creating a spectral file.
		Confirm that Filter Set G5 modules are installed and used for analysis.
	Too much DNA in reaction	Use recommended amount of template DNA (1.0 ng).
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 or use the AmpF*l*STR® MiniFiler™Kit.
	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. Repeat test.

Table 14   Troubleshooting *(continued)*

Observation	Possible causes	Recommended actions
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.

Safety

B

This appendix covers:

Chemical safety

Chemical hazard warning

⚠ **WARNING! CHEMICAL HAZARD.** Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

⚠ **WARNING! CHEMICAL HAZARD.** All chemicals in the instrument, including liquid in the lines, are potentially hazardous. Always determine what chemicals have been used in the instrument before changing reagents or instrument components. Wear appropriate eyewear, protective clothing, and gloves when working on the instrument.

⚠ **WARNING! CHEMICAL HAZARD.** Four-liter reagent and waste bottles can crack and leak. Each 4-liter 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.

⚠ **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 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 124.)
- 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 **www.appliedbiosystems.com**, click **Support**, then select **MSDS**.

2. In the Keyword Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.

3. Find the document of interest, right-click the document title, then select any of the following:
   • **Open** – To view the document
   • **Print Target** – To print the document
   • **Save Target As** – To download a PDF version of the document to a destination that you choose

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

Chemical waste safety

Chemical waste hazards

⚠ **WARNING! 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 a 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

General biohazard

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

Chemical alerts

For the definitions of the alert words **IMPORTANT**, **CAUTION**, **WARNING**, and **DANGER**, see "Safety alert words" on page vii.

General alerts for all chemicals

Avoid contact with skin, eyes, and/or clothing. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Specific chemical alerts

⚠ **CAUTION! CHEMICAL HAZARD. AmpF***ℓ***STR**® Identifiler® Plus PCR Amplification Kit may cause eye, skin, and respiratory tract irritation. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

⚠ **WARNING! CHEMICAL HAZARD. POP-4™ Polymer** for 3130/3130*xl* Genetic Analyzers causes skin, eye, and respiratory tract irritation.

⚠ **WARNING! CHEMICAL HAZARD. Running Buffer, 10✕** causes skin, eye, and respiratory tract irritation.

⚠ **WARNING! CHEMICAL HAZARD. Hi-Di™ Formamide** is harmful if swallowed, inhaled or absorbed through skin, and causes irritation to skin, eyes, and respiratory tract. It affects the central nervous system and may affect the reproductive system.

⚠ **WARNING! CHEMICAL HAZARD. POP-4™ Polymer** for 3100/3100-*Avant* Genetic Analyzers is irritating to eyes, respiratory system, and skin. It causes adverse cardiovascular effects. It contains a known or suspected reproductive toxin and a known or suspected mutagen.

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Documentation

Related documentation

For additional documentation, see "How to obtain support" on page ix.

Document title	Part number
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® 3100/3100-Avant Genetic Analyzers Protocols for Processing AmpFlSTR PCR Amplification Kit PCR Products User Bulletin	4332345
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
Applied Biosystems 3130/3100xl DNA Analyzers User Guide	4331468
Quantifiler® Kits: Quantifiler® Human DNA Quantification Kit and Quantifiler® Y Human Male DNA Quantification Kit User's Manual	4344790
AmpFlSTR® Identifiler® PCR Amplification Kit User's Manual	4323291
GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide	4338775
GeneMapper® ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial	4335523
Installation Procedures and New Features for GeneMapper® ID Software v3.2 User Bulletin	4352543
GeneMapper® ID-X Software Version 1.0 Getting Started Guide	4375574
GeneMapper® ID-X Software Version 1.0 Quick Reference Guide	4375670
GeneMapper® ID-X Software Version 1.0 Reference Guide	4375671
Veriti® 96-Well Thermal Cycler AmpFlSTR Kit Validation User Bulletin	4440754

Portable document format (PDF) versions of this guide and the documents listed above are available at **www.appliedbiosystems.com**

Note: To open the user documentation available from the Applied Biosystems web site, use the Adobe® Acrobat® Reader® software available from **www.adobe.com**

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techpubs@appliedbiosystems.com

IMPORTANT! The e-mail address above is for submitting comments and suggestions relating *only* to documentation. To order documents, download PDF files, or for help with a technical question, see "How to obtain support" on page ix.

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