applied biosystems

AmpFℓSTR™ SGM Plus™ PCR Amplification Kit

USER GUIDE

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ThermoFisher SCIENTIFIC

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Revision history: Pub. No. 4309589

Revision	Date	Description
J	24 August 2018	Updated branding and trademarks, no technical changes.
H	August 2012	• Add validation experiments and results for buffer and enzyme kit component changes. • Update instrument and software instructions.
G	March 2012	Change to copyright page information.
F	2011	Change to copyright page information.
E	2006	Change to copyright page information.
D	2006	—
C	2005	—
B	2001	—
A	1999	New document.

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Contents

About This Guide

IMPORTANT! Before using this product, read and understand the information the "Safety" appendix in this document.

Revision history

Revision	Date	Description
A	1999	New document.
B	2001	—
C	2005	—
D	2006	—
E	2006	Change to limited licensing information.
F	2011	Change to limited licensing information.
G	March 2012	Change to copyright page information.
H	August 2012	• Add validation experiments and results for buffer and enzyme kit component changes. • Update instrument and software instructions.

Purpose

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

User attention words

Five user attention words may appear in this document. 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 or accurate chemistry kit use.

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.

1 Overview

Product overview

Purpose

The AmpFℓSTR™ SGM Plus™ PCR Amplification Kit is a short tandem repeat (STR) multiplex assay that amplifies 10 tetranucleotide repeat loci (D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01, FGA) and the Amelogenin gender-determining marker in a single PCR amplification.

Product description

The SGM Plus™ Kit contains all the necessary reagents for the amplification of human genomic DNA.

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

- Applied Biosystems 3100/3100-*Avant* Genetic Analyzer
- Applied Biosystems 3130/3130*xl* Genetic Analyzer
- Applied Biosystems 3500/3500xL 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 SGM Plus™ Kit employs the same primer sequences for all loci common to other AmpFℓSTR™ kits (except the MiniFiler™ kit), but does not include the degenerate unlabeled primer for the D8S1179 locus that was included in later AmpFℓSTR™ kits (for more information, see the *AmpFℓSTR™ Identifiler PCR Amplification Kit User Guide*).

Loci amplified by the kit

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

Table 1 SGM Plus™ Kit loci and alleles

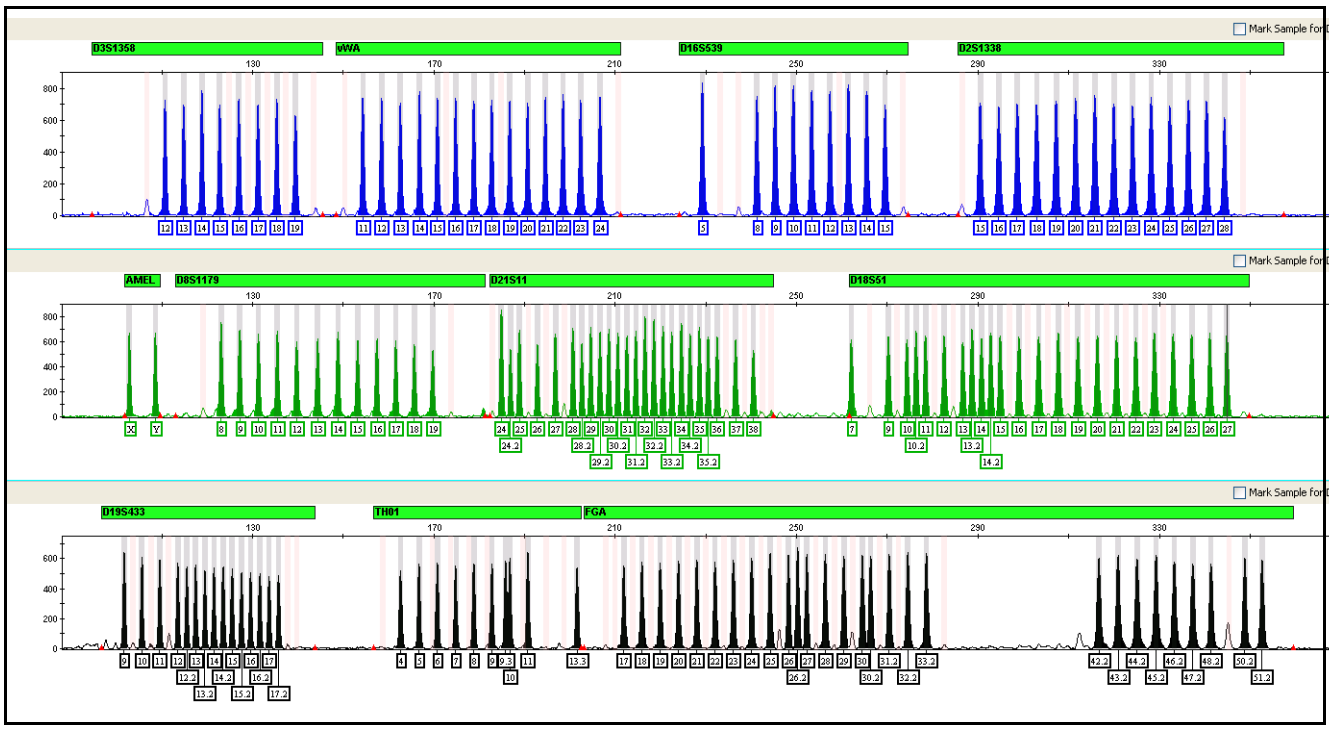
Locus designation	Chromosome location	Alleles included in AmpFℓSTR™ SGM Plus™ Allelic Ladder	Dye label	Control DNA 007
D3S1358	3p	12, 13, 14, 15, 16, 17, 18, 19	5-FAM™	15, 16
vWA	12p12-pter	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24		14, 16
D16S539	16q24-qter	5, 8, 9, 10, 11, 12, 13, 14, 15		9, 10
D2S1338	2q35–37.1	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28		20, 23
Amelogenin	X: p22.1-22.3 Y: p11.2	X, Y	JOE™	X, Y
D8S1179†	8	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19		12, 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		28, 31
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		12, 15
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
TH01	11p15.5	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3		7, 9.3
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		24, 26

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

Allelic ladder profile

Figure 1 shows the allelic ladder for the SGM Plus™ Kit. See "Allelic ladder requirements" on page 25 for information on ensuring accurate genotyping.

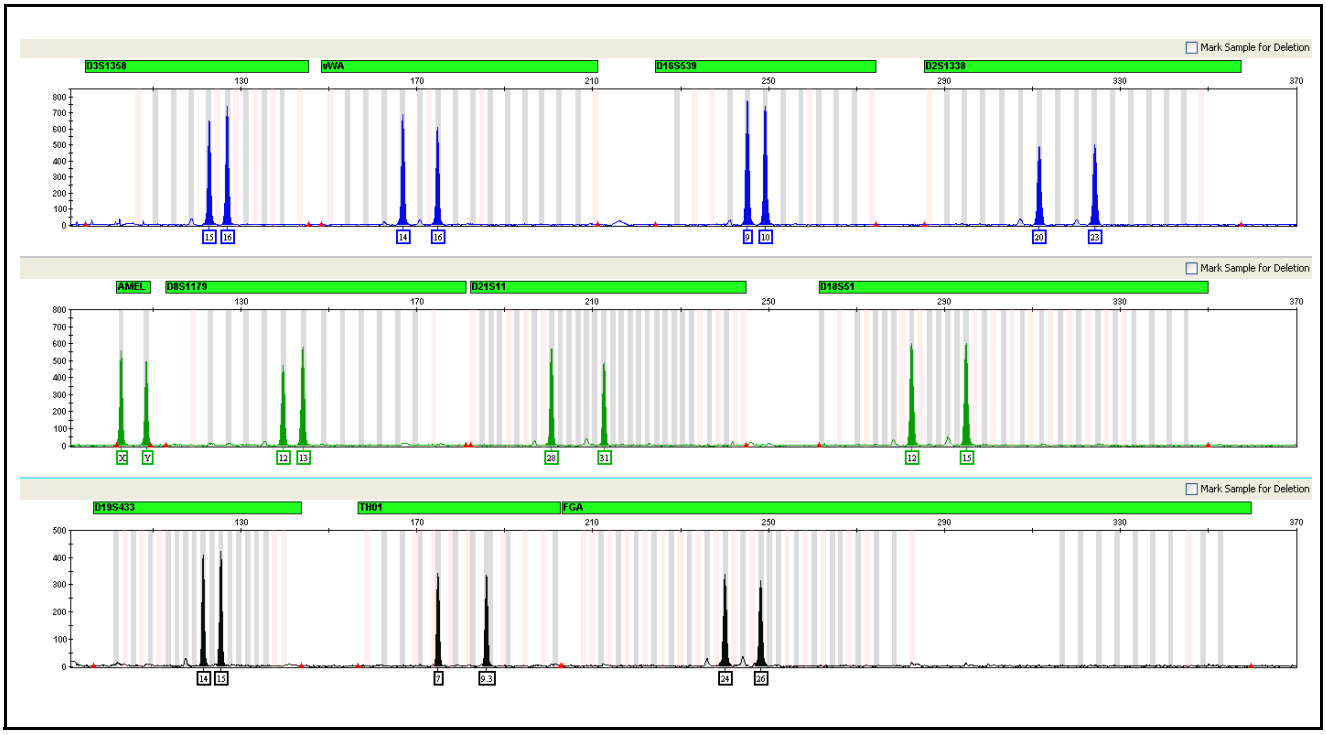
Figure 1 GeneMapper™ *ID-X* Software plot of the AmpFℓSTR™ SGM Plus™ Allelic Ladder



Control DNA 007 profile

Figure 2 shows amplification of Control DNA 007 using the SGM Plus™ Kit.

Figure 2 1 ng of Control DNA 007 amplified with the SGM Plus™ Kit and analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer



Workflow overview

Perform PCR

Extract DNA


AutoMate *Express*™ System + PrepFiler™ Express Kit

Quantify DNA

Quantifiler™ Duo DNA Quantification Kit

Prepare reactions

AmpF*l*STR™ SGM Plus™ PCR Amplification Kit

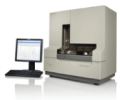
Perform PCR

GeneAmp™ PCR System 9700 Cycler

Veriti™ 96-Well Thermal Cycler

Perform electro-phoresis

3100/3100-*Avant* Genetic Analyzer



3130/3130*xl* Genetic Analyzer

3500/3500xL Genetic Analyzer

310 Genetic Analyzer

Analyze data

GeneMapper™ *ID-X* or GeneMapper™ *ID* Software

Instrument and software overview

This section provides information about the Data Collection Software versions required to run the AmpFℓSTR™ SGM 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

Table 2 Software specific to each instrument

Instrument	Operating system	Data Collection Software	Analysis software
3500/3500xL	• Windows™ XP • Windows Vista™	3500 Series Data Collection Software v1.0	GeneMapper™ *ID-X* Software v1.2 or higher
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	
	• Windows™ NT • Windows™ 2000	3.0	

Note: We conducted validation studies for the AmpFℓSTR™ SGM Plus™ PCR Amplification Kit using the Applied Biosystems 310 Genetic Analyzer running Mac Operating System™. This configuration is now obsolete.

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.

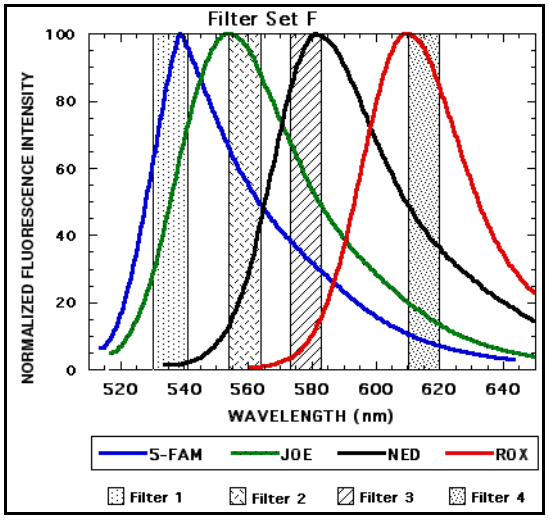
Multicomponent analysis is the process that separates the four different fluorescent dye colors into distinct spectral components. The three dyes used in the SGM Plus™ Kit to label samples are 5-FAM™, JOE™, and NED™ dyes. The fourth dye, ROX™ dye, is used to label the GeneScan™ 500 ROX™ Size Standard.

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 Applied Biosystems 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 5-FAM™ dye emits at the shortest wavelength and it is displayed as blue, followed by JOE™ (green), NED™ (yellow), and ROX™ (red) dyes.

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 four dyes used in the AmpFℓSTR™ SGM Plus™ Kit



Materials and equipment

Kit contents and storage

The AmpFℓSTR™ SGM Plus™ PCR Amplification Kit (Part no. 4307133) contains materials sufficient to perform 100 amplifications at 50 µL/amplification.

IMPORTANT! The fluorescent dyes attached to the primers are light sensitive. Protect the primer set, amplified DNA, allelic ladder, and size standard from light when not in use. Keep freeze-thaw cycles to a minimum.

Component	Description	Volume	Storage
AmpFℓSTR™ PCR Reaction Mix	Contains $MgCl_2$, dNTPs, and bovine serum albumin in buffer with 0.05% sodium azide.	2 tubes, 1.1 mL each	–15 to –25°C on receipt, 2 to 8°C after initial use
AmpFℓSTR™ SGM Plus ™ Primer Set	Contains fluorescently labeled primers and non-labeled primers.	1 tube, 1.1 mL	
AmpFℓSTR™ SGM Plus™ Allelic Ladder	Contains amplified alleles. See Table 1 on page 12 for a list of alleles included in the allelic ladder.	1 tube, 0.05 mL	
AmpFℓSTR™ Control DNA 007	Contains 0.10 ng/µL human male 007 DNA in 0.05% sodium azide and buffer†. See Table 1 on page 12 for profile.	1 tube, 0.3 mL	
AmpliTaq Gold™ DNA Polymerase	Contains enzyme, with an activity of 5 U/µL.	2 tubes, 0.05 mL/tube	–15 to –25°C

† The AmpFℓSTR™ Control DNA 007 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 AmpFℓSTR™ Control DNA 007 is not designed to be used as a DNA quantitation control, and you may see variation from the labelled concentration when quantitating aliquots of the AmpFℓSTR™ Control DNA 007.

Standards for samples

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

- **AmpFℓSTR™ Control DNA 007** – A positive control for evaluating the efficiency of the amplification step and STR genotyping using the AmpFℓSTR™ SGM Plus™ Allelic Ladder.
- **GeneScan™ 500 ROX™ Size Standard** – Used for obtaining sizing results. This standard, which has been evaluated as an internal size standard, yields precise sizing results for SGM Plus™ Kit PCR products. Order the GeneScan™ 500 ROX™ Size Standard (Part no. 4322682) separately.
- **AmpFℓSTR™ SGM Plus ™ Allelic Ladder** – Allelic ladder developed by Life Technologies for accurate characterization of the alleles amplified by the SGM Plus™ Kit. The AmpFℓSTR™ SGM Plus™ Allelic Ladder contains most of the alleles reported for the 10 autosomal loci. Refer to Table 1 on page 12 for a list of the alleles included in the AmpFℓSTR™ SGM Plus™ Allelic Ladder.

2 Perform PCR

Required user-supplied reagents

In addition to the SGM 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 SGM Plus™ Kit is 1.0–2.5 ng in a maximum input volume of 20 µL for 28 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

Life Technologies provides several kits for quantifying DNA in samples. See the reference cited in the following table for details about these kits.

Product	Description
Quantifiler™ Human DNA Quantification Kit (Part no. 4343895) *and* Quantifiler™ Y Human Male DNA Quantification Kit (Part no. 4343906) For more information, see *Quantifiler™ Human DNA Quantification Kits User's Manual* (Pub. no. 4344790)	**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, and one TaqMan™ MGB probe labeled with VIC™ dye for detecting the amplified IPC.
Quantifiler™ Duo DNA Quantification Kit (Part no. 4387746) For more information, see *Quantifiler™ Duo DNA Quantification Kit User's Manual* (Pub. no.4391294)	**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.

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
AmpFℓSTR™ PCR Reaction Mix	21.0 µL
AmpFℓSTR™ SGM Plus ™ Primer Set	11.0 µL
AmpliTaq Gold™ DNA Polymerase	1.0 µL

Note: The volumes above include a slight overfill to provide excess volume for the loss that occurs during reagent transfers.

2. Prepare reagents. Thaw the AmpFℓSTR™ PCR Reaction Mix and the SGM Plus™ Primer Set, then vortex all reagent tubes including the enzyme for 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 these reagents.

3. Pipette the required volumes of components into an appropriately sized polypropylene tube to create a master mix.
4. Vortex the master mix for 3 seconds, then centrifuge briefly.
5. Dispense 30 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 20 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 1.0–2.5 ng of total DNA is in a final volume of 20 µL. Add 20 µL of the diluted sample to the reaction mix.
Positive control	Add 20 µL of 007 control DNA (0.1 ng/µL).

The final reaction volume (sample or control plus master mix) is 50 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. Centrifuge the tubes or plate at 3000 rpm for ~20 seconds in a tabletop centrifuge (with plate holders if using 96-well plates).
9. Amplify the samples in a GeneAmp™ PCR System 9700 with the silver or gold-plated silver 96-well block or a Veriti™ 96-Well Thermal Cycler.
Note: The SGM 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 SGM Plus™ Kit.

Select the correct PCR cycle number

All AmpFℓSTR™ 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 AmpFℓSTR™ SGM Plus™ PCR Amplification Kit is optimized for 28 cycles of amplification only.

The results of developmental validation studies are shown in "Developmental Validation" on page 66.

Perform PCR

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* (Pub. no.4440754).

Initial incubation step	Melt	Anneal	Extend	Final extension	Final hold
HOLD	CYCLE (28)			HOLD	HOLD
95°C 11 min	94°C 1 min	59°C 1 min	72°C 1 min	60°C 45 min	25°C ∞

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

IMPORTANT! If using the 9700 thermal cycler with silver or gold-plated silver block and adhesive clear film instead of caps to seal the plate wells, be sure to place a MicroAmp™ compression pad (Part no. 4312639) on top of the plate to prevent evaporation during thermal cycling. The Veriti™ Thermal Cycler does not require a compression pad.

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

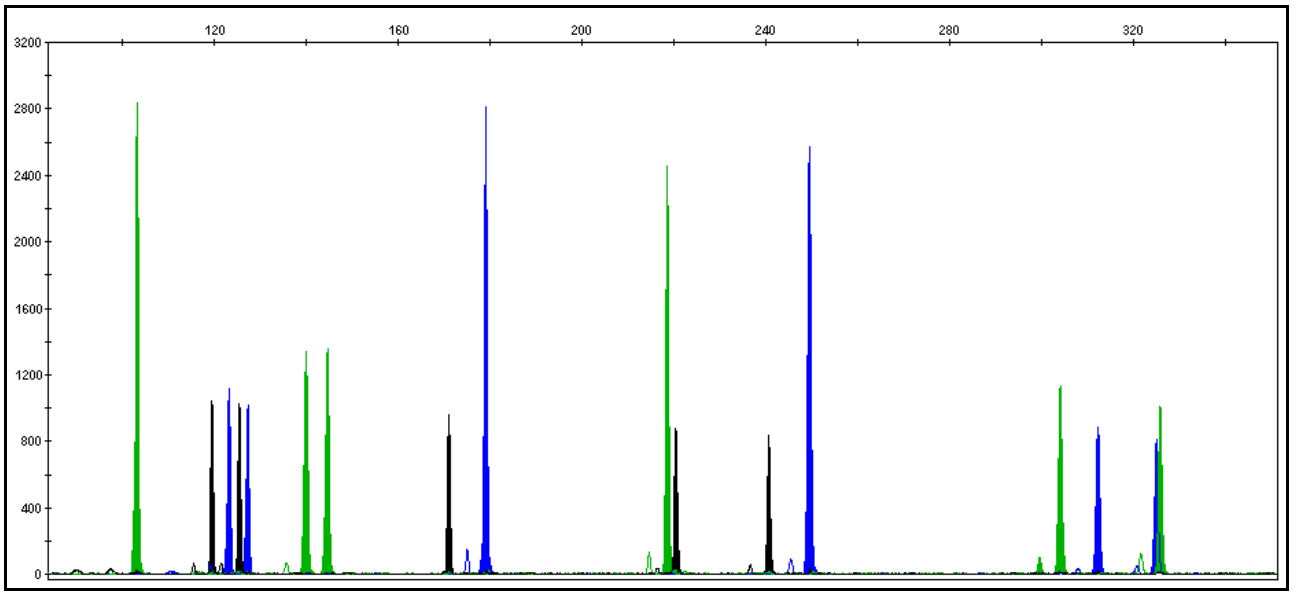
IMPORTANT! Store the amplified products so that they are protected from light.

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. Our studies indicate that a 1.2-mm bloodstained disc contains approximately 5–20 ng DNA. An appropriate cycle number for this high quantity of DNA is 25 cycles as determined by our 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 a MicroAmp™ tube for 25 cycles.

Figure 4 AmpFℓSTR™ SGM Plus™ Kit PCR Amplification Kit results from a 1.2-mm FTA bloodstain disc (25-cycle amplification), analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer



3 Electrophoresis

Allelic ladder requirements

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

Instrument	Number of allelic ladders to run	One injection equals	Number of samples per allelic ladder(s)
3100-*Avant* or 3130	1 per 4 injections	4 samples	15 samples + 1 allelic ladder
3100 or 3130*xl*	1 per injection	16 samples	15 samples + 1 allelic ladder
3500	1 per 3 injections	8 samples	23 samples + 1 allelic ladder
3500xL	1 per injection	24 samples	23 samples + 1 allelic ladder
310	1 per 10 injections	1 sample	9 samples + 1 allelic ladder

IMPORTANT! Variation in laboratory temperature can cause changes in fragment migration speed and sizing variation between both single- and multiple-capillary runs (with larger size variations seen between samples injected in multiple-capillary runs). We recommend the above 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.

It is critical to genotype using 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.

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

"Ordering Information" on page 117 lists the required materials not supplied with the AmpFℓSTR™ SGM Plus™ PCR Amplification Kit.

IMPORTANT! The fluorescent dyes attached to the primers are light sensitive. Protect the primer set, amplified DNA, allelic ladder, and size standard from light when not in use. Keep freeze-thaw cycles to a minimum.

Electrophoresis software setup and reference documents

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

Genetic Analyzer	Data Collection Software	Operating System	Run modules and conditions	References
Applied Biosystems 3130/3130*xl*	3.0	Windows™ XP	• HIDFragmentAnalysis36_POP4_1 Injection conditions: – 3130 = 3 kV/5 sec – 3130*xl* = 3 kV/10 sec • Dye Set F	*Applied Biosystems 3130/3130xl Genetic Analyzers Using Data Collection Software v3.0, Protocols for Processing* AmpFℓSTR™ *PCR Amplification Kit PCR Products User Bulletin* (Pub. no. 4363787)
Applied Biosystems 3100	2.0	Windows™ 2000	• HIDFragmentAnalysis36_POP4_1 Injection condition: 3kV/10 sec • Dye Set F	*3100/3100-Avant Genetic Analyzers Using Data Collection Software v2.0, Protocols for Processing* AmpFℓSTR™ *PCR Amplification Kit PCR Products User Bulletin* (Pub. no. 4350218)
	1.1	Windows™ NT	• GeneScan36_POP4DyeSetF Injection condition: 3kV/10 sec • GS500Analysis.gsp	*3100/3100-Avant Genetic Analyzers Protocols for Processing* AmpFℓSTR™ *PCR Amplification Kit PCR Products User Bulletin* (Pub. no. 4332345)
Applied Biosystems 3100-*Avant*	1.0	Windows™ NT	• GeneScan36A_POP4DyeSetFModule Injection condition: 3 kV/5sec • GS500Analysis.gsp	*3100/3100-Avant Genetic Analyzers Protocols for Processing* AmpFℓSTR™ *PCR Amplification Kit PCR Products User Bulletin* (Pub. no. 4332345)

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

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 size standard needed to prepare the samples:

Reagent	Volume per reaction
GeneScan™ 500 ROX™ Size Standard	0.5 µL
Hi-Di™ Formamide	8.5 µL

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 experiments and results.

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 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, then place on the autosampler.
9. Ensure that a plate record is completed and link the plate record to the plate.
10. Start the electrophoresis run.

Section 3.2 3500/3500xL Series instruments

Set up the 3500/3500xL instrument for electrophoresis

Reagents and parts

"Ordering Information" on page 117 lists the required materials not supplied with the AmpFℓSTR™ SGM Plus™ PCR Amplification Kit.

IMPORTANT! The fluorescent dyes attached to the primers are light sensitive. Protect the primer set, amplified DNA, allelic ladder, and size standard from light when not in use. Keep freeze-thaw cycles to a minimum.

Electrophoresis software setup and reference documents

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

Genetic Analyzer	Data Collection Software	Operating System	Run modules and conditions	References
Applied Biosystems 3500	3500 Data Collection Software v1.0	Windows™ XP *or* Windows Vista ™	• HID36_POP4 Injection conditions: 1.2kV/15 sec • Dye Set F	*Applied Biosystems 3500/3500xL Genetic Analyzer User Guide* (Pub. no. 4401661) *Applied Biosystems 3500 and 3500xL Genetic Analyzers Quick Reference Card* (Pub. no. 4401662)
Applied Biosystems 3500xL			• HID36_POP4 Injection conditions: 1.2kV/24 sec • Dye Set F	

Prepare samples for electrophoresis on the 3500/3500xL instrument

Prepare the samples for electrophoresis on the 3500/3500xL instrument immediately before loading.

1. Calculate the volume of Hi-Di™ Formamide and size standard needed to prepare the samples:

Reagent	Volume per reaction
GeneScan™ 500 ROX™ Size Standard	0.5 µL
Hi-Di™ Formamide	8.5 µL

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, or each MicroAmp™ optical strip tube, 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 or strip tubes with the appropriate septa, then centrifuge to ensure that the contents of each well are collected at the bottom.
6. Heat the reaction plate or strip tubes in a thermal cycler for 3 minutes at 95°C.
7. Immediately put the plate or strip tubes on ice for 3 minutes.
8. Prepare the plate assembly, then place on the autosampler.
9. Ensure that a plate record is completed and link the plate record to the plate.
10. Start the electrophoresis run.

Section 3.3 310 Instrument

Set up the 310 instrument for electrophoresis

Reagents and parts

"Ordering Information" on page 117 lists the required materials not supplied with the AmpFℓSTR™ SGM Plus™ PCR Amplification Kit.

IMPORTANT! The fluorescent dyes attached to the primers are light sensitive. Protect the primer set, amplified DNA, allelic ladder, and size standard from light when not in use. Keep freeze-thaw cycles to a minimum.

Electrophoresis software setup and reference documents

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

Data Collection Software	Operating System	Run modules and conditions	References
3.1† *or* 3.0†	Windows™ XP *or* Windows™ NT and Windows™ 2000	• GS STR POP4 (1mL) Injection condition: 15 kV/5 sec	*310 Genetic Analyzer User's Manual (Windows)* (Pub. no. 4317588) *310 Protocols for Processing* AmpFℓSTR™ *PCR Amplification Kit Products with Microsoft Windows NT Operating System: User Bulletin* (Pub. no. 4341742)

† We conducted concordance studies for the SGM Plus™ Kit using this configuration.

Prepare samples for electrophoresis on the 310 instrument

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

1. Calculate the volume of Hi-Di™ Formamide and size standard needed to prepare the samples:

Reagent	Volume per reaction
GeneScan™ 500 ROX™ Size Standard	0.75 µL
Hi-Di™ Formamide	24.5 µL

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 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

   Note: For blank wells, add 25 µL of Hi-Di™ Formamide.
5. Seal the tubes with the appropriate septa, then briefly centrifuge to ensure that the contents of each tube are mixed and collected at the bottom.
6. Heat the tubes in a thermal cycler for 3 minutes at 95°C.
7. Immediately place the tubes on ice for 3 minutes.
8. Place the sample tray on the autosampler.
9. Ensure that an injection list is prepared.
10. Start the electrophoresis run.

4 Data Analysis

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 "Instrument and software overview" on page 16 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.

Set up GeneMapper™ *ID* Software for data analysis

File names

The file names shown in this section may differ from the file names you see when you download or import files. If you need help determining the correct files to use, contact your local Life Technologies Human Identification representative, or go to **www.lifetechnologies.com/support** ▸ **Software, Patches & Updates** ▸ **GeneMapper™ *ID* Software**.

Workflow

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

- Import panels and bins into the Panel Manager, as explained in "Import panels and bins" on page 35.
- Create an analysis method, as explained in , "Create an analysis method" on page 38.
- Create a size standard, as explained in "Create size standard" on page 43.
- Define custom views of analysis tables.
  Refer to Chapter 1 of the *GeneMapper™ ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial* (Pub. no. 4335523) for more information.
- Define custom views of plots.
  Refer to Chapter 1 of the *GeneMapper™ ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial* (Pub. no. 4335523) for more information.

Import panels and bins

To import the SGM Plus™ panel and bin set into the GeneMapper™ *ID* Software v3.2.1 database:

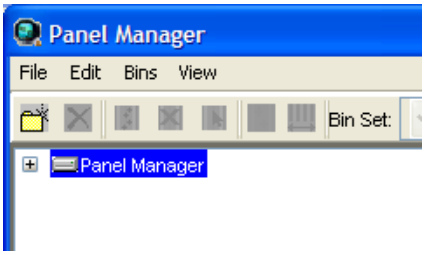
1. Start the GeneMapper™ *ID* Software, then log in with the appropriate user name and password.

   IMPORTANT! If you need logon instructions, refer to page 2-7 of the *GeneMapper™ ID Software Version 3.1 Human Identification Analysis User Guide* (Pub. no. 4338775).

2. Select **Tools** ▸ **Panel Manager**.

3. Find, then open the folder containing the panels and bins:

   a. Select **Panel Manager** in the navigation pane.

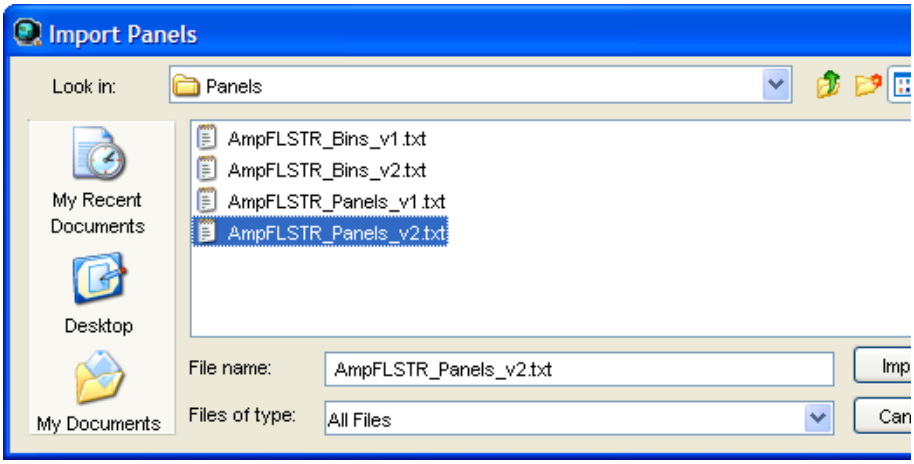
   

   b. Select **File** ▸ **Import Panels** to open the Import Panels dialog box.

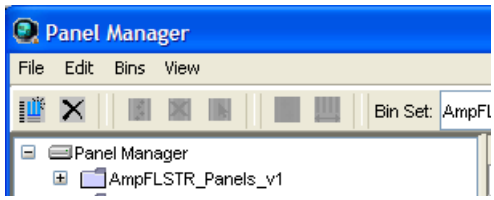
   c. Navigate to, then open the ***x***:\Applied Biosystems\GeneMapper\Panels folder, where *x* is the drive on which the GeneMapper™ *ID* Software is installed.

4. Select **AmpFLSTR_Panels_v2.txt**, then click **Import**.

   Note: Importing this file creates a new folder in the navigation pane of the Panel Manager, AmpFLSTR_Panels_v2. This folder contains the panels and associated markers.

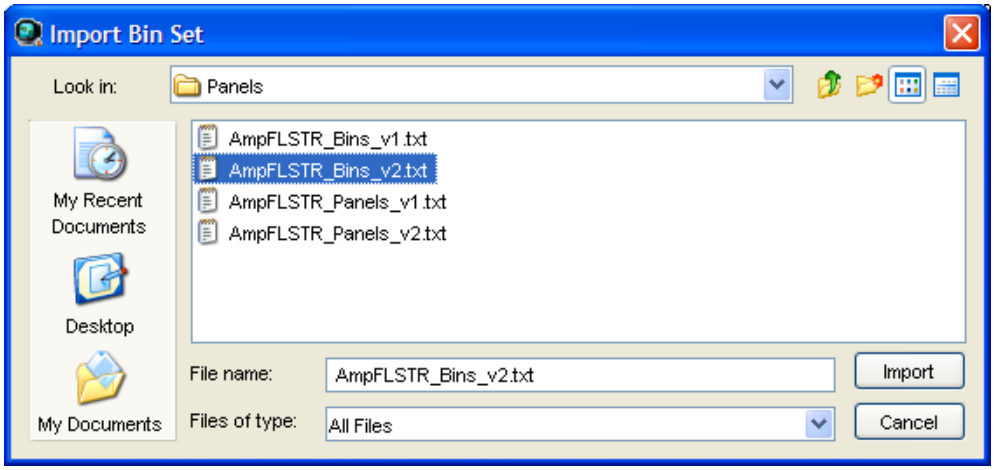
   

5. Import AmpFLSTR_Bins_v2.txt:
   a. Select the **AmpFLSTR_Panels_v2** folder in the navigation pane.

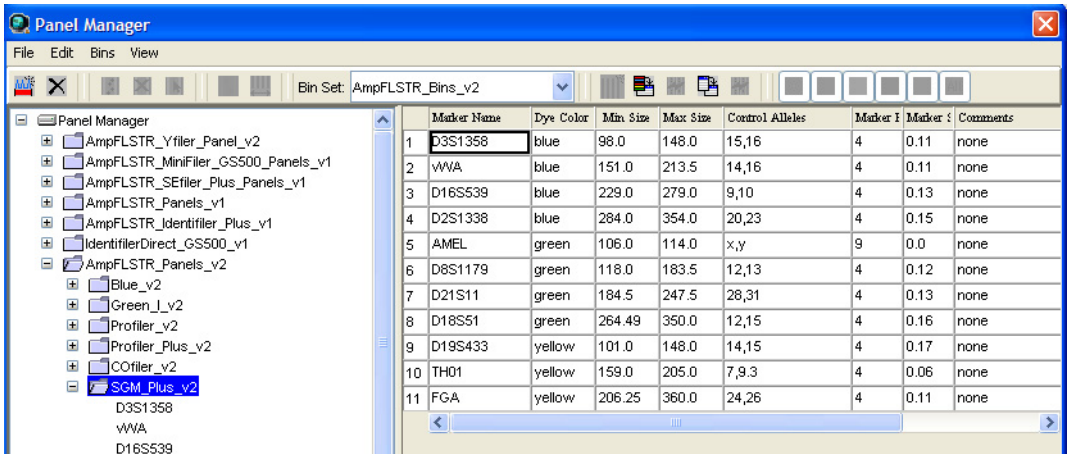


   b. Select **File ▸ Import Bin Set** to open the Import Bin Set dialog box.
   c. Navigate to, then open the ***x***:\Applied Biosystems\GeneMapper\Panels folder.
   d. Select **AmpFLSTR_Bins_v2.txt**, then click **Import**.

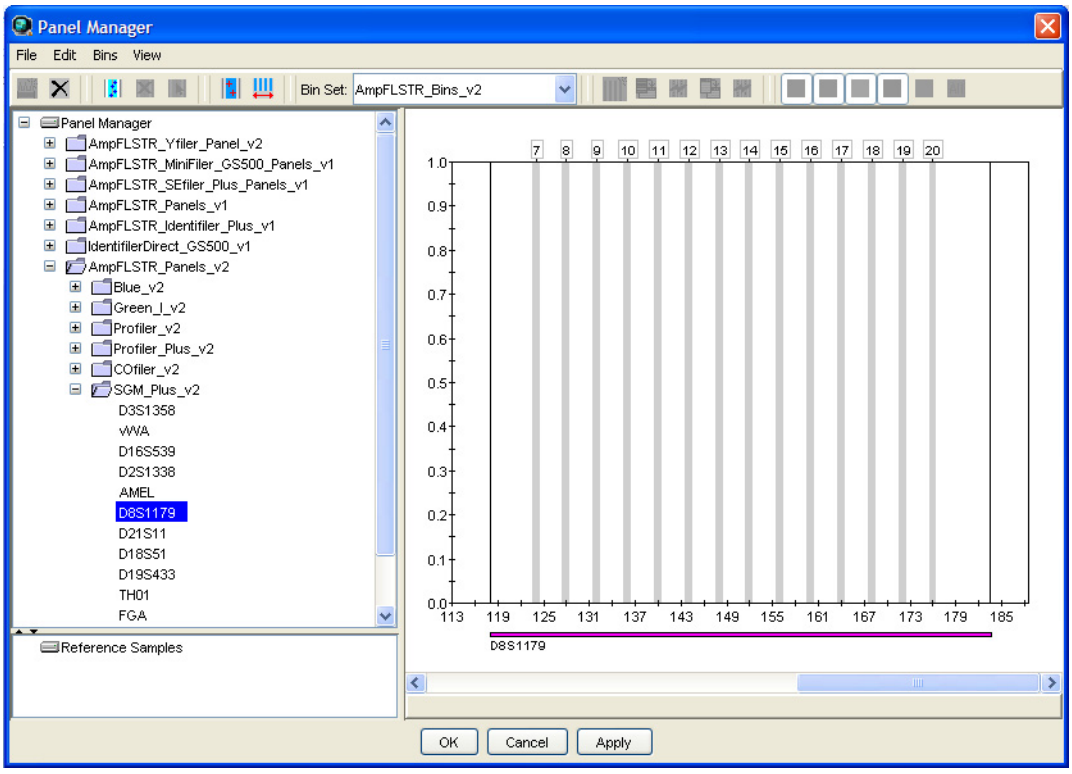
   Note: Importing this file associates the bin set with the panels in the AmpFLSTR_Panels_v2 folder.



6. View the imported panels in the navigation pane:
   a. Double-click the **AmpFLSTR_Panels_v2** folder.
   b. Double-click the **SGM_Plus_v2** folder to display the panel information in the right pane and the markers below it.



7. View the markers and display the Bin view in the navigation pane:
   a. Double-click the **AmpFLSTR_Panels_v2** folder to display its list of kits in the right pane.
   b. Double-click the **SGM_Plus_v2** folder to display its list of markers below it.
   c. Select **D8S1179** to display the Bin view for the marker in the right pane.



8. Click **Apply**, then **OK** to add the SGM_Plus_v2 panel and bin set to the GeneMapper™ *ID* Software database.

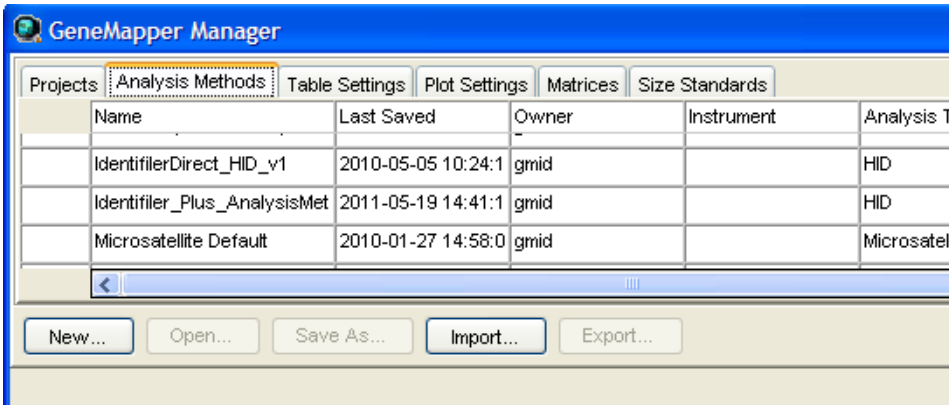
IMPORTANT! If you close the Panel Manager without clicking OK, the panels and bins are not imported into the GeneMapper™ *ID* Software database.

Create an analysis method

The HID Advanced analysis method for the SGM Plus™ Kit uses the AmpFLSTR_Bins_v2 file described in step 5 on page 36.

Use the following procedure to create a HID analysis method for the SGM Plus™ Kit.

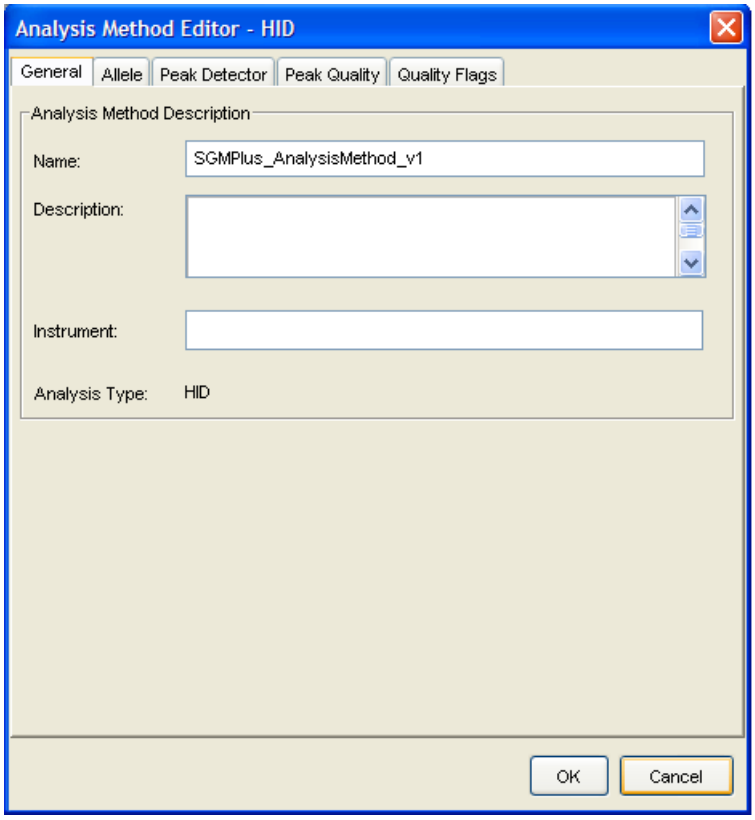
1. Select **Tools** ▸ **GeneMapper Manager** to open the GeneMapper Manager.



2. Select the **Analysis Methods** tab, then click **New** to open the New Analysis Method dialog box.
3. Select **HID** and click **OK** to open the Analysis Method Editor with the General Tab selected.
4. The figures below show the settings for each tab of the Analysis Method Editor. Configure settings as shown unless the instructions state otherwise.

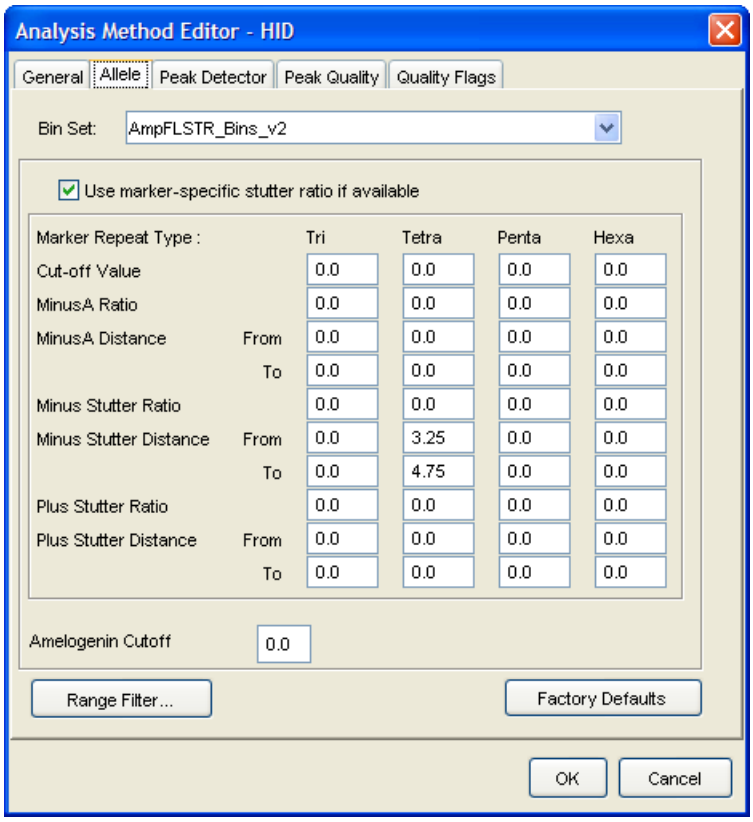
   Note: The Analysis Method Editor closes when you save your settings. To complete this step quickly, do not save the analysis method until you finish entering settings in all of the tabs.
5. After you enter settings in all tabs, click **Save**.

General tab settings



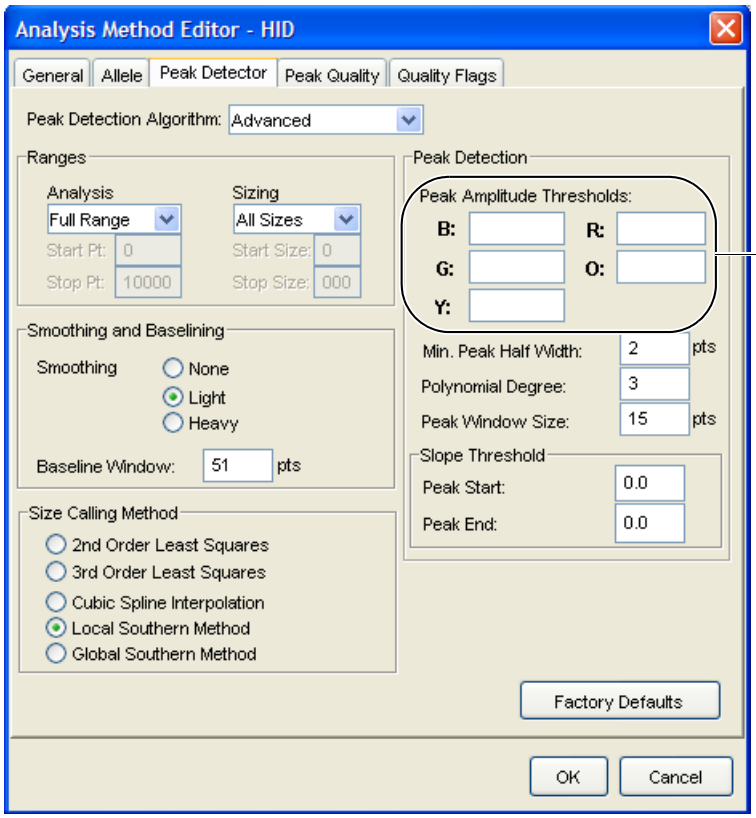
In the Name field, either type the name as shown, or enter a name of your choosing. The Description and Instrument fields are optional.

Allele tab settings



- In the Bin Set field, select the **AmpFLSTR_Bins_v2** bin set imported previously and configure the stutter distance parameters as shown.
- 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.
- Specify the stutter ratio:
  - To apply the stutter ratios listed in the Allele tab for single-source data, deselect the "Use marker-specific stutter ratio if available" check box (selected by default). Perform appropriate internal validation studies to determine the appropriate filter setting to use.
    Note: Applying global stutter ratios may reduce the editing required for single-source sample data.
  - To apply the stutter ratios contained in the AmpFLSTR_Panels_v2.txt file, select the "Use marker-specific stutter ratio if available" check box (selected by default). Perform appropriate internal validation studies to determine the appropriate filter setting to use.

Peak Detector tab settings



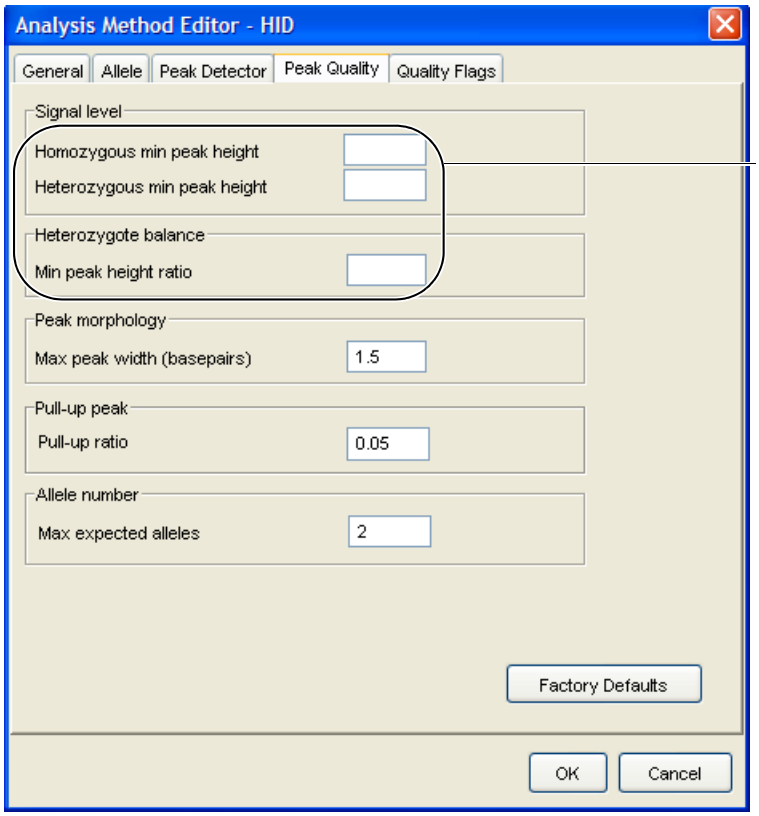
Perform internal validation studies to determine settings

IMPORTANT! Perform the appropriate internal validation studies to determine the peak amplitude thresholds for interpretation of SGM Plus™ Kit data.

Fields include:

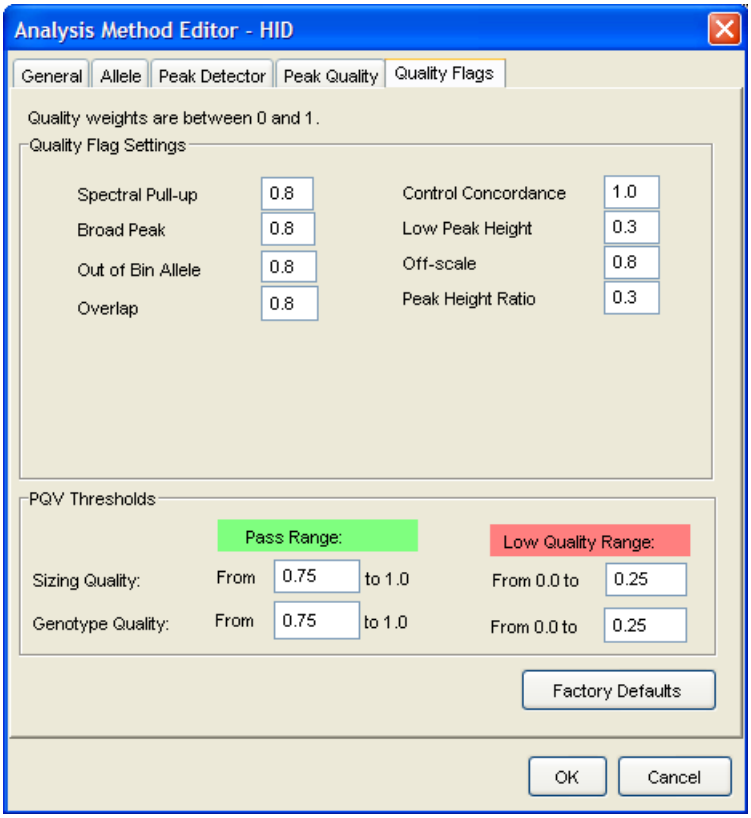
- **Peak amplitude thresholds** – The software uses these parameters to specify the minimum peak height, in order to limit the number of detected peaks. Although GeneMapper™ *ID* Software displays peaks that fall below the specified amplitude in electropherograms, the software does not label or determine the genotype of these peaks.
- **Size calling method** – The SGM Plus™ Kit has been validated using the Local Southern sizing method. Before using other sizing methods, perform internal validation studies.

Peak Quality tab settings



IMPORTANT! Perform the appropriate internal validation studies to determine the minimum heterozygous and homozygous minimum peak height thresholds and the minimum peak height ratio threshold for interpretation of SGM Plus™ Kit data.

Quality Flags tab settings



IMPORTANT! The values shown are the software defaults and are the values we used during developmental validation. Perform the appropriate internal validation studies to determine the appropriate values for interpretation of SGM Plus™ Kit data.

Create size standard

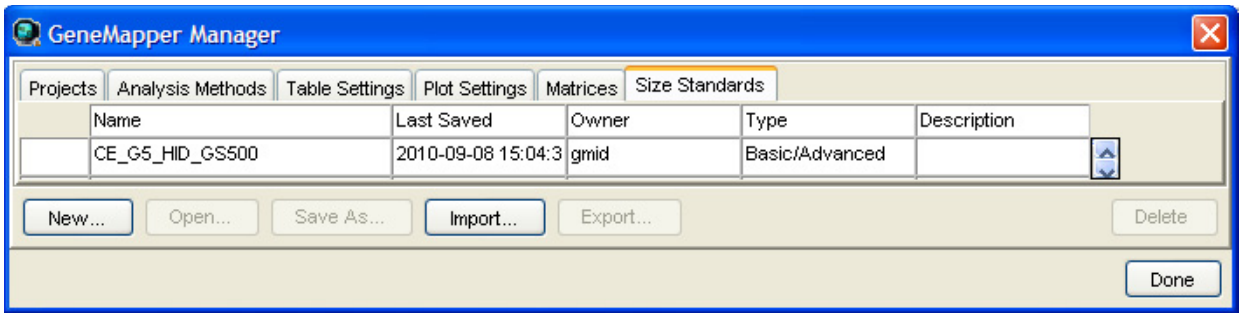
The GeneScan™ 500 ROX™ Size Standard for the SGM Plus™ Kit uses the following size standard peaks in its definitions: 75, 100, 139, 150, 160, 200, 300, 340, 350, 400, and 450.

Note: The 250-nt peak in the GeneScan™ 500 ROX™ Size Standard is not included in the size standard definition. This peak can be used as an indicator of precision within a run.

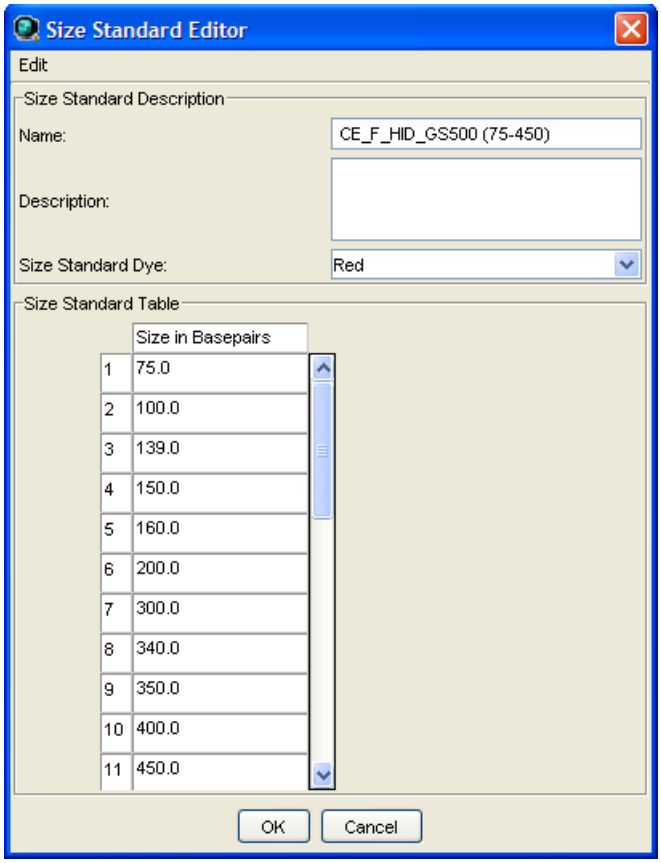
Use the following procedure to create the appropriate size standard:

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

2. Select the **Size Standards** tab, click **New**, select the **Basic or Advanced** radio button, then click **OK**.



3. Enter a name (for example, CE_F_HID_GS500(75-450)). In the Size Standard Dye field, select **Red**. In the Size Standard Table, enter the sizes specified in on page 43. The example below is for the GeneScan™ 500 ROX™ Size Standard.



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

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	SGMPlus_AnalysisMethod_v1 (or the name of the analysis method you created)
Panel	SGM_Plus_v2
Size Standard	CE_F_HID_GS500(75-450)† (or the name of the size standard you created)

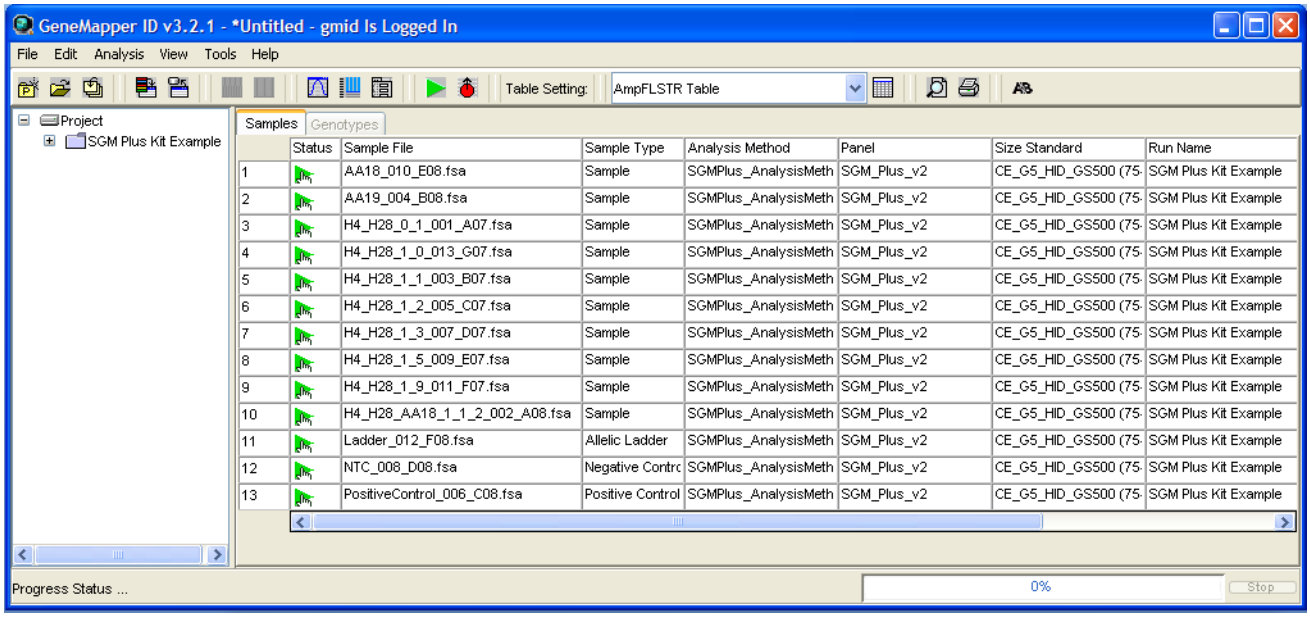
† The SGM Plus™ Kit was originally validated using the GeneScan™ 500 ROX™ Size Standard. If you use the GeneScan™ 400 HD Size Standard as an alternative, perform the appropriate internal validation studies to support the use of this size standard with the SGM Plus™ Kit.

Note: For more information about how the Size Caller works, refer to the *GeneScan™ Analysis Software for the Windows™ NT Operating System Overview of the Analysis Parameters and Size Caller User Bulletin* (Pub. no. 4335617).

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 indicated
     - With text messages on the left

- The table displays the row of the sample currently being analyzed in green (or red if analysis failed for the sample).
- The Genotypes tab becomes available after analysis (see the figure on the next page).

Project window after analysis



For more information about any of these tasks, refer to the GeneMapper™ *ID* Software *Version 3.1 Human Identification Analysis User Guide* (Pub. no. 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

For details about GeneMapper™ *ID* Software features, allele filters, peak detection algorithms, and project editing, refer to:

- *GeneMapper™ ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial (Pub. no. 4335523)*
- *GeneMapper™ ID Software Version 3.1 Human Identification Analysis User Guide (Pub. no. 4338775)*
- *Installation Procedures and New Features for GeneMapper™ ID Software Software Version v3.2 User Bulletin* (Pub. no. 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 or .hid file. Using GeneMapper™ *ID-X* Software v1.0.1 or higher you can then analyze and interpret the data from the .fsa or .hid files.

Instruments

Refer to "Instrument and software overview" on page 16 for a list of compatible instruments.

Before you start

When using GeneMapper™ *ID-X* Software v1.0.1 or higher 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-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 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.

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

Panel, bin, and stutter file version

The file names shown in this section may differ from the file names you see when you download or import files. If you need help determining the correct files to use, contact your local Life Technologies Human Identification representative, or go to **www.lifetechnologies.com/support** ▸ **Software, Patches & Updates** ▸ **GeneMapper™ *ID-X* Software**.

The instructions and examples in this section refer to the latest version of panel, bin, and stutter file available at the time of publication.

Before using the software for the first time

Before you use GeneMapper™ *ID-X* Software (v1.0.1 or higher for .fsa files, v1.2 or higher for .hid files) to analyze data for the first time, you must do the following:

1. Check the version of panel, bin, and stutter files installed with the GeneMapper™ *ID-X* Software as explained in "Check panel, bin, and stutter file version" below.
2. Check **www.lifetechnologies.com/support** ▸ **Software, Patches & Updates** ▸ **GeneMapper™ *ID-X* Software** to determine if newer files are available.
3. If updated files are available, download and import the files into the GeneMapper™ *ID-X* Software, as explained in "Import panels, bins, and marker stutter" on page 50.
   Note: When downloading new versions of analysis files, refer to the associated Read Me file for details of changes between software file versions. If you have validated previous file versions for data analysis, conduct the appropriate internal verification studies before using new file versions for operational analysis.
4. Create an analysis method, as explained in "Create an analysis method" on page 55.
5. Define custom views of analysis tables.
   Refer to Chapter 1 of the *GeneMapper™ ID-X Software Version 1.0 Getting Started Guide* (Pub. no. 4375574) for more information.
6. Define custom views of plots.
   Refer to Chapter 1 of the *GeneMapper™ ID-X Software Version 1.0 Getting Started Guide* (Pub. no. 4375574) for more information.

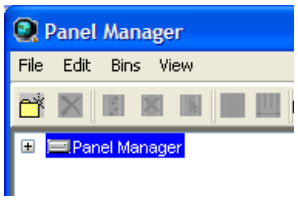
Check panel, bin, and stutter file version

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

   IMPORTANT! For logon instructions, refer to the *GeneMapper™ ID-X Software Version 1.0 Getting Started Guide* (Pub. no. 4375574).

2. Select **Tools** ▸ **Panel Manager**.

3. Check the version of files imported into the Panel Manager:



   a. Select **Panel Manager** in the navigation pane.
   b. Expand the Panel Manager folder and any sub-folders to identify the analysis file version already installed for your kit choice.

4. Check the version of files available for import into the Panel Manager:
   a. Select **Panel Manager**, then select **File ▸ Import Panels** to open the Import Panels dialog box.
   b. Navigate to, then open the Panels folder and check the version of panel, bin, and stutter files installed.

5. If newer versions are available on the website, download and import as described below.

Import panels, bins, and marker stutter

To import the SGM Plus™ Kit panel, bin set, and marker stutter from our web site into the GeneMapper™ *ID-X* Software database:

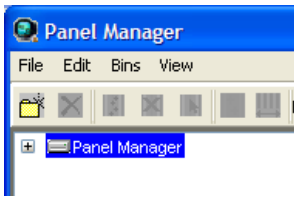
1. Download and open the file containing panels, bins, and marker stutter:
   a. Go to **www.lifetechnologies.com/support ▸ Software, Patches & Updates ▸ GeneMapper™ *ID-X* Software**. Download the file **AmpFLSTR Analysis Files GMIDX**.
   b. Unzip the file.

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

IMPORTANT! For logon instructions, refer to the *GeneMapper™ ID-X Software Version 1.0 Getting Started Guide* (Pub. no. 4375574).

3. Select **Tools ▸ Panel Manager**.

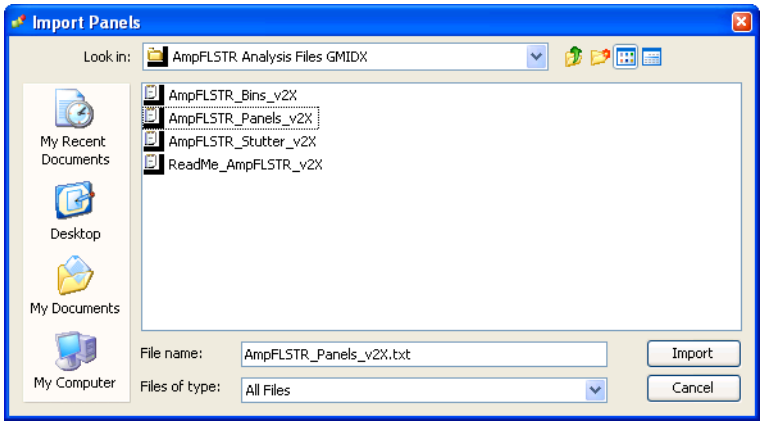
4. Find, then open the folder containing the panels, bins, and marker stutter:
   a. Select **Panel Manager** in the navigation pane.



   b. Select **File ▸ Import Panels** to open the Import Panels dialog box.
   c. Navigate to, then open the **AmpFLSTR Analysis Files GMIDX** folder that you unzipped in step 1 on page 50.

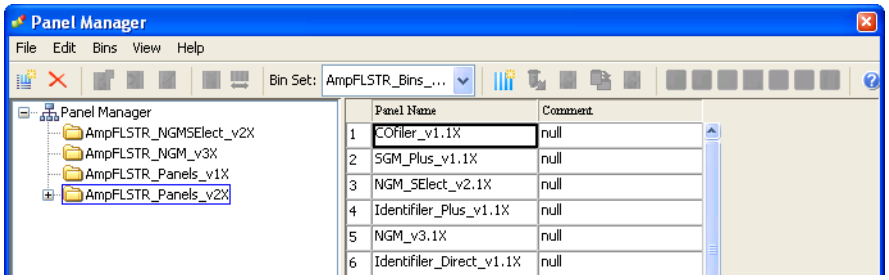
5. Select **AmpFLSTR_Panels_v2X** (or the version you installed), then click **Import**.

   Note: Importing this file creates a new folder in the navigation pane of the Panel Manager "AmpFLSTR_Panels_v2X". This folder contains panels for multiple AmpFℓSTR™ kits and associated markers.



6. Import AmpFLSTR_Bins_v2X.txt:

   a. Select the **AmpFLSTR_Panels_v2X** folder in the navigation pane.

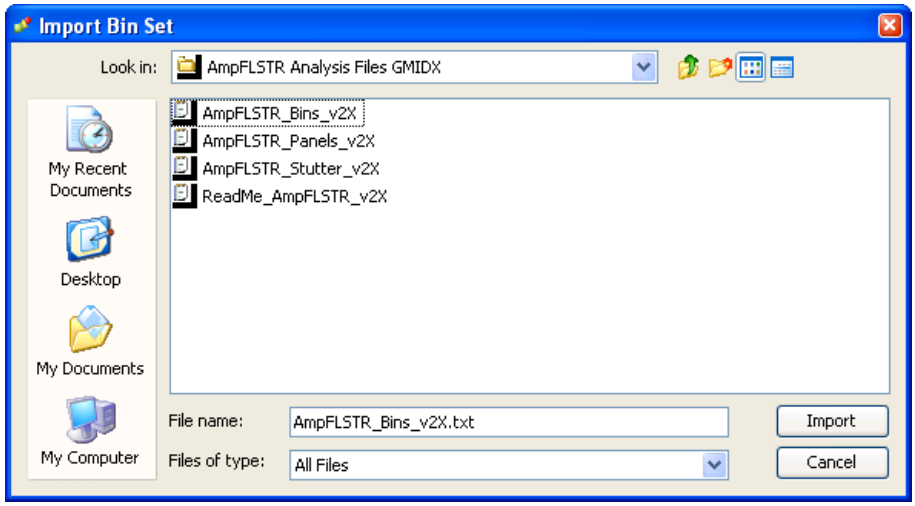


   b. Select **File** ▸ **Import Bin Set** to open the Import Bin Set dialog box.

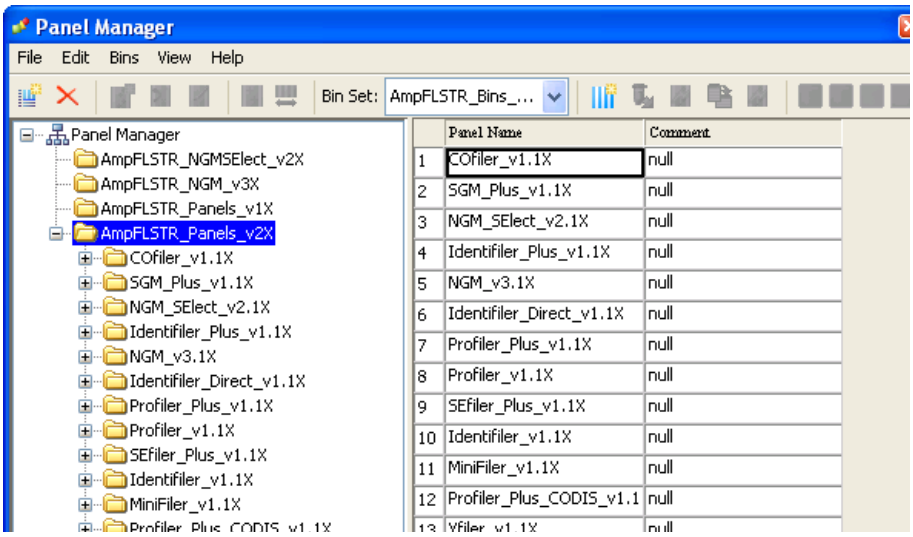
   c. Navigate to, then open the **AmpFLSTR Analysis Files GMIDX** folder.

d. Select **AmpFLSTR_Bins_v2X.txt**, then click **Import**.

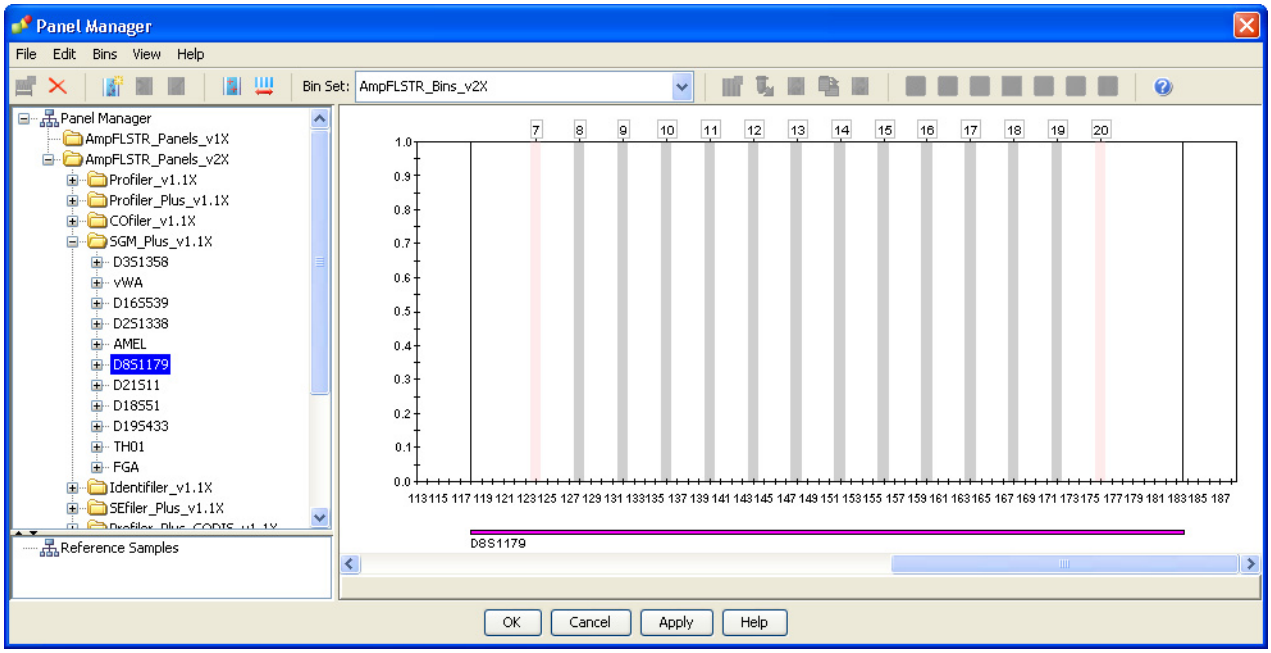
Note: Importing this file associates the bin set with the panels in the AmpFLSTR_Panels_v2X folder.



7. View the imported panels in the navigation pane:
   a. Double-click the **AmpFLSTR_Panels_v2X** folder.
   b. Double-click the **SGM_Plus_v1.1X** folder to display the panel information in the right pane.

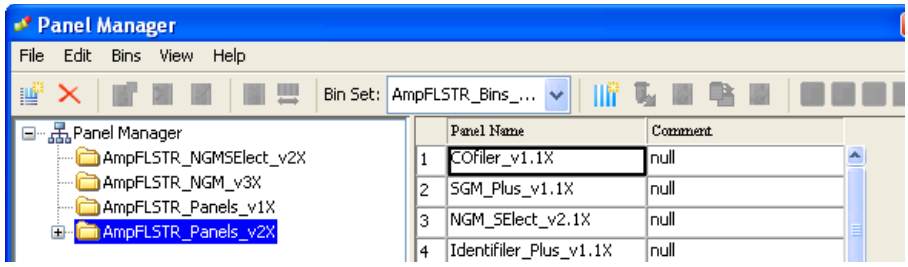


8. Select and expand **SGM_Plus_v1.1X** in the navigation pane, then select **D8S1179** to display the Bin view for the marker in the right pane.



9. Import AmpFLSTR_Stutter_v2X:

   a. Select the **AmpFLSTR_Panels_v2X** folder in the navigation panel.

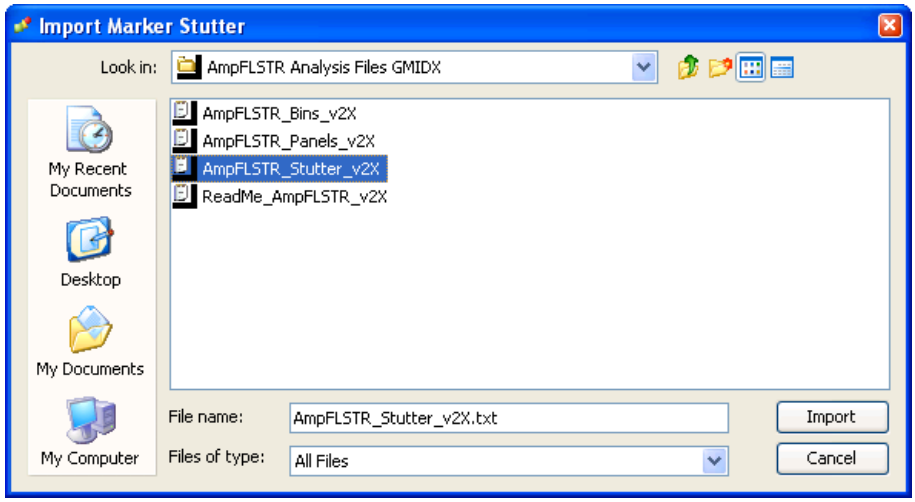


   b. Select File ▸ **Import Marker Stutter** to open the Import Marker Stutter dialog box.

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

d. Select **AmpFLSTR_Stutter_v2X**, then click **Import**.

Note: Importing this file associates the marker stutter ratio with the bin set in the AmpFLSTR_Panels_v2X folder.

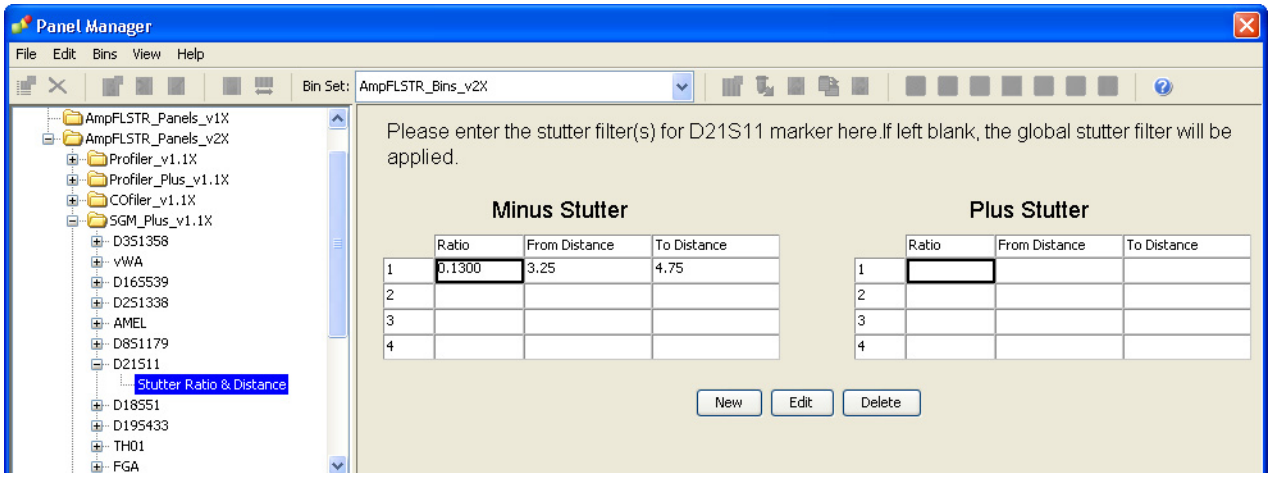


10. View the imported marker stutters in the navigation pane:

a. Double-click the **AmpFLSTR_Panels_v2X** folder to display its list of kits in the right pane.

b. Double-click the **SGM_Plus_v1.1X** folder to display its list of markers below it.

c. Double-click **D21S11** to display the Stutter Ratio & Distance view for the marker in the right pane.



11. Click **Apply**, then **OK** to add the SGM Plus™ Kit panel, bin set, and marker stutter to the GeneMapper™ *ID-X* Software database.

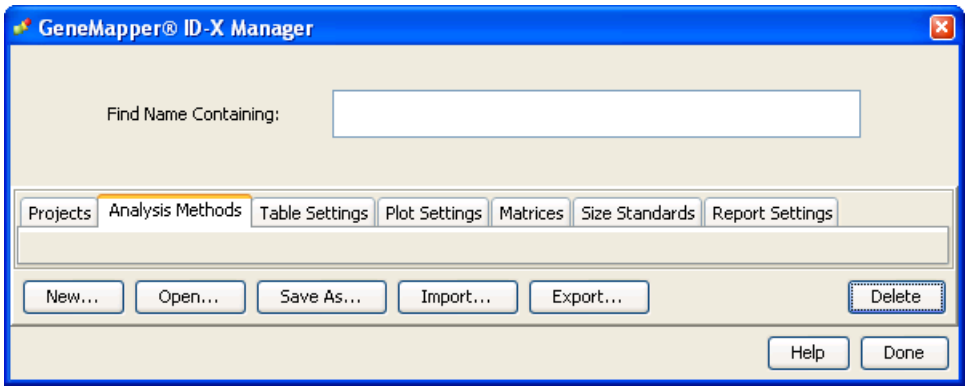
IMPORTANT! If you close the Panel Manager without clicking **Apply**, the panels, bin sets, and marker stutter will not be imported into the GeneMapper™ *ID-X* Software database.

Create an analysis method

Use the following procedure to create an analysis method for the SGM Plus™ Kit.

IMPORTANT! Analysis methods are version-specific, so you must create an analysis method for each version of the software. For example, an analysis method created for GeneMapper™ *ID-X* version 1.2 is not compatible with earlier versions of GeneMapper™ *ID-X* Software or with GeneMapper™ *ID* Software version 3.2.1.

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



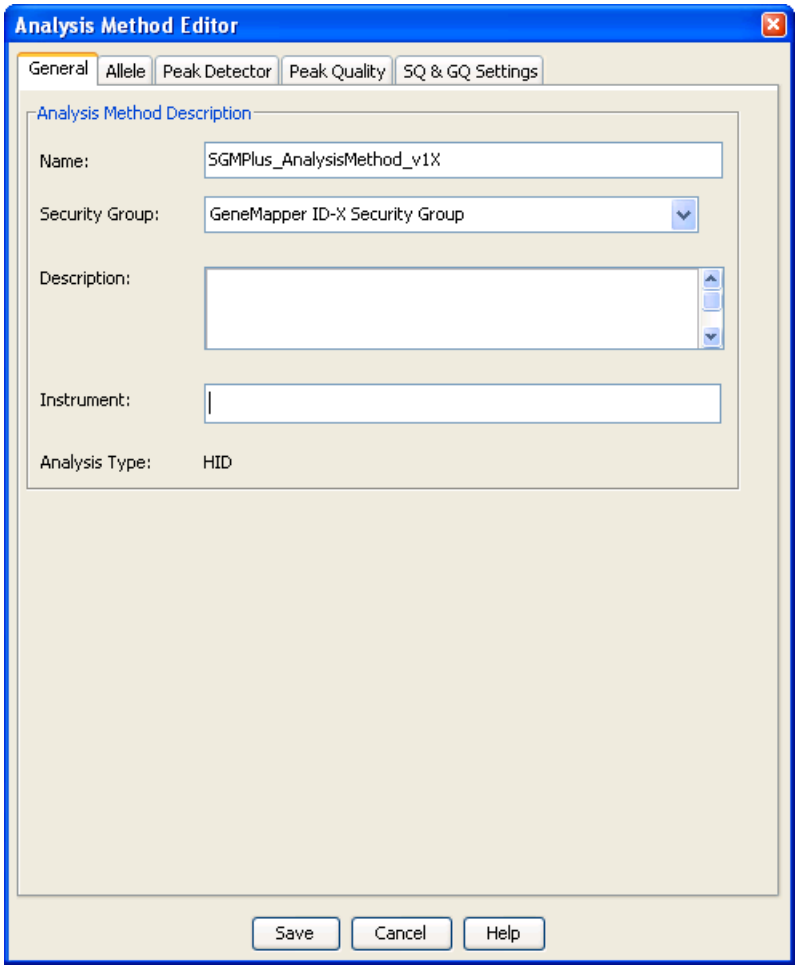
2. Select the **Analysis Methods** tab, then click **New** to open the Analysis Method Editor with the **General** tab selected.

   The figures below show the settings for each tab of the Analysis Method Editor. Configure the Analysis Method Editor tab settings as shown in the figures below, unless the instructions state otherwise.

   Note: The Analysis Method Editor closes when you save your settings. To complete this step quickly, do not save the analysis method until you finish entering settings in all of the tabs.

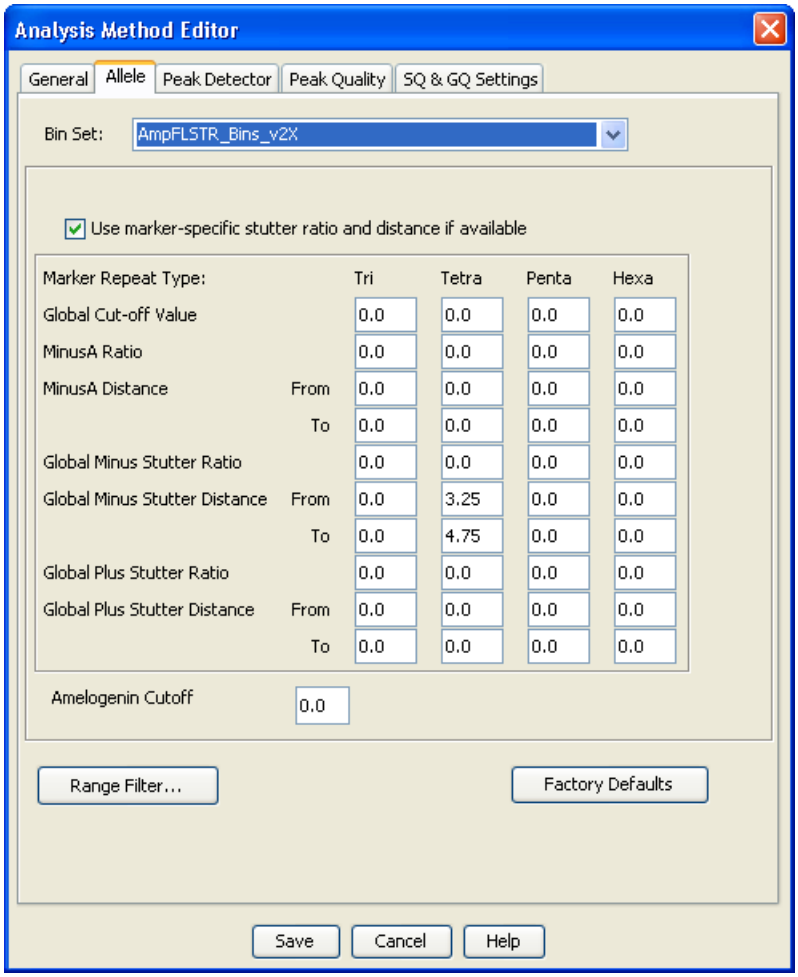
3. After you enter settings in all tabs, click **Save**.

General tab settings



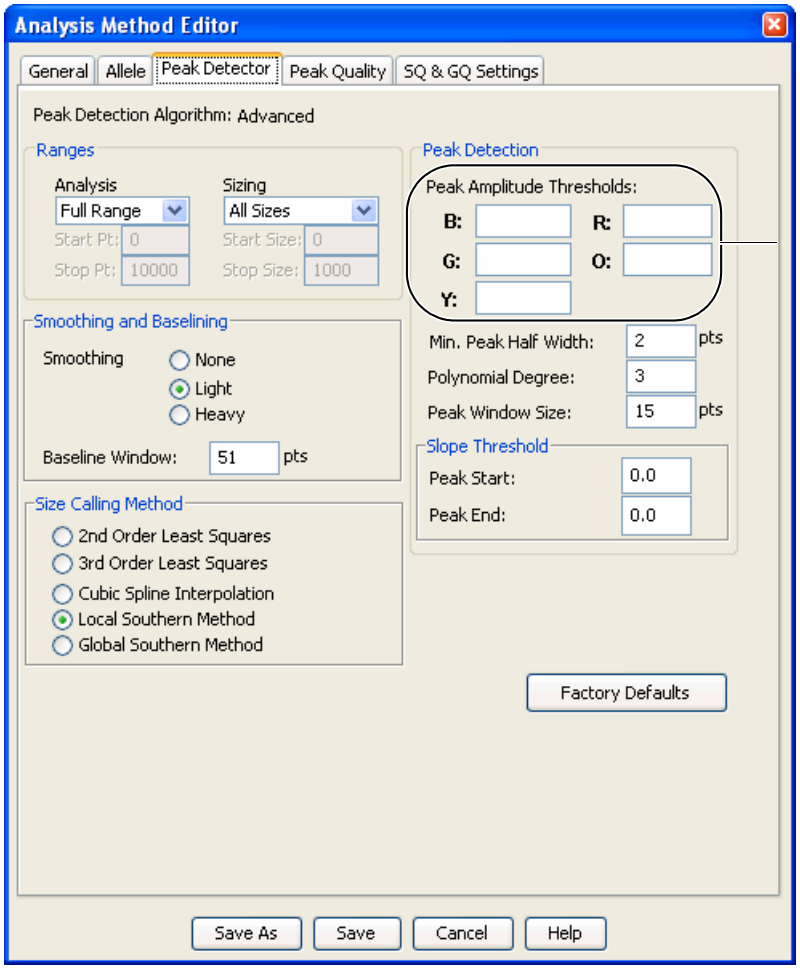
In the Name field, either type the name as shown or enter a name of your choosing. In the Security Group field, select the Security Group appropriate to your software configuration from the dropdown list. The Description and Instrument fields are optional.

Allele tab settings



- In the Bin Set field, select the **AmpFLSTR_Bins_v2X** bin set and configure the stutter distance parameters as shown.
- GeneMapper™ *ID-X* Software v1.0.1 or higher allows you to specify 4 types of marker repeat motifs: tri, tetra, penta and hexa. You can enter parameter values for each type of repeat in the appropriate column.
- Specify the stutter ratio:
  - To apply the stutter ratios listed in the Allele tab for single-source data, deselect the "Use marker-specific stutter ratio if available" check box (selected by default). Perform appropriate internal validation studies to determine the appropriate filter setting to use.
    Note: Applying global stutter ratios may reduce the editing required for single-source sample data.
  - To apply the stutter ratios contained in the AmpFLSTR_Stutter_v2X file, select the "Use marker-specific stutter ratio if available" check box (selected by default). Perform appropriate internal validation studies to determine the appropriate filter setting to use.

Peak Detector tab settings



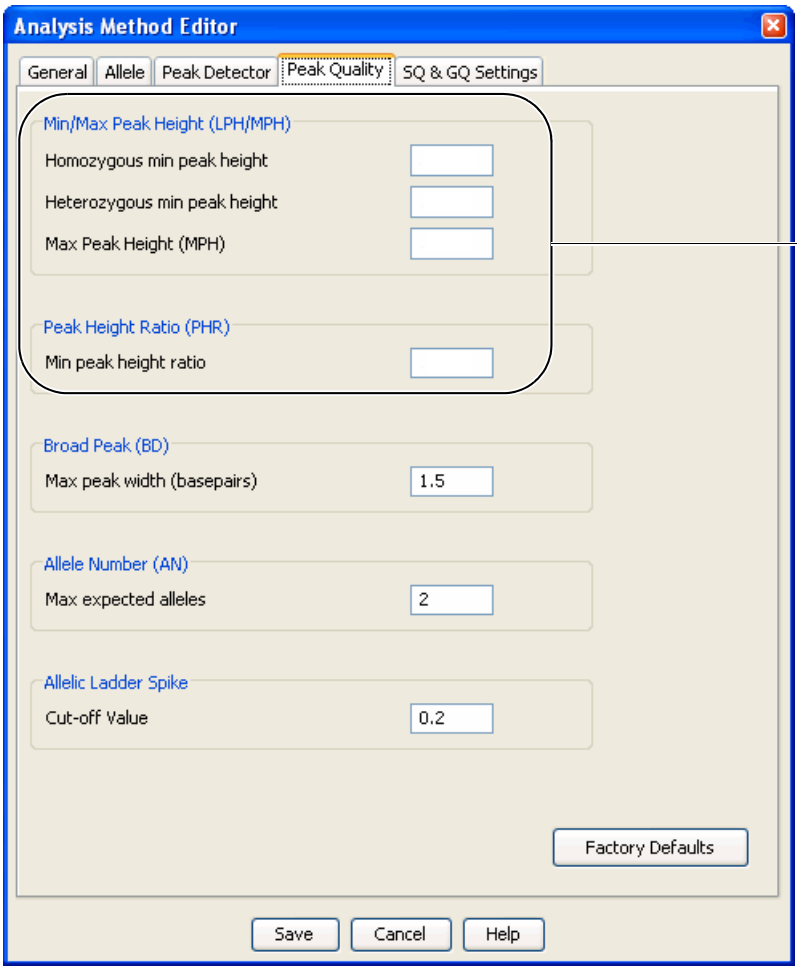
Perform internal validation studies to determine settings

IMPORTANT! Perform the appropriate internal validation studies to determine the appropriate peak amplitude thresholds for interpretation of SGM Plus™ Kit data.

Fields include:

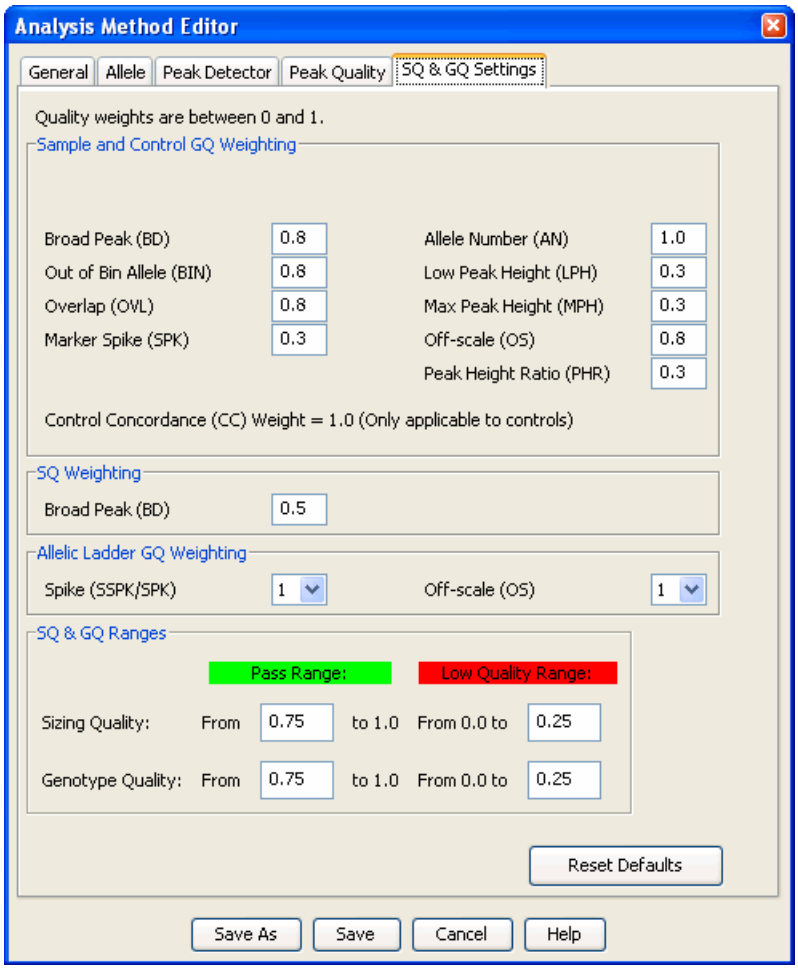
- **Peak amplitude thresholds** – The software uses these parameters to specify the minimum peak height, in order to limit the number of detected peaks. Although GeneMapper™ *ID-X* Software displays peaks that fall below the specified amplitude in electropherograms, the software does not label or determine the genotype of these peaks.
- **Size calling method** – The SGM Plus™ Kit has been validated using the Local Southern sizing method. Before using other sizing methods, perform internal validation studies.
- **Normalization** – A Normalization checkbox is available on this tab in GeneMapper™ *ID-X* Software v1.2 for use in conjunction with data run on the Applied Biosystems 3500 Series Genetic Analyzers. Normalization cannot be applied to 4-dye data, so this feature is not for use with SGM Plus™ Kit data.

Peak Quality tab settings



IMPORTANT! Perform the appropriate internal validation studies to determine the minimum heterozygous and homozygous minimum peak height thresholds, maximum peak height threshold and the minimum peak height ratio threshold for interpretation of SGM Plus™ Kit data.

SQ & GQ tab settings



IMPORTANT! The values shown are the software defaults and are the values we used during developmental validation. Perform appropriate internal validation studies to determine the appropriate values to use.

Create size standard (optional)

The CE_F_HID_GS500 (75–450) size standard definition is installed with the software for use with the SGM Plus™ Kit and contains the following size standard peaks:

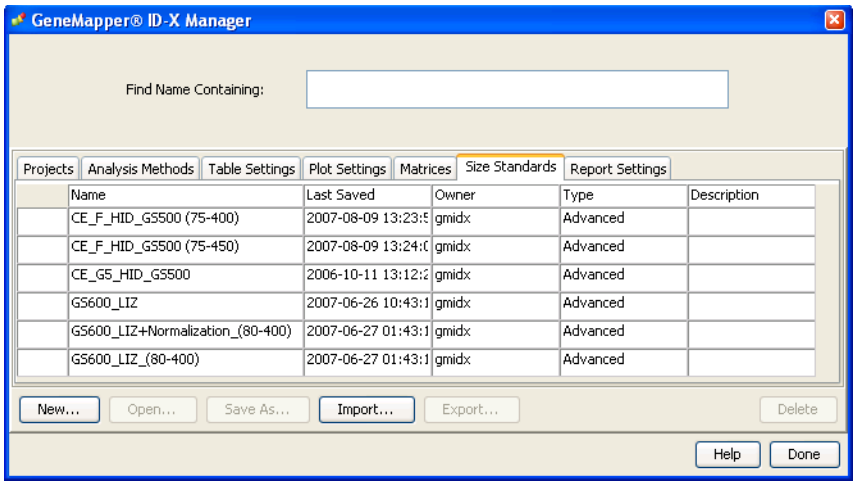
GeneScan™ 500 ROX™ Size Standard
75, 100, 139, 150, 160, 200, 300, 340, 350, 400, and 450

Note: The 250-nt peak in the GeneScan™ 500 ROX™ Size Standard is not included in the size standard definition. This peak can be used as an indicator of precision within a run.

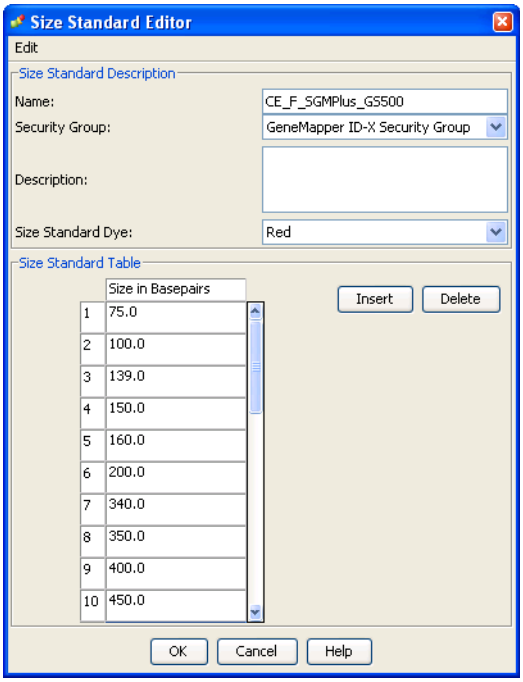
Use the following procedure if you want to create your own size standard:

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

2. Select the **Size Standards** tab, then click **New**.



3. Enter a name. In the Size Standard Dye field, select **Red**. In the Size Standard Table, enter the sizes specified in on page 60. The example below is for the GeneScan™ 500 ROX™ Size Standard.



Analyze and edit sample files with GeneMapper™ *ID-X* Software

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.

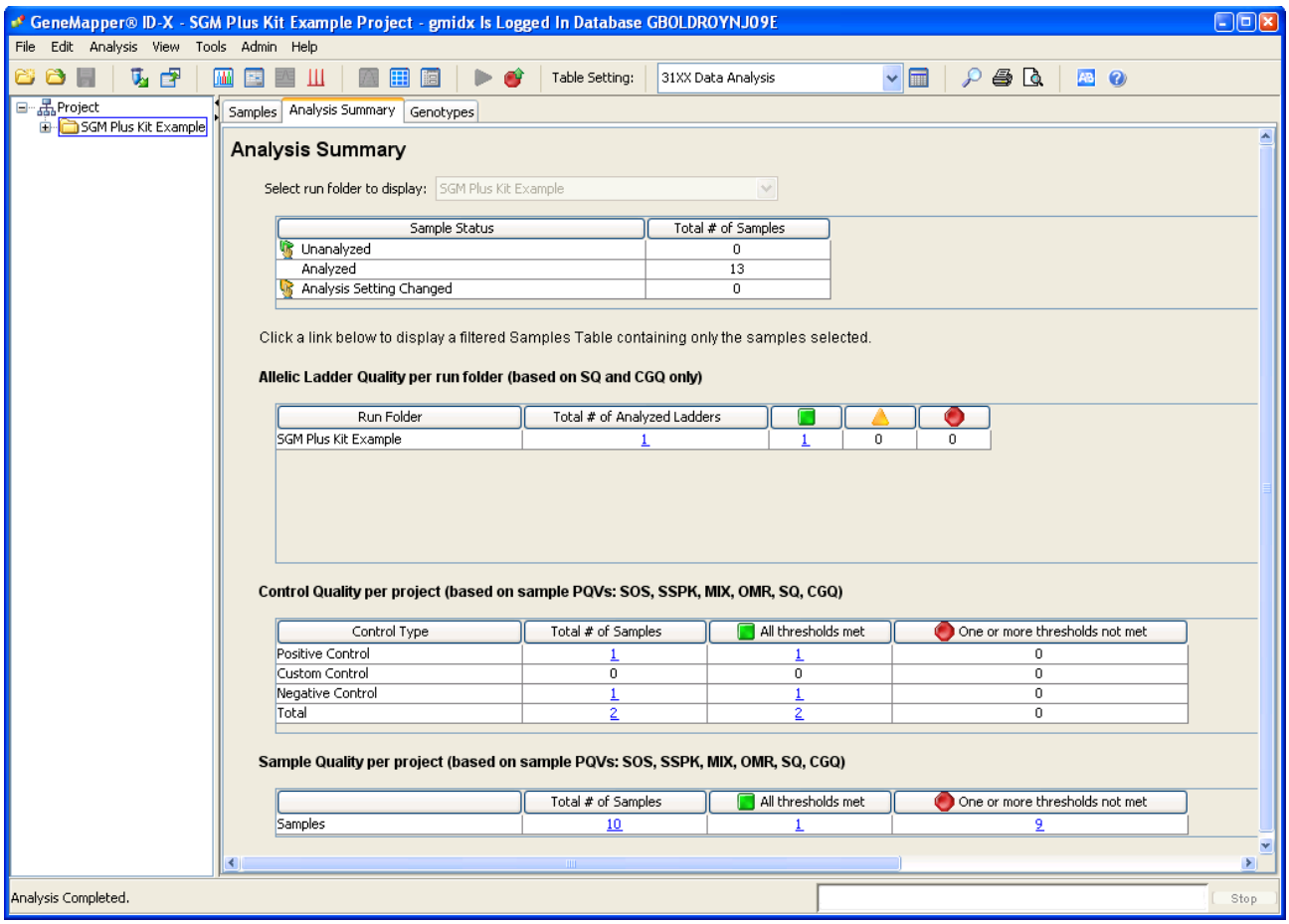
Parameter	Settings
Sample Type	Select the sample type.
Analysis Method	SGMPlus_AnalysisMethod_v1X (or the name of the analysis method you created)
Panel	SGM_Plus_v1.1X
Size Standard	CE_F_GS500(75-450)† (or the name of the size standard you created)

† The SGM Plus™ Kit was originally validated using the GeneScan™ 500 ROX™ Size Standard. If you use the GeneScan™ 400 HD Size Standard as an alternative, perform the appropriate internal validation studies to support the use of this size standard with the SGM Plus™ Kit.

Note: For more information about how the Size Caller works, refer to the *GeneScan™ Analysis Software for the Windows™ NT Operating System Overview of the Analysis Parameters and Size Caller User Bulletin* (Pub. no. 4335617).

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 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 is displayed and the Genotypes tab becomes available upon completion of the analysis.

Analysis summary window after analysis



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

For more information, refer to:

- *GeneMapper™ ID-X Software Version 1.0 Getting Started Guide* (Pub. no. 4375574)
- *GeneMapper™ ID-X Software Version 1.0 Quick Reference Guide* (Pub. no. 4375670)
- *GeneMapper™ ID-X Software Version 1.0 Reference Guide* (Pub. no. 4375671)
- *GeneMapper™ ID-X Software Version 1.1(Mixture Analysis) Getting Started Guide* (Pub. no. 4396773)
- *GeneMapper™ ID-X Software Version 1.2 Reference Guide* (Pub. no. 4426481)
- *GeneMapper™ ID-X Software Version 1.2 Quick Reference Guide* (Pub. no. 4426482)

5 Experiments and Results

Section 5.1 Developmental Validation

Overview

This section provides results of the developmental validation experiments we performed using the SGM Plus™ Kit.

These studies meet and exceed those recommended in the Technical Working Group on DNA Analysis Methods (TWGDAM) guidelines as well as the DNA Advisory Board (DAB) Quality Assurance Standards, effective October 1, 1998 (Technical Working Group on DNA Analysis Methods, 1995; DNA Advisory Board, Federal Bureau of Investigation, U.S. Department of Justice, 1998). These studies also address the guidelines outlined in the ENFSI DNA Working Group Quality Assurance Programme for DNA Laboratories.

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; Wallin *et al.,* 1998).

Experiment conditions

This chapter discusses many of the experiments we performed and provides examples of results obtained. We chose conditions that produced optimum PCR product yield and that met reproducible performance standards. It is our opinion that while these experiments are not exhaustive, they are appropriate for a manufacturer of STR kits intended for forensic and/or parentage testing use. Each laboratory using the SGM Plus™ Kit should perform their own internal validation studies.

Developmental validation

DAB 8.1.1 Developmental Validation

"Developmental validation that is conducted shall be appropriately documented." (DNA Advisory Board, 1998).

Critical reagent concentrations and reaction conditions (such as thermal cycling parameters, AmpliTaq Gold™ DNA polymerase activation, cycle number) to produce reliable, locus-specific amplification and appropriate sensitivity have been determined.

PCR components

The concentration of each component of the SGM Plus™ Kit was examined. The PCR components are Tris-HCl (pH 8.3), KCl, dNTPs, primers, AmpliTaq Gold™ DNA Polymerase, $MgCl_2$, bovine serum albumin, and sodium azide. The concentration for a particular component was established to be in the window that meets the reproducible performance characteristics of specificity and sensitivity.

After the optimal concentration was determined for a single component, the others were tested sequentially until it was determined that each component was at the optimal concentration relative to the concentrations of the other components in the master mix. The optimized SGM Plus™ Kit provides the required degree of specificity such that it is specific to primates for the species tested (with the exception of the amelogenin locus, see "Nonhuman studies" on page 84) and does not produce nonspecific mispriming artifacts.

Thermal cycler parameters

Thermal cycling parameters were established for amplification of the SGM Plus™ Kit in the DNA Thermal Cycler 480 and GeneAmp™ PCR Systems 2400, 9600, and 9700. Thermal cycling times and temperatures met GeneAmp™ PCR Instrument specifications. Annealing and denaturation temperature windows were tested around each setpoint to verify that a ±2°C window (DNA Thermal Cycler 480) or ±1.5°C window (GeneAmp PCR System 2400, 9600, and 9700) yielded specific PCR product with the desired sensitivity of at least 1 ng of AmpFℓSTR™ Control DNA 007.

SGM Plus™ Kit reactions were amplified for 27, 28, 29, 30, and 31 cycles on the GeneAmp PCR System 9600 using 1.5 ng of three DNA samples. As expected, PCR product increased with the number of cycles. Peak heights for all loci were above 150 RFU, even at 27 cycles; off-scale data was collected for several allele peaks at 31 cycles. See "DNA quantification" on page 19 for information on off-scale data.

While none of the cycle numbers tested produced nonspecific peaks, 28 cycles was found to give optimal sensitivity when the amplified products were examined on Applied Biosystems instruments. Additionally, the cycle number was set to avoid detection of low quantities of DNA (35 pg or less). At 28 cycles, 2.0 ng ofAmpFℓSTR™ Control DNA 007 amplifies reliably and specifically following the conditions outlined in this manual.

AmpliTaq Gold™ DNA Polymerase activation

The thermal cycler program for the SGM Plus™ Kit includes an initial incubation to allow for AmpliTaq Gold™ DNA polymerase activation. Polymerase activation times and temperatures were tested in the DNA Thermal Cycler 480 and the GeneAmp PCR Systems 2400, 9600, and 9700.

Five activation times (5, 8, 11, 14 and 17 minutes) were tested. A plateau in fluorescent signal (RFU) of the SGM Plus™ Kit loci was reached at ~8 minutes and was maintained through the 17–minute timepoint. The 11–minute activation time was determined to be optimal based on this plateau (within a ± >25% window).

A 95°C activation temperature (for 11 minutes) was determined to be optimal for the reactions. These conditions produced maximum PCR product yield and a window in which reproducible performance characteristics were met.

Accuracy, reproducibility, and precision

DAB 8.1.2 Accuracy

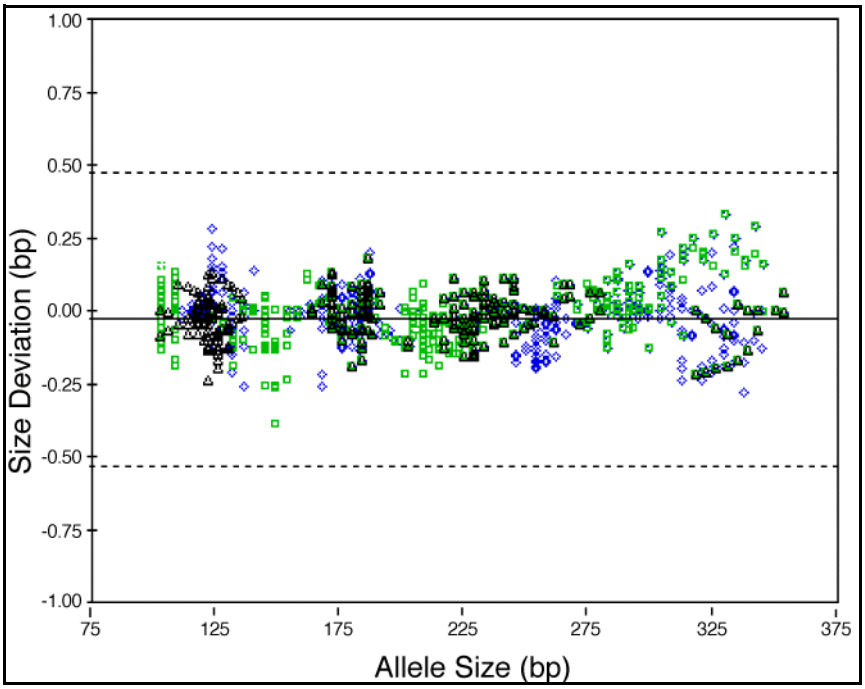
"Novel forensic DNA methodologies shall undergo developmental validation to ensure the accuracy, precision and reproducibility of the procedure." (DAB, 1998).

Laser-induced fluorescence detection systems of length polymorphism at short tandem repeat loci is not a novel methodology (Holt *et al.*, 2001 and Wallin *et al.*, 2001). However, accuracy and reproducibility of SGM Plus™ Kit profiles have been determined from various sample types.

Accuracy

Figure 5 illustrates the size differences that are typically observed between sample alleles and AmpFℓSTR™ SGM Plus™ Allelic Ladder alleles on the Applied Biosystems 310 Genetic Analyzer with POP-4™ polymer. The x-axis in Figure 5 represents the nominal base pair sizes for a single injection of AmpFℓSTR™ SGM Plus™ Allelic Ladder, and the dashed lines parallel to the x-axis represent the ±0.5-bp windows. The y-axis is the deviation of each sample allele size from the corresponding allelic ladder allele size. The data include a total of 892 alleles from 30 population database samples and two injections of allelic ladder. In this representative example, all sample alleles are within 0.5 bp of a corresponding allelic ladder allele.

Figure 5 Size deviation of 30 samples and two allelic ladders from one injection of allelic ladder on a single Applied Biosystems 310 instrument run.



The AmpFℓSTR™ SGM Plus™ Allelic Ladder contains the majority of alleles for the D3S1358, vWA, D16S539, D2S1338, amelogenin, D8S1179, D21S11, D18S51, D19S433, TH01, and FGA loci. However, alleles not found in the AmpFℓSTR™ SGM Plus™ Allelic Ladder do exist. These "off-ladder" alleles may contain full and/or partial repeat units. An "off-ladder" allele should flag itself by not falling inside the ±0.5 bp window of any known allelic ladder allele.

Note: If a sample allele peak is found to be >0.5 bp from the corresponding allelic ladder peak, then the sample must be rerun to verify the result.

Reproducibility

The reproducible nature of purified DNA samples from various individuals (>10), used routinely at Life Technologies, as well as validation samples processed during characterization of AmpFℓSTR™ PCR Amplification Kits (for example, from body fluid mixture studies, environmental studies, matrix studies, nonprobative studies, CEPH family DNA sets) was without exception. All samples yielded the correct genotype.

Precision and size windows

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

- An "off-ladder" allele, i.e., an allele of a size that is not represented in the AmpFℓSTR™ SGM Plus™ Allelic Ladder (go to **http://www.cstl.nist.gov/strbase/**for examples of known off-ladder alleles)
- An allele that does correspond to an allelic ladder allele, but whose size is just outside a window because of measurement error

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

Table 3 on page 70 indicates typical precision results obtained from 30 database samples and three AmpFℓSTR™ SGM Plus™ Allelic Ladder samples analyzed on the Applied Biosystems 310 Genetic Analyzer (47-cm capillary, POP-4™ polymer, GeneScan™ 500 ROX™ Size Standard). These results were obtained within a set of injections on a single capillary.

As indicated above, sample alleles may occasionally size outside of the ±0.5-bp window for a respective allelic ladder allele because of measurement error. The frequency of such an occurrence is lowest in detection systems with the smallest standard deviations in sizing. Figure 5 on page 68 illustrates the tight clustering of allele sizes obtained on the Applied Biosystems 310 Genetic Analyzer, where the standard deviation in sizing is typically less than 0.15 bp. The instance of a sample allele sizing outside of the ±0.5-bp window because of measurement error is relatively rare when the standard deviation in sizing is approximately 0.15 bp or less (Smith, 1995).

For sample alleles that do not size within a ±0.5-bp window, the PCR product must be rerun to distinguish between a true off-ladder allele versus measurement error of a sample allele that corresponds with an allele in the allelic ladder.

It is important to note that while the precision within a set of capillary injections is very good, the determined allele sizes vary between platforms. Cross-platform sizing differences arise from a number of conditions, including type and concentration of polymer, run temperature, and electrophoresis conditions. Variations in sizing can also be found between runs on the same instrument and between runs on different instruments because of these conditions. We strongly recommend that the allele sizes obtained be compared to the sizes obtained for known alleles in the AmpFℓSTR™ SGM Plus™ Allelic Ladder from the same run and then converted to genotypes (see "Allelic ladder requirements" on page 25). For more information on precision and genotyping, see Lazaruk *et al.*, 1998.

Table 3 Example of precision results on the Applied Biosystems Genetic Analyzer

Allele	n	Mean	S.D.
D3S1358			
12	3	111.25	0.02
13	4	115.38	0.01
14	8	119.36	0.03
15	17	123.35	0.10
15	10	127.48	0.09
17	16	131.58	0.07
18	10	135.78	0.09
19	5	139.92	0.08
vWA			
11	3	154.59	0.04
12	3	158.80	0.01
13	3	163.00	0.02
14	11	167.22	0.11
15	7	171.18	0.05
16	13	175.15	0.07
17	12	179.18	0.06
18	16	183.15	0.06
19	9	187.09	0.07
20	3	190.94	0.04
21	3	194.88	0.05
22	3	198.73	0.06
23	3	202.55	0.06
24	3	206.83	0.04
D16S539			
5	3	229.29	0.06
8	4	241.27	0.01
9	8	245.25	0.08
10	6	249.33	0.07
11	19	253.27	0.07
12	22	257.31	0.06
13	12	261.36	0.07
14	3	265.51	0.04
15	3	269.57	0.03
D2S1338			
15	4	290.55	0.03
16	4	294.67	0.02

Allele	n	Mean	S.D.
17	11	298.75	0.06
18	8	303.12	0.07
19	11	307.53	0.07
20	10	311.86	0.10
21	4	316.20	0.05
22	7	320.39	0.12
23	9	324.68	0.15
24	11	328.72	0.09
25	10	332.93	0.11
26	4	336.92	0.12
27	3	340.89	0.06
28	3	344.57	0.08
Amelogenin			
X	29	103.48	0.06
Y	18	109.13	0.07
D8S1179			
8	3	123.69	0.02
9	6	127.73	0.03
10	7	131.80	0.08
11	6	135.92	0.40
12	9	140.20	0.06
13	19	144.72	0.10
14	14	149.23	0.10
15	14	153.70	0.07
16	3	158.08	0.02
17	3	162.27	0.06
18	3	166.38	0.04
19	3	170.48	0.04
D21S11			
24	3	185.21	0.03
24.2	3	187.21	0.04
25	3	189.17	0.03
26	3	193.08	0.05
27	3	197.01	0.06
28	11	200.82	0.07
28.2	3	202.78	0.06
29	12	204.77	0.05
29.2	3	206.71	0.04

Allele	n	Mean	S.D.
30	21	208.74	0.07
30.2	4	210.67	0.08
31	7	212.68	0.04
31.2	7	214.61	0.07
32	5	216.67	0.03
32.2	5	218.57	0.06
33	4	220.58	0.07
33.2	7	222.53	0.05
34	3	224.55	0.04
34.2	5	226.53	0.04
35	3	228.54	0.04
35.2	3	230.47	0.05
36	3	232.56	0.04
37	3	236.51	0.03
38	3	240.43	0.02
D18S51			
7	3	262.31	0.04
9	3	270.55	0.01
10	4	274.64	0.07
10.2	3	276.67	0.04
11	4	278.77	0.03
12	9	282.88	0.08
13	12	286.98	0.05
13.2	3	289.03	0.03
14	15	291.13	0.06
14.2	3	293.22	0.03
15	7	295.31	0.03
16	13	299.46	0.06
17	9	303.96	0.08
18	4	308.38	0.08
19	6	312.77	0.12
20	3	317.11	0.13
21	4	321.35	0.09
22	3	325.58	0.13
23	3	329.78	0.17
24	3	333.95	0.13
25	3	338.05	0.09
26	3	341.93	0.15

Allele	n	Mean	S.D.
27	3	345.59	0.10
D19S433			
9	3	102.01	0.05
10	3	105.93	0.04
11	3	109.82	0.07
12	6	113.68	0.06
12.2	3	115.73	0.04
13	16	117.62	0.04
13.2	4	119.65	0.06
14	22	121.57	0.08
14.2	6	123.60	0.07
15	14	125.54	0.06
15.2	5	127.62	0.06
16	7	129.54	0.08
16.2	3	131.59	0.06
17	3	133.60	0.05
17.2	3	135.62	0.03
TH01			
4	3	163.12	0.01
5	3	167.19	0.05
6	16	171.20	0.06
7	7	175.25	0.04
8	7	179.25	0.09
9	14	183.23	0.08
9.3	22	186.26	0.05
10	3	187.21	0.04
11	3	191.13	0.03
13.3	3	201.87	0.06
FGA			
17	3	212.48	0.02
18	5	216.51	0.07
19	7	220.52	0.06
20	10	224.54	0.06
21	16	228.52	0.07
22	14	232.60	0.07
23	12	236.62	0.03
24	8	240.64	0.07
25	8	244.74	0.06

Allele	n	Mean	S.D.
26	3	248.78	0.04
26.2	3	250.83	0.03
27	3	252.77	0.02
28	3	256.89	0.01
29	3	260.99	0.06
30	3	265.10	0.05
30.2	3	266.95	0.05
31.2	3	271.05	0.03
32.2	3	275.17	0.05
33.2	3	279.27	0.03
42.2	3	317.77	0.13
43.2	3	322.01	0.11
44.2	3	326.21	0.10
45.2	3	330.39	0.10
46.2	3	334.44	0.10
47.2	3	338.55	0.08
48.2	3	342.41	0.04
50.2	3	349.77	0.00
51.2	3	353.44	0.04

Extra peaks in the electropherogram

Overview

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

Stutter products

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

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

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

- For each SGM Plus™ Kit locus, the percent stutter generally increases with allele length, as shown in Figure 6 through Figure 8 on page 76 through page 76. Smaller alleles display a lower level of stutter relative to the longer alleles within each locus. This is reflected in Figure 6 through Figure 8, where minimal data points are plotted for some smaller alleles, because stutter was not detected for many of these samples.
- For the alleles within a particular locus, the percent stutter is generally greater for the longer allele in a heterozygous sample (this is related to the first point above).
- Each allele within a locus displays a percent stutter that is quite reproducible; the average of the standard deviation values measured for each allele at each locus is: 0.4% for TH01, 0.6% for D3S1358, vWA and FGA, 0.8% for D8S1179, D21S11 and D18S51, 0.9% for D16S539, and 1% for D19S433 and D2S1338. The expected range of percent stutter for any particular allele can be estimated as ± 3 standard deviations from the mean. For example, if the percent stutter for a particular allele averages 5% for multiple replicates, and if the average standard deviation at the allele is 0.5%, then the expected range in percent stutter for this allele is (5 ± 1.5%) = 3.5–6.5%. This range also provides an estimate of the maximum expected stutter percent for each allele.
- The highest percent stutter observed for any TH01 allele was <4%, for any D3S1358, D8S1179, vWA or FGA allele <9%, for any D16S539 or D21S11 allele <10%, for any D2S1338 allele <12%, for any D18S51 allele <13%, and for any D19S433 allele <14%.
- An upper-limit stutter percent interpretational threshold can be estimated for each locus as 3 standard deviations above the highest percent stutter observed at the locus (see above two observations). Peaks at the stutter position that are above this threshold are not expected to be observed in single-source samples and therefore can be noted for closer examination. The upper-limit threshold values for each locus are as follows: 6% (TH01), 11% (D3S1358, vWA, FGA), 12% (D8S1179), 13% (D16S539, D21S11), 15% (D2S1338), 16% (D18S51), and 17% (D19S433). For evaluation of mixed samples, see "DAB 8.1.2.2 Mixture Studies" on page 77.
- The percent stutter does not change significantly with the quantity of input DNA, for on-scale data with a minimum analyzed peak height of 150 relative fluorescence units (RFU). The measurement of percent stutter may be unnaturally high for main peaks that are off-scale. Loading or injecting less of the PCR product will yield accurate quantitation. See "DNA quantification" on page 19 for information on off-scale data.

Figure 6 Stutter percentages for the D3S1358, vWA, D16S539, and D2S1338 loci

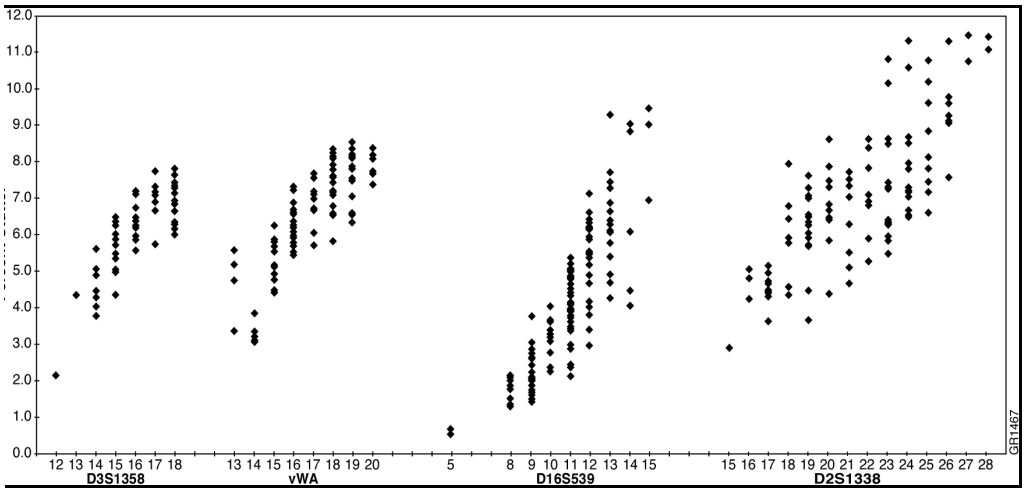


Figure 7 Stutter percentages for the D8S1179, D21S11, and D18S51 loci.

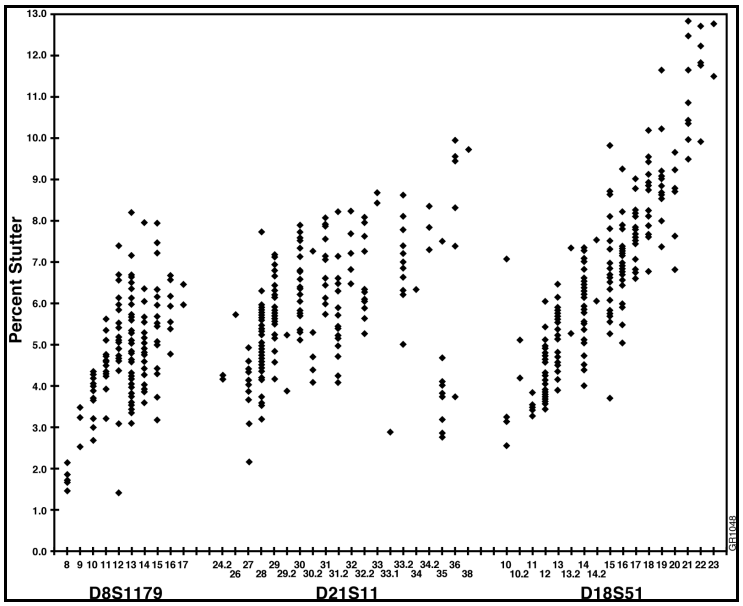
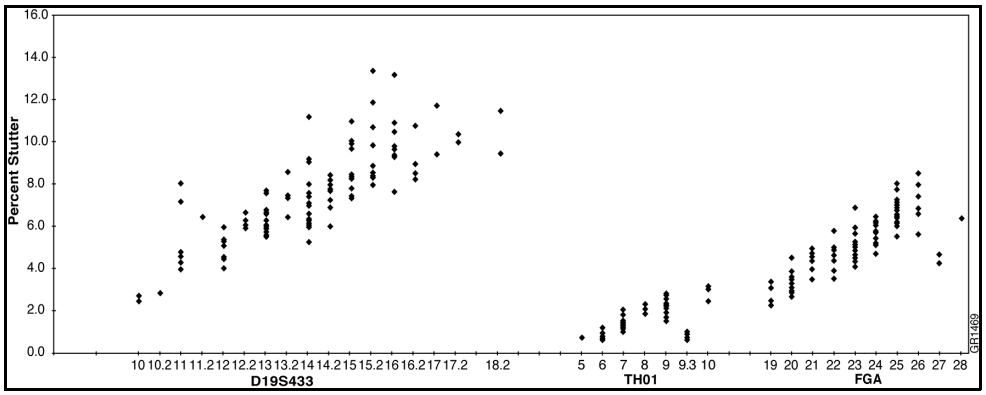


Figure 8 Stutter percentages for the D19S433, TH01, and FGA loci.



Addition of 3′ A nucleotide

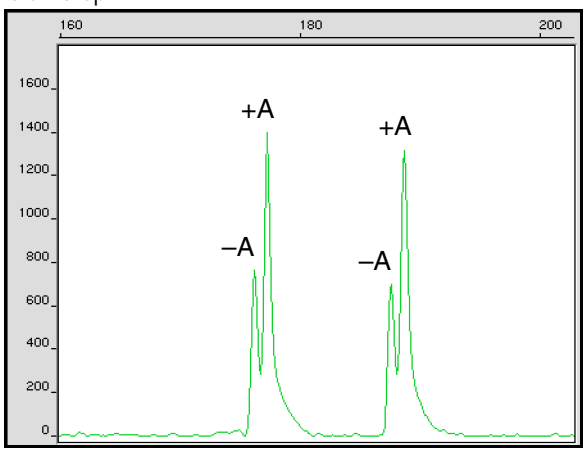
AmpliTaq Gold™ enzyme, like many other DNA polymerases, can catalyze the addition of a single nucleotide (predominately adenosine) to the 3′ ends of double-stranded PCR products (Clark, 1988, Magnuson *et al.*, 1996).This non-template addition results in a PCR product that is one base pair longer than the actual target sequence, and the PCR product with the extra nucleotide is referred to as the "+A" form.

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

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

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

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



Lack of full A nucleotide addition may be observed in SGM Plus™ Kit results when the amount of input DNA is greater than approximately 5.0 ng. The reason for this is that more time is needed for AmpliTaq Gold™ DNA Polymerase to add the A nucleotide to all molecules as more PCR product is generated. Amplification of too much input DNA will also result in off-scale data (see "DNA quantification" on page 19 for more information on off-scale data).

DAB 8.1.2.2 Mixture Studies

"Species specificity, sensitivity, stability and mixture studies are conducted." (DAB, 1998).

Evidence samples may contain DNA from more than one individual. The possibility of multiple contributors should be considered when interpreting the results. We recommend 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.

In the discussion below, a peak is defined as any peak that is greater than 150 RFU. We recommend a minimum peak height threshold to avoid typing less than 35 pg of input DNA (see "Effect of DNA quantity on results" on page 85).

Detection of mixed samples

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

- 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 (see "Stutter products" on page 74 and Figure 6 through Figure 8).
- Significantly imbalanced alleles for a heterozygous genotype
  The peak height ratio is defined as the height of the lower peak (in RFU) divided by the height of the higher peak (in RFU), expressed as a percentage. Mean peak height ratios and standard deviations observed for alleles in the SGM Plus™ Kit loci in unmixed population database samples are as follows:

Allele	Mean Peak Height Ratio	Number of Observations (n)
D3S1358	93 ± 4%	68
vWA	93 ± 5%	74
D16S539	92 ± 6%	70
D2S1338	91 ± 6%	87
Amelogenin	90 ± 6%	46
D8S1179	92 ± 6%	93
D21S11	91 ± 7%	95
D18S51	91 ± 6%	100
D19S433	92 ± 6%	97
TH01	92 ± 6%	70
FGA	93 ± 5%	80

- For all 11 loci, the mean peak height ratios indicate that the two alleles of a heterozygous individual are generally very well balanced. Ratios <70% are rare in normal, unmixed samples.

  If the peak height ratio is <70% 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. Reproducible imbalance at only one locus may indicate a mixture of significantly overlapping genotypes. Other possible causes of imbalance at a locus are degraded DNA, presence of inhibitors, extremely low amounts of input DNA, or the presence of an allele containing a rare sequence that does not amplify as efficiently as the other allele. Amplification and analysis of additional loci may assist in the interpretation of the sample.

Resolution of genotypes in mixed samples

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

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

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

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

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

Limit of detection of the minor component

Mixtures of two DNA samples were examined at various ratios (1:1 to 1:20). The total amount of genomic input DNA mixed at each ratio was 1 ng.

The samples were amplified in a GeneAmp™ PCR System 9600 and were electrophoresed and detected using a 377 DNA Sequencer.

The results of the mixed DNA samples, shown separately in Figure 10 on page 80, are displayed in Figure 11 on page 81 where sample A was the minor component and sample B was the major component.

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

Allele	Genotype	
	Sample A	Sample B
Amelogenin	X, Y	X, Y
D19S433	14,15	15,15
D3S1358	15, 16	14,17
D8S1179	12, 13	12,14
vWA	14, 16	15,17
TH01	7, 9.3	7, 8
D21S11	28, 31	30, 30.2
FGA	24, 26	22, 22
D16S539	9, 10	11, 12
D18S51	12, 15	15, 16
D2S1338	20, 23	19, 25

For these 1 ng total DNA mixture studies, the limit of detection is when the minor component is present at approximately 1/20 of the concentration of the major component. The limit of detection for the minor component is influenced by the combination of genotypes in the mixture.

The following figures show the reference samples and the two DNA samples used for this study.

Figure 10 Reference samples for mixture study shown in next figure

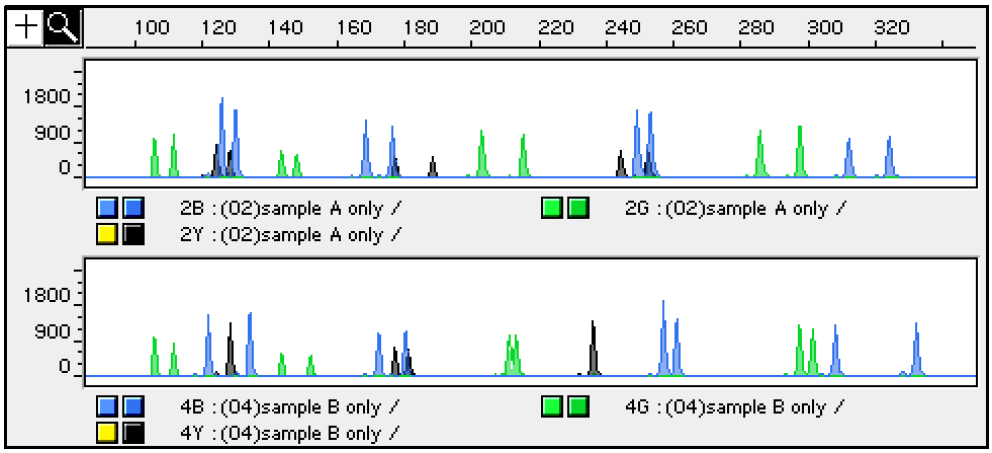
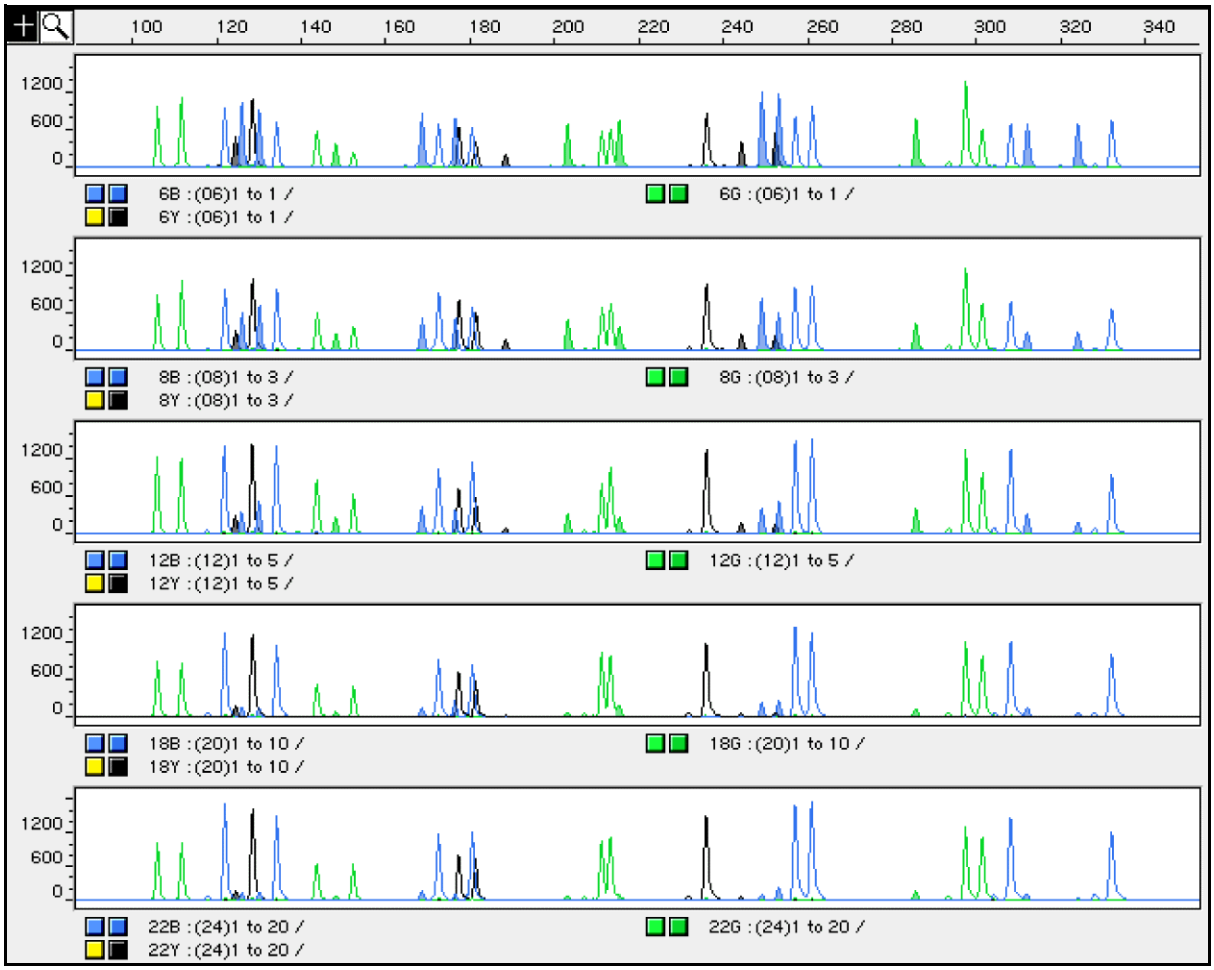


Figure 11 Results of the two DNA samples from previous figure mixed together at defined ratios and amplified with the SGM Plus™ Kit. The A:B ratios shown are 1:1, 1:3, 1:5, 1:10, and 1:20 (top to bottom). Alleles attributable only to the minor component are highlighted.



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

Mixed stains were prepared and DNA was extracted at the Santa Clara County Crime Laboratory, San Jose, CA, following the phenol/chloroform procedure (differential lysis was performed on stains containing semen). At Applied Biosystems approximately 3 ng of DNA was amplified from each dried fluid mixture in a GeneAmp PCR System 9600 and detected using either the Applied Biosystems 310 Genetic Analyzer or the Applied Biosystems 377 DNA Sequencer.

The limit of detection of the minor genotype component of each body fluid mixture ratio was determined. The limit of detection is defined here as the ratio below which a mixture is not recognized but rather appears to contain DNA from a single source (the major contributor of the mixture). The limit of detection occurred in blood:blood mixtures when the minor component was present at one tenth the volume of the major genotype.

In epithelial cell fractions of differential extractions of semen:blood or semen:saliva stains, blood was detectable at one fiftieth and saliva at one tenth the volume of semen. Sperm DNA carryover into the epithelial cell fraction of these stains was detectable at one-tenth the volume of blood or saliva. In sperm fractions, the male genotype was detectable from every semen:blood or semen:saliva mixture with no trace of the female DNA.

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

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

Analysis of sexual assault DNA mixture evidence

SGM Plus™ Kit reactions with DNA extracted from adjudicated sexual assault evidence were examined to assess the performance of the SGM Plus™ Kit on typical casework samples comprised of mixed body fluids. These samples were extracted, amplified, and analyzed in collaboration with the Santa Clara County Crime Laboratory.

DNA extracts from three adjudicated sexual assault cases were prepared by DNA analysts at the Santa Clara County Crime Laboratory. Sexual assault evidence materials were processed using the differential lysis and organic extraction procedure, while victim/suspect reference blood samples were processed using the Chelex™ extraction procedure. Following amplification with the SGM Plus™ Kit reagents, the PCR products were analyzed using the Applied Biosystems 377 DNA Sequencer.

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

Characterization of loci

DAB 8.1.2.1 Documentation

"Documentation exists and is available which defines and characterizes the locus." (DAB, 1998).

Nature of the polymorphisms

The primers for the amelogenin locus flank a six-base pair deletion within intron 1 of the X homologue. Amplification results in 107-bp and 113-bp products from the X and Y chromosomes, respectively. (Sizes are the actual base pair size according to sequencing results, including 3' A nucleotide addition.) The remaining SGM 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–bp repeat units (see Table 1 on page 12).

Alleles in the AmpFℓSTR™ SGM Plus™ Allelic Ladder, alleles containing partial repeat units, population database, and nonhuman primate DNA samples have been subjected to DNA sequencing at Life Technologies. In addition, other groups in the forensic 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; Möller and Brinkmann, 1995; Barber *et al.*, 1996; Barber and Parkin, 1996; Brinkmann *et al.*, 1998; Momhinweg *et al.*, 1998; Watson *et al.*, 1998). Among the various sources of sequence data on the SGM Plus™ Kit loci, there is consensus on the repeat patterns and structure of the STRs (see Table 1 on page 12).

D19S433 alleles have been sequenced at Life Technologies, including those in the AmpFℓSTR™ SGM Plus™ Allelic Ladder. Sequenced D19S433 alleles that contain a 2–bp repeat unit result from a deletion of the last two nucleotides (AG) in the second tetranucleotide repeat unit (AAAG) of the repeat motif (see Table 1 on page 12).

Inheritance

The SGM Plus™ Kit loci have been validated by family studies in order to demonstrate their mode(s) of inheritance.

The Centre d'Etude du Polymorphisme Humain (CEPH) has collected DNA from 39 families of Utah Mormon, French Venezuelan, and Amish descent. These DNA sets have been extensively studied all over the world and are routinely used to characterize the mode of inheritance of various DNA loci. Each family set contains three generations, generally including four grandparents, two parents, and several offspring. Consequently, the CEPH family DNA sets are ideal for studying inheritance patterns (Begovich *et al.*,1992).

Four CEPH family DNA sets were examined. DNA input of 1.5 ng from each sample was amplified using the SGM Plus™ Kit, followed by analysis using an Applied Biosystems 377 DNA Sequencer. The families examined included #1331 (11 offspring), #13291 (9 offspring), #13292 (9 offspring), and #13294 (8 offspring), representing 37 meiotic divisions. The results confirmed that the loci are inherited according to Mendelian rules, as has been reported in the literature (Nakahori *et al.*,1991; Edwards *et al.*,1992; Kimpton *et al.*,1992; Mills *et al.*,1992; Sharma and Litt, 1992; Li *et al.*,1993; Straub *et al.*,1993).

Mapping

The SGM Plus™ Kit loci D3S1358, vWA, D16S539, D2S1338, amelogenin, D8S1179, D21S11, D18S51, D19S433, TH01, and FGA have been mapped and the chromosomal locations have been published (Nakahori *et al.*, 1991; Edwards *et al.*,1992; Kimpton *et al.*,1992; Mills *et al.*,1992; Sharma and Litt,1992; Li *et al.*,1993; Straub *et al.*,1993; Barber and Parkin,1996). They are listed in Table 1 on page 12.

Population studies

Population distribution data of the 11 SGM Plus™ Kit loci have been established in different racial and/or ethnic groups. These loci were amplified and typed for 200 U.S. Caucasian and 195 African-American individuals. For more information regarding analysis of these samples, see "Population data" on page 92

Species specificity

DAB 8.1.2.2 Species Specificity

"Species specificity, sensitivity, stability and mixture studies are conducted." (DAB, 1998).

Nonhuman studies

Nonhuman DNA may be present in forensic casework samples. The SGM Plus™ Kit provides the required degree of specificity such that it is specific to primates for the species tested (with the exception of the amelogenin locus).

The following experiments were conducted to investigate interpretation of SGM Plus™ Kit results from nonhuman DNA sources.

The extracted DNA samples were amplified in SGM Plus™ Kit reactions and analyzed using the Applied Biosystems 377 DNA Sequencer.

- **Primates** – Gorilla, chimpanzee, and macaque (2.5 ng each).
- **Non-primates** – Hamster, rat, rabbit, cat, dog, pig, chicken, cow, and fish (50 ng each).
- **Bacteria and yeast** – *Legionella*, *Escherichia*, *Listeria*, *Neisseria*, *Vibrio*, *Citrobacter*, *Salmonella*, *Candida*, *Saccharomyces* (equivalent to ~50 ng human DNA), and *Rhodotorula*.

The primate DNA samples all amplified, producing fragments within the 75–350 base pair region (Wallin *et al.*,1998). The primate samples were subsequently sequenced by Life Technologies scientists. The data revealed significant sequence homology between the primate and human DNA for the SGM Plus™ Kit loci (Meyer *et al.*, 1995; Andersen *et al.*, 1996).

The bacteria, yeast, hamster, rat, rabbit, cat, chicken, and fish samples did not yield detectable product. The dog, pig, and cow samples produced a 103-bp fragment. This 102-bp fragment was also amplified using the amelogenin primers alone. This confirms amplification of the product obtained by Buel *et al.*(1995). The 103-bp fragment is 4 bp shorter than the primate 107-bp X-specific product (including +A addition).

Sensitivity

DAB 8.1.2.2 Sensitivity

"Species specificity, sensitivity, stability and mixture studies are conducted." (DAB, 1998).

Effect of DNA quantity on results

The amount of input DNA added to the PCR reaction should be 1.0–2.5 ng. The DNA sample should be quantitated prior to amplification using a system such as the Quantifiler™ Human DNA Quantitation Kit (Part no. 4343895). Figure 12 on page 86 shows the effect of different amounts of AmpFℓSTR™ Control DNA 007.

The final DNA concentration should be in the range of 0.05–0.125 ng/µL so that 1.0–2.5 ng of DNA will be added to the PCR reaction in a volume of 20 µL. If the sample contains degraded DNA, amplification of additional DNA may be beneficial.

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

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

  Off-scale data is a problem for two reasons:

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

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

  Identification of off-scale peaks and multicomponent analysis are discussed in "DNA quantification" on page 19 and "About multicomponent analysis" on page 16.

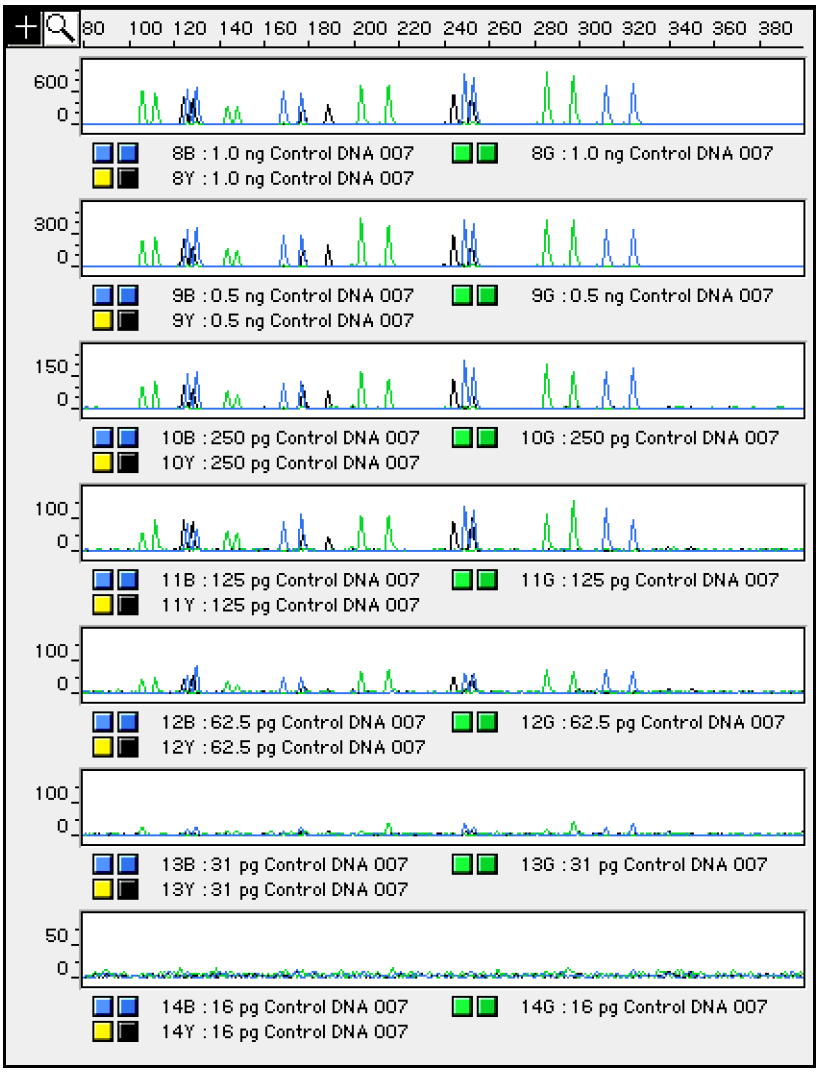
- Incomplete A nucleotide addition

To avoid these issues, the sample can be re-amplified using less DNA.

When the total number of allele copies added to the PCR is extremely low, unbalanced amplification of the two alleles of a heterozygous individual may occur (Wallin *et al.*, 1998; Walsh *et al.*, 1992). This is due to stochastic fluctuation in the ratio of the two different alleles (Sensabaugh *et al.*, 1991). The PCR cycle number and amplification conditions have been specified to produce low peak heights for a sample containing produce peak heights of <150 RFU for a sample containing 35 pg human genomic DNA (corresponding to ten total allele copies). Peak heights <150 RFU should be interpreted with caution.

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

Figure 12 Effect of amplifying various amounts of AmpFℓSTR™ Control DNA 007 ranging from 16 pg to 1 ng. Note that the y-axis scale differs in many of these panels.



Stability

DAB 8.1.2.2 Stability

"Species specificity, sensitivity, stability and mixture studies are conducted." (DAB, 1998).

Lack of amplification of some loci

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

Differential and preferential amplification

Differential amplification can be defined as the difference in the degree of amplification of each locus within a co-amplified system, such that one or more loci may amplify to a greater extent compared to the other loci. Preferential amplification is used in this guide to describe differences in the amplification efficiency of two alleles at a single locus and is observed when the peak height ratio between the two alleles is <70% (see "DAB 8.1.2.2 Mixture Studies" on page 77).

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

DNA samples containing either D2S1338 alleles separated by 28–40 bp or containing FGA alleles separated by 90–100 bp were analyzed in several of our studies to determine the potential for preferential amplification.

In assessing potential for differential and preferential amplification, the following four variables were examined:

- Low template copy number
- Effect of PCR inhibitor in a DNA sample
- Degraded DNA
- Amplification denaturation and annealing temperatures

Low template copy number

To determine if the amount of input DNA may result in either differential or preferential amplification, varying quantities of three DNA samples were amplified in duplicate reactions. One nanogram, 0.5 ng, 0.25 ng, 0.125 ng, 0.06 ng, 0.03 ng, and 0.015 ng of AmpFℓSTR Control DNA 007 and samples containing either widespread D2S1338 or FGA alleles were amplified and then analyzed using the Applied Biosystems 377 DNA Sequencer.

No loci in any of the samples tested differentially amplified at any of the eleven loci. Preferential amplification was not observed between the widespread D2S1338 alleles.

For the sample containing widespread FGA alleles, the duplicate dilution series are denoted here as A and B. In dilution series A, for the sample containing 0.06 ng template DNA, the shorter of the two widespread FGA alleles (270 bp) was detected and the longer allele (334 bp) was not. However, the shorter allele peak height (26 RFU) was significantly below the recommended minimum threshold of 150 RFU (see "Effect of DNA quantity on results" on page 85). In dilution series A, preferential amplification was not seen at any of the higher template DNA concentrations.

Moreover, in dilution series B, for the sample containing widespread FGA alleles, such preferential amplification did not occur at 0.06 ng. But, at 0.125 ng of input DNA, a peak height ratio of 55% was detected between the widespread FGA alleles. Both FGA alleles in this sample (93 and 51 RFU) were below the recommended minimum threshold of 150 RFU (see "Effect of DNA quantity on results" on page 85).

For dilution series A and B, at 0.25 ng and above, both FGA alleles amplified in equal proportions; at 0.03 ng neither allele was detectable. Taken together, these data underscore the importance of interpreting single peaks of low fluorescence signal with caution and on a case-by-case basis.

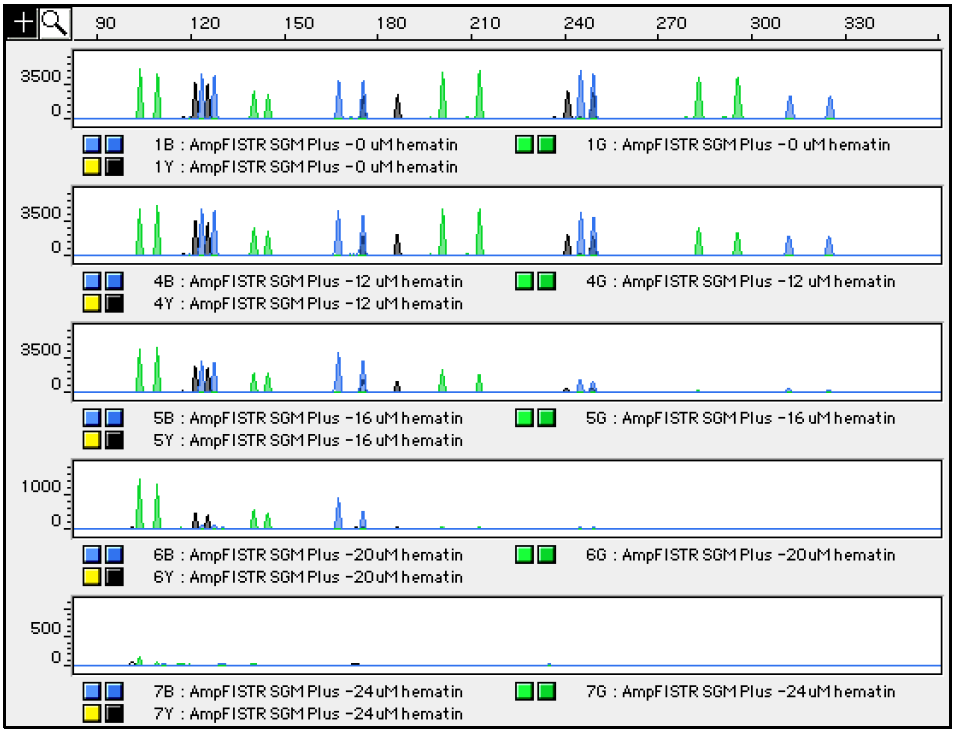
Effect of inhibitors

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

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

To examine the effects of hematin on the SGM Plus™ Kit amplification results, DNA samples were amplified using the SGM Plus™ Kit reagents (including the BSA-containing PCR reaction mix) in the presence of varying concentrations of purified hematin. The concentrations of hematin used were 0 µM,12 µM, 16 µM, 20 µM, and 24 µM. When the amount of hematin was increased to a concentration that started to inhibit the PCR, D2S1338 and D18S51 were the first loci to drop out in each experiment, followed by FGA (Figure 13).

Figure 13 DNA amplified with the SGM Plus™ Kit in the presence of varying concentrations of hematin: 0, 12 µM, 16 µM, 20 µM, and 24 µM

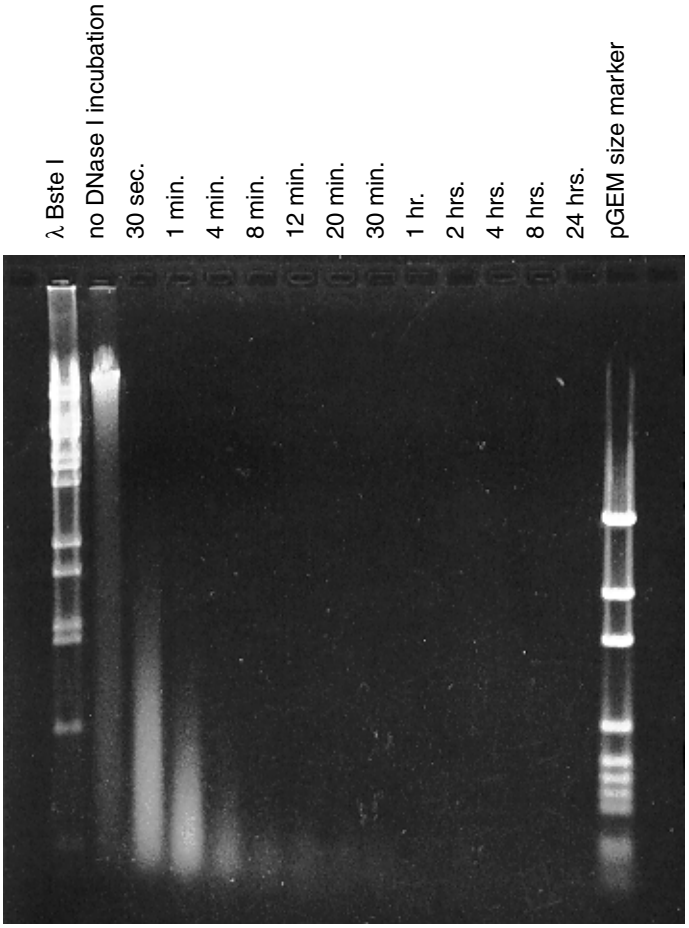


Degraded DNA

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

Degraded DNA was prepared to examine the potential for differential amplification of loci. High molecular weight DNA was incubated with the enzyme DNase I for varying amounts of time. The DNA was examined by agarose gel analysis to determine the average size of the DNA fragments at each timepoint. Gel analysis results of the degraded DNA are shown in Figure 14.

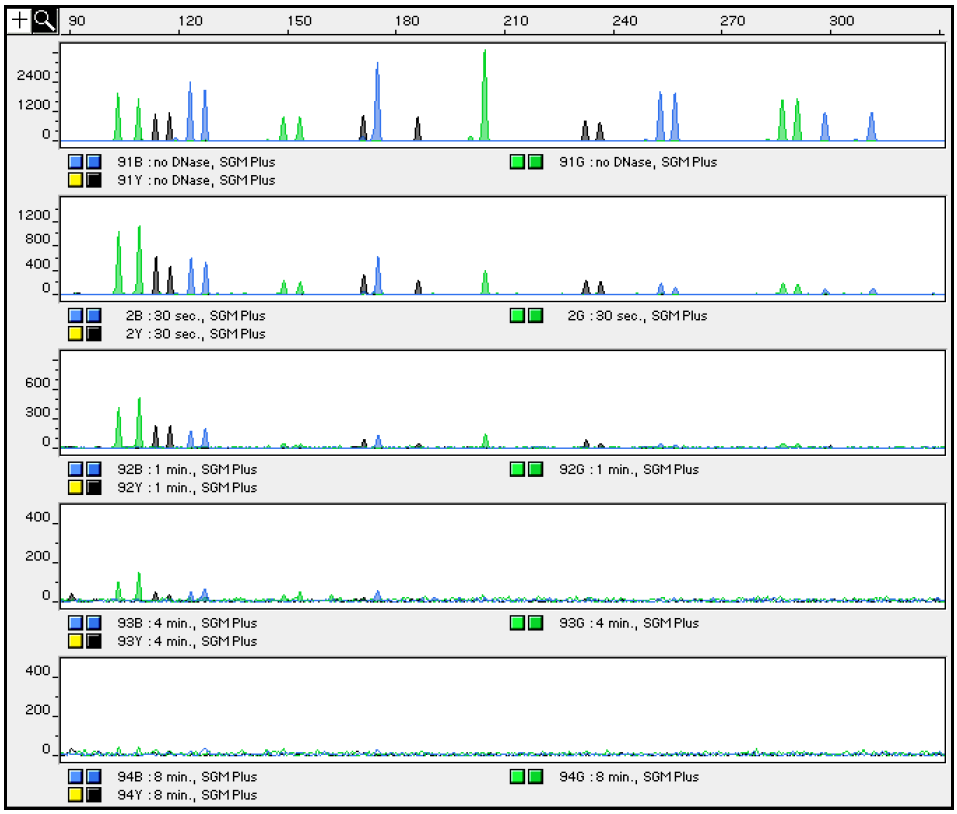
Figure 14 Agarose gel of degraded genomic DNA



Four (4) ng of degraded DNA (or 2 ng undegraded DNA) was amplified using the SGM Plus™ Kit (all 11 primer pairs together) and also in reactions containing each locus-specific primer pair individually.

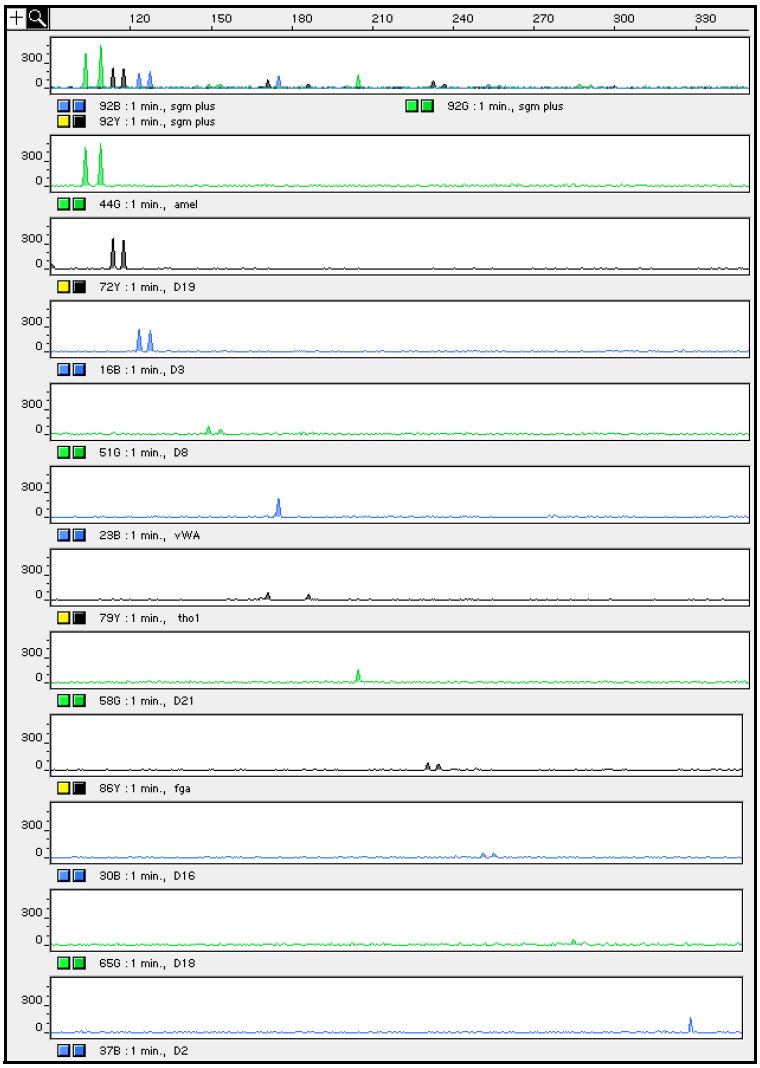
The electropherograms in Figure 15 show the SGM Plus™ Kit amplification results of a DNA sample with no DNase I treatment (2 ng amplified) and those of the 30-second, 1-, 4-, and 8-minute incubations (approximately 4 ng amplified).

Figure 15 Amplifications of DNA incubated for various times with DNase I



The loci failed to amplify in the order of decreasing size as the extent of degradation progressed: D2S1338 was the first locus to drop out, followed by D18S51 and D16S539 and so forth. A similar result at each timepoint was obtained whether the DNA samples were amplified for each locus alone or co-amplified with the SGM Plus™ Kit (Figure 16).

Figure 16 Multiplex and single-locus amplifications of the DNA sample incubated for 1 minute with DNase I



Denaturation and annealing temperatures

The effects of denaturation and annealing temperatures on the amplification of SGM Plus™ Kit loci were examined using 1–2 ng of three DNA samples: AmpFℓSTR Control DNA 007 and samples containing either widespread D2S1338 or FGA alleles.

The denaturation temperatures tested were 92, 94, and 96°C (DNA Thermal Cycler 480) and 92.5, 94, and 95.5 °C (GeneAmp Detection Systems 2400, 9600, and 9700), all for 1-minute hold times. The annealing temperatures tested were 57, 59, 61, and 63°C, also for 1-minute hold times, in the DNA Thermal Cycler 480 and the GeneAmp PCR Systems 2400, 9600 and 9700. The PCR products were analyzed using the Applied Biosystems 310 Genetic Analyzer.

Neither preferential nor differential amplification was observed in the denaturation temperature experiments. Of the tested annealing temperatures, 57, 59, and 61°C did not induce any differential amplification. At 63°C, the yield of the majority of loci was significantly reduced. This should pose no problem with routine thermal cycler calibration and when following the recommended amplification protocol. Preferential amplification was not observed at any of the tested annealing temperatures.

Matrix studies

Analysts at the Santa Clara Crime Laboratory prepared a panel of blood and semen specimens deposited on a variety of commonly encountered substrates. Blood samples from two donors were deposited individually on wool, cotton, nylon, metal, glass, leather (one donor), and blue denim. Semen samples from two donors were deposited individually on wool, cotton, nylon, leather, blue denim, acetate, vinyl upholstery, facial tissue, and a condom with spermicide (5% nonoxynol-9).

Specimens were stored at room temperature and at specified time points a sampling of each stain was removed for extraction. The blood and semen stains were extracted using the organic extraction procedure. Additionally, a portion of each blood specimen was extracted using the Chelex™ method followed by Centricon™-100 ultrafiltration. Extracted samples were then stored at –15 to –25°C for 4–5 years.

The 1–week and 1–year time points were analyzed by Life Technologies scientists. The samples were amplified using SGM Plus™ Kit reagents and analyzed using the Applied Biosystems 310 Genetic Analyzer. A complete 11-locus genotype was obtained for 47 of the 49 blood and semen samples exposed to the tested matrices for 1 week or 1 year. Some alleles in two of the 1–year samples were of low signal intensity and were either too weak to be genotyped or were interpreted with caution. No non-specific artifacts were generated and all samples yielded expected genotypes.

Population data

DAB 8.1.2.3 Population Data

"Population distribution data are documented and available." (DAB, 1998).

DAB 8.1.2.3.1 Population Distribution Data

"The population distribution data would include the allele and genotype distributions for the locus or loci obtained from relevant populations. Where appropriate, databases should be tested for independence expectations." (DAB, 1998).

Overview

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

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

Population samples used in these studies

The SGM Plus™ Kit was used to generate the population data provided in this section. Samples were collected from individuals throughout the United States with no geographical preference.

Population	Number of samples	Samples provided by
African-American	195	Laboratory Corporation of America
U.S. Caucasian	200	

Allele frequencies

Table 4 shows the SGM Plus™ Kit allele frequencies in two populations, listed as percentages.

Table 4 SGM Plus™ Kit allele frequencies

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
D3S1358		
9	0.26†	‡
11	0.26†	0.25†
12	0.51†	0.25†
13	‡	0.50†
14	11.80	11.25
15	27.95	28.25
15.2	0.26†	‡
16	32.31	22.25
17	21.80	22.25
18	4.62	14.50
19	0.26†	0.50†
vWA		
11	0.26†	‡
12	‡	‡
13	1.54	‡
14	7.70	8.50
15	22.05	8.25
16	26.92	19.75
17	16.92	25.00
18	13.85	25.75
19	8.46	11.00
20	2.05	1.50
21	‡	0.25†
22	0.26†	‡
23	‡	‡
24	‡	‡

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
D16S539		
5	0.26†	‡
8	3.85	1.5
9	18.21	10.75
10	11.28	4.25
11	30.00	29.75
12	18.46	34.25
13	15.64	17.5
14	2.05	1.75
15	0.26†	0.25†
D2S1338		
15	‡	0.25†
16	5.64	4.25
17	11.03	17.75
18	2.82	8.75
19	16.41	12.75
20	8.72	13.50
21	13.59	3.25
22	12.31	3.75
23	8.72	10.75
24	8.46	10.00
25	8.72	12.00
26	2.82	2.75
27	0.51†	‡
28	0.26†	0.25†
D8S1179		
8	‡	1.75
9	0.51†	1.00†
10	3.08	8.00
11	4.36	6.25
12	10.51	14.25
13	19.74	34.75
14	33.33	18.75
15	21.03	13.00
16	6.41	2.00
17	1.03†	0.25†
18	‡	‡
19	‡	‡

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
D21S11		
24	‡	‡
24.2	‡	0.50†
25	‡	‡
26	0.26†	‡
27	5.90	3.75
28	21.80	16.25
28.2	‡	‡
29	20.00	20.75
29.2	‡	0.50†
29.3	0.26†	‡
30	16.15	26.25
30.2	2.56	2.50
31	8.97	5.50
31.2	5.90	10.50
32	0.77†	1.25†
32.2	6.92	7.25
33	0.51†	0.25†
33.1	0.26†	‡
33.2	4.10	4.00
34	0.26†	‡
34.2	‡	0.75†
35	3.59	‡
35.2	‡	‡
36	1.54	‡
37	‡	‡
38	0.26†	‡
D18S51		
7	‡	‡
9	‡	‡
10	0.51†	0.50†
10.2	0.51†	‡
11	1.54	2.00
12	5.64	14.25
13	4.10	15.00
13.2	0.51†	‡
14	6.67	16.75
14.2	0.51†	‡

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
15	17.69	14.25
16	17.44	14.00
17	16.41	10.50
18	11.03	6.00
19	10.26	3.75
20	4.87	1.50
21	1.03†	1.00†
22	1.03†	0.25†
23	0.26†	0.25†
24	‡	‡
25	‡	‡
26	‡	‡
27	‡	‡
D19S433		
9	‡	‡
10	0.77†	‡
10.2	0.26†	‡
11	7.70	0.25†
11.2	0.26†	‡
12	10.51	6.50
12.2	4.10	‡
13	26.92	26.00
13.2	4.62	2.25
14	21.80	32.75
14.2	5.90	3.25
15	7.44	18.25
15.2	4.87	2.50
16	0.77†	5.25
16.2	3.33	2.00
17	‡	0.50†
17.2	0.51†	0.25†
18.2	0.26†	0.25†
TH01		
4	‡	‡
5	0.26†	‡
6	13.08	25.25
7	40.00	16.00
8	22.82	9.25

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
9	13.08	13.75
9.3	10.00	35.00
10	0.77†	0.75†
11	‡	‡
13.3	‡	‡
FGA		
16.2	0.26†	‡
17	0.26†	‡
18	0.51†	1.50
19	4.62	6.25
19.2	0.26†	‡
20	4.36	16.25
20.2	‡	0.75†
21	13.33	17.75
21.2	0.26†	‡
22	18.97	16.50
22.2	0.26†	0.50†
23	18.97	14.00
24	16.41	13.25
25	12.31	11.25
26	4.10	1.50
26.2	‡	‡
27	4.10	0.50†
28	0.77†	‡
29	0.26†	‡
30	‡	‡
30.2	‡	‡
31.2	‡	‡
32.2	‡	‡
33.2	‡	‡
34.2	‡	‡
42.2	‡	‡
43.2	‡	‡
44.2	‡	‡
45.2	‡	‡
46.2	‡	‡
47.2	‡	‡
48.2	‡	‡

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
50.2	‡	‡
51.2	‡	‡

† A minimum allele frequency of 1.3% is suggested by the National Research Council in forensic calculations using either of these African American or U.S. Caucasian databases analyzed using the SGM Plus™ Kit.

‡ Presen t in the AmpFℓSTR™ SGM Plus™ Allelic Ladder but not observed in the respective population database samples.

Analyzing the two databases

Analysis across both databases of 790 total chromosomes (per locus) revealed a total of 11 different D3S1358 alleles, 11 different vWA alleles, 9 different D16S539, 14 different D2S1338 alleles, 10 different D8S1179 alleles, 21 different D21S11 alleles, 17 different D18S51 alleles, 17 D19S433 alleles, 7 different TH01 alleles, and 18 different FGA alleles.

In addition to the alleles that were observed and recorded in the Applied Biosystems databases, other known alleles (listed in Table 1, "SGM Plus™ Kit loci and alleles" on page 12) have either been published or reported to us by other laboratories.

Independent allele frequencies

Independence of allelic frequencies within a locus can be expressed by the Hardy-Weinberg (HW) relationship. Approximation of HW expectations in a sample population allows estimation of genotypic frequencies (HW proportions) from observed allelic frequencies using the HW equation (expanded binomial square law) (Hartl and Clark, 1989; Weir, 1996).

Several biostatistical tests were used to survey HW relationships at the SGM Plus™ Kit STR loci in each sample population. Independence was found between alleles within each locus, as p values >0.05 were obtained from the homozygosity test (Chakraborty *et al.*, 1988; Nei and Roychoudhury, 1974; Nei, 1978), likelihood-ratio test (Edwards *et al.*, 1992; Weir, 1992), and Guo-Thompson exact test (Guo and Thompson, 1992).

Additionally, allele frequency data were analyzed for independence based on the total number of observed distinct homozygous and heterozygous genotype classes (Nei, 1978; Chakraborty *et al.*, 199). Observed values were within two standard errors of expected values for each locus. These sets of data demonstrate that appropriate estimations of SGM Plus™ Kit genotype frequencies are generated from allele frequencies observed in the Life Technologies African-American and U.S. Caucasian databases.

Random association

Existence of random association (linkage equilibrium) between all 10 STR loci was established through two separate statistical tests. Results of the first test, which considers the observed variance of the number of heterozygous loci (Brown *et al.*, 1980; Budowle *et al.*, 1995) indicate that in both population samples, all SGM Plus™ Kit loci are inherited independently.

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

Low frequency alleles

Some alleles of the SGM Plus™ Kit loci occur at a low frequency (less than five times in either database). For these alleles, a minimum frequency of 0.013 (five divided by 2n, where n equals the number of individuals in the database) was assigned for the SGM Plus™ Kit African-American and U.S. Caucasian databases, as suggested in the 1996 report of the Committee on DNA Forensic Science (National Research Council, 1996). These databases are summarized in Table 4, "SGM Plus™ Kit allele frequencies" on page 93. The minimum reportable genotype frequency at each locus is then 1.69×10^{-4}, giving a minimum combined multilocus genotype frequency of 1.90×10^{-38} for both the African-American and U.S. Caucasian databases.

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.

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

Additional 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 SGM 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 5 shows the Probability of Identity (P_I) values of the SGM Plus™ Kit loci individually and combined.

Table 5 Probability of Identity values for the SGM Plus™ Kit STR loci

Locus	African-American	U.S. Caucasian
D3S1358	0.102	0.078
vWA	0.058	0.065
D16S539	0.066	0.103
D2S1338	0.021	0.024
D8S1179	0.075	0.067
D21S11	0.033	0.045
D18S51	0.028	0.030
D19S433	0.039	0.078
TH01	0.102	0.094
FGA	0.035	0.036
Combined	7.91×10^{-14}	2.99×10^{-13}

The P_I value is the probability that two individuals selected at random will have an identical SGM Plus™ Kit genotype (Sensabaugh, 1982). The P_I values for the populations described in this section are then approximately $1/1.3 \times 10^{13}$ (African-American) and $1/3.3 \times 10^{12}$ (U.S. Caucasian).

Of 18,915 and 19,900 pairs of SGM Plus™ Kit profiles represented by the African-American and U.S. Caucasian databases, respectively, no 10-locus matches were observed.

Linkage disequilibrium between the SGM Plus™ Kit loci and unique loci included in the AmpFℓSTR™ Profiler™ PCR Amplification Kit (TPOX, CSF1PO, D5S818, D13S317, and D7S820) was not detected. The combination of these 15 AmpFℓSTR™ kit loci offers an average probability of identity of approximately $1/2.2 \times 10^{18}$ (African-American) and $1/2.1 \times 10^{17}$ (U.S. Caucasian).

Probability of paternity exclusion

Table 6 shows the Probability of Paternity Exclusion (P_E) values of the SGM Plus™ Kit STR loci individually and combined.

Table 6 Probability of paternity exclusion values for the SGM Plus™ Kit loci

Locus	African-American	U.S. Caucasian
D3S1358	0.5260	0.5797
vWA	0.6394	0.6170
D16S539	0.6146	0.5252
D2S1338	0.7818	0.7678

Locus	African-American	U.S. Caucasian
D8S1179	0.5930	0.6128
D21S11	0.7281	0.6835
D18S51	0.7518	0.7414
D19S433	0.7031	0.5865
TH01	0.5250	0.5418
FGA	0.7202	0.7173
Combined	0.99998	0.99997

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 SGM Plus™ Kit STR loci (Chakraborty and Stivers, 1996).

The P_E value offered by the combination of the 10 AmpFℓSTR™ kit loci (D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01, FGA) amplified by the AmpFℓSTR SGM Plus and Profiler kits is approximately 0.9999997 for the African American database and approximately 0.9999992 for the U.S. Caucasian database.

Section 5.2 Performance Validation After Buffer and Enzyme Component Replacement

Overview

As part of an ongoing program to exercise greater control over raw materials used in the AmpFℓSTR™ PCR Amplification Kits, manufacturing of the AmpliTaq Gold™ enzyme and 10× PCR Buffer II (Tris-KCl buffer) components is transitioning from Roche Molecular Systems to Life Technologies. Manufacturing of both components by Life Technologies will be conducted according to the same specifications used previously by Roche. The in-house components are established raw materials in our next generation kits (for example, the NGM™, NGM SElect™ and Identifiler™ Plus Kits).

Experiments

We performed studies to compare the performance of the SGM Plus™ Kit containing the in-house components (updated kit) with the performance of the original kit, focussing on studies most relevant to forensic DNA testing (see SWGDAM Guidelines effective January 1, 2011). These studies, while not exhaustive, are in our opinion appropriate for a manufacturer.

Our studies compared the performance of two Roche-manufactured enzyme and buffer lots (Control mixes) with three new lots of buffer and two new lots of enzyme manufactured by Life Technologies (Test mixes). Studies were performed using Test mixes containing both the enzyme and buffer manufactured by Life Technologies.

Test Material	Control A mix	Control B mix	Test A mix	Test B mix	Test C mix
Buffer	Control Buffer Lot 1	Control Buffer Lot 2	Test Buffer Lot 1	Test Buffer Lot 2	Test Buffer Lot 3
Enzyme	Control Enzyme Lot 1	Control Enzyme Lot 2	Test Enzyme Lot 1	Control Enzyme Lot 2	Test Enzyme Lot 2

Each of the five mixes listed above were used to conduct reproducibility, sensitivity, and inhibition studies. All amplifications were performed using a GeneAmp™ PCR System 9700 with either silver or gold-plated silver block using the recommended amplification conditions and cycle number for the SGM Plus™ Kit. All data was run on an Applied Biosystems 3130*xl* Genetic Analyzer running Data Collection Software v3.0 and analyzed using GeneMapper™ *ID-X* Software. Subsequent data analysis was performed using Minitab™ Statistical Software.

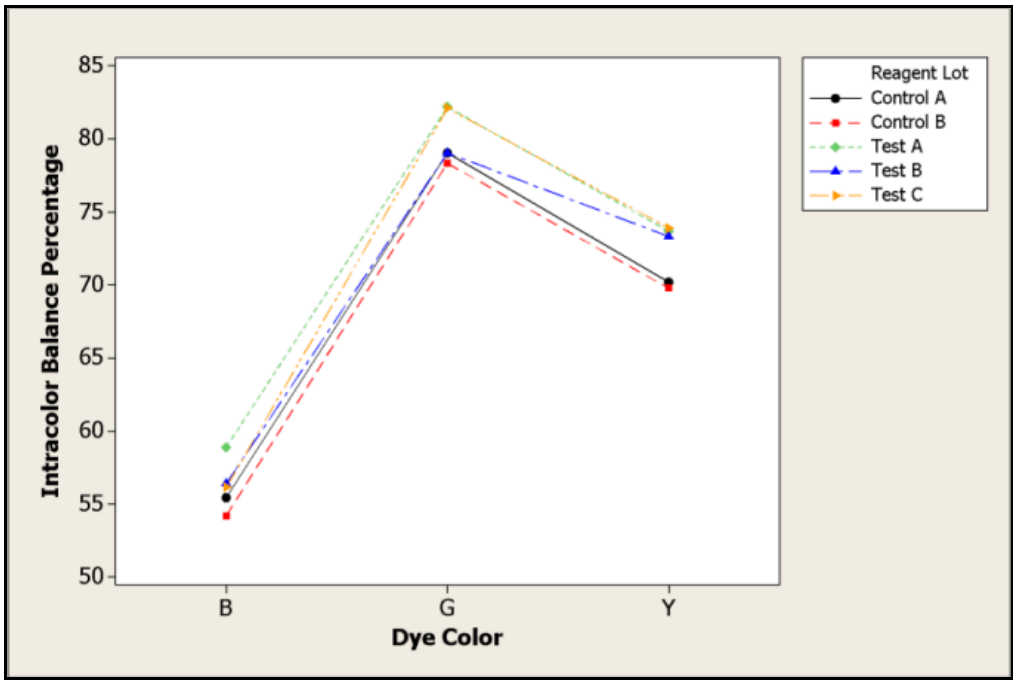
Reproducibility study

For the reproducibility study, 12 replicates of control DNA 007 at 2 ng input and three negative control replicates were amplified. The results were evaluated for intracolor balance, stutter percentage, and the presence, signal intensity, and location of artifacts.

Intracolor balance

No significant difference (<10% increase or decrease) in the level of intracolor balance was observed between the Test and Control mixes (Figure 17).

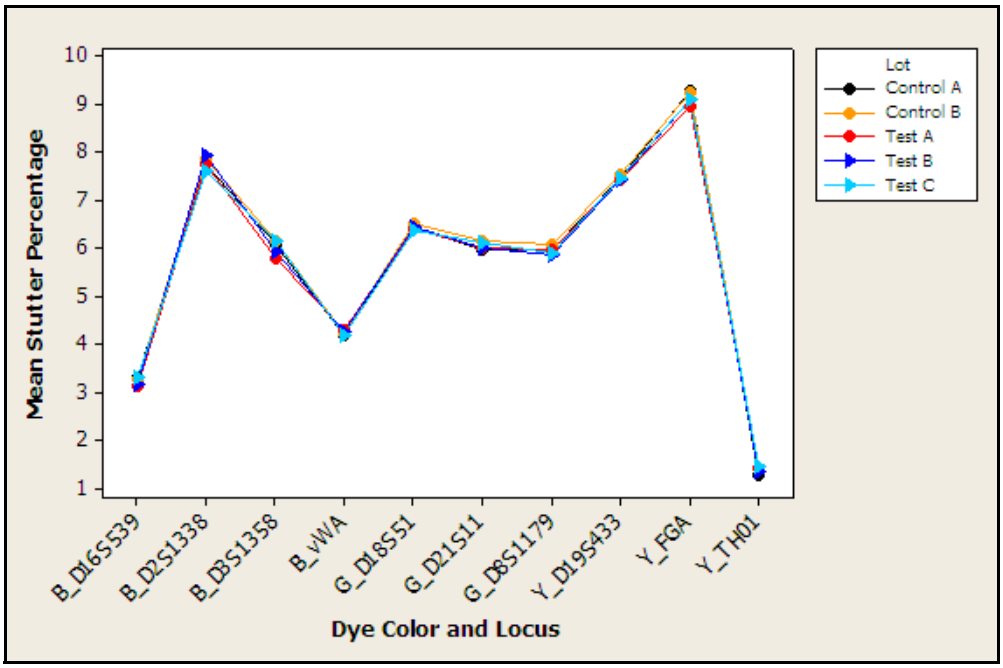
Figure 17 Reproducibility study: intracolor balance



Stutter percentages

Stutter percentage results for each marker were comparable across all Test and Control mixes (Figure 18).

Figure 18 Reproducibility study: mean stutter percentage



Artifacts

Known artifacts observed showed the same morphology, signal intensity, and location in all Test and Control mixes and do not exceed 50 RFU, except for the NED™ dye artifacts highlighted in Figure 19. No new artifacts were observed in the Test mixes.

Figure 19 Reproducibility study: known artifacts (Y-scale 100 RFU)

VIC™ dye labeled artifacts at ~70 bp and ~115 bp

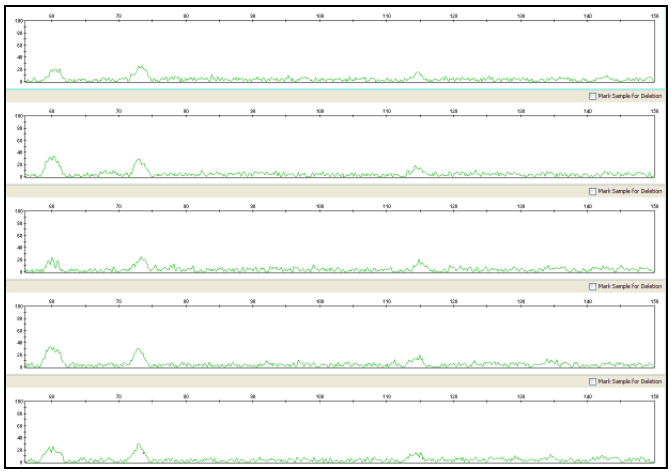
Control A

Control B

Test A

Test B

Test C



NED™ dye labeled artifact at ~88 bp (highlighted peaks >50 RFU)

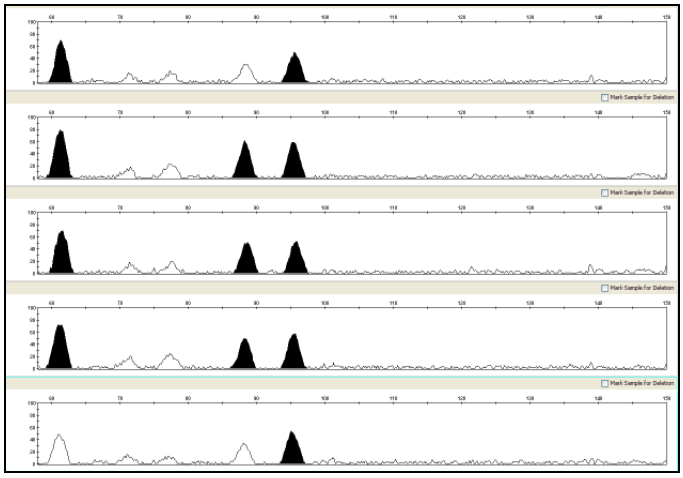
Control A

Control B

Test A

Test B

Test C



FAM™ dye labeled artifact at ~110 bp

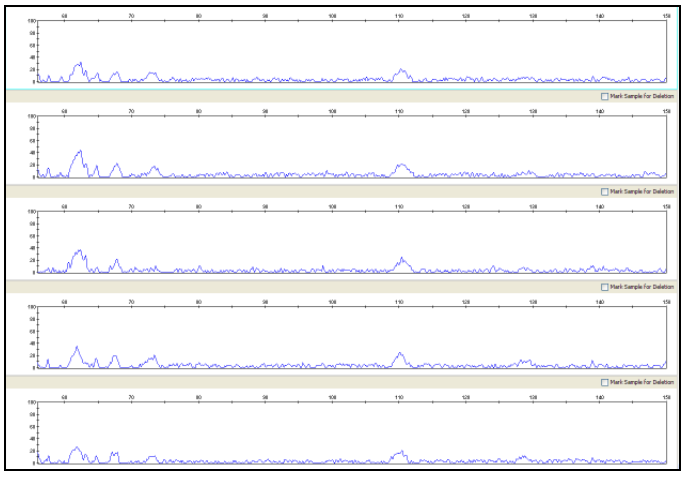
Control A

Control B

Test A

Test B

Test C



Sensitivity study

For the sensitivity study, dilution series of three genomic DNA samples were amplified: 2 ng (three replicates), 1 ng, 0.5 ng, and 0.25 ng (four replicates each). The results were evaluated for mean peak height, degree of linearity between input DNA concentration and peak height, level of allelic dropout at 250 pg, and genotype concordance.

Mean peak height

Overall mean peak height observations were consistent between all Test and Control mixes (Figure 20) demonstrating equivalent performance (Figure 21). Sample IBB286 produced a wider spread between data points. However, this spread did not correlate to a difference in Test versus Control mixes and is likely due to variability in sample set up.

Figure 20 Sensitivity study: mean peak heights for three genomic DNA samples

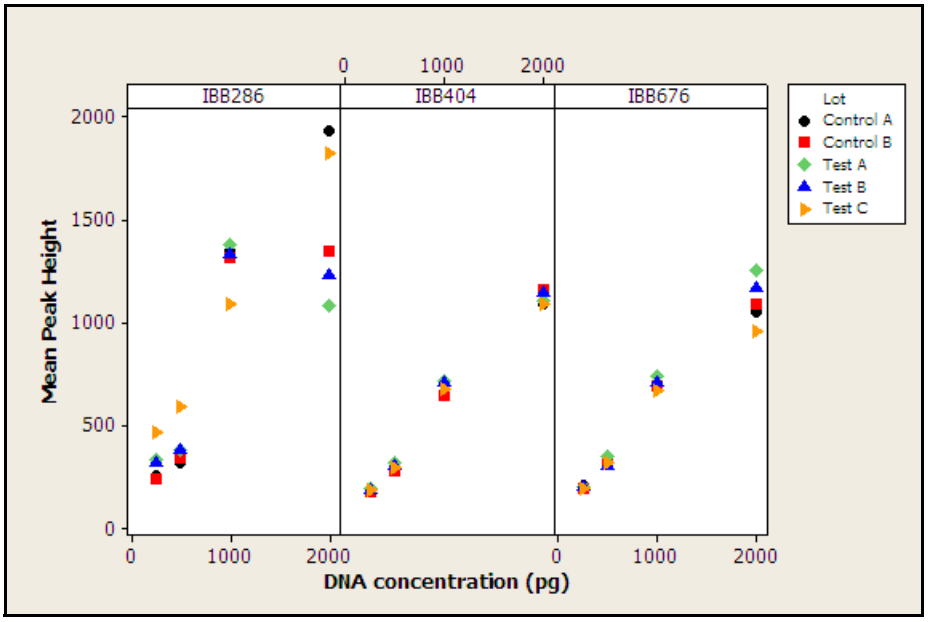
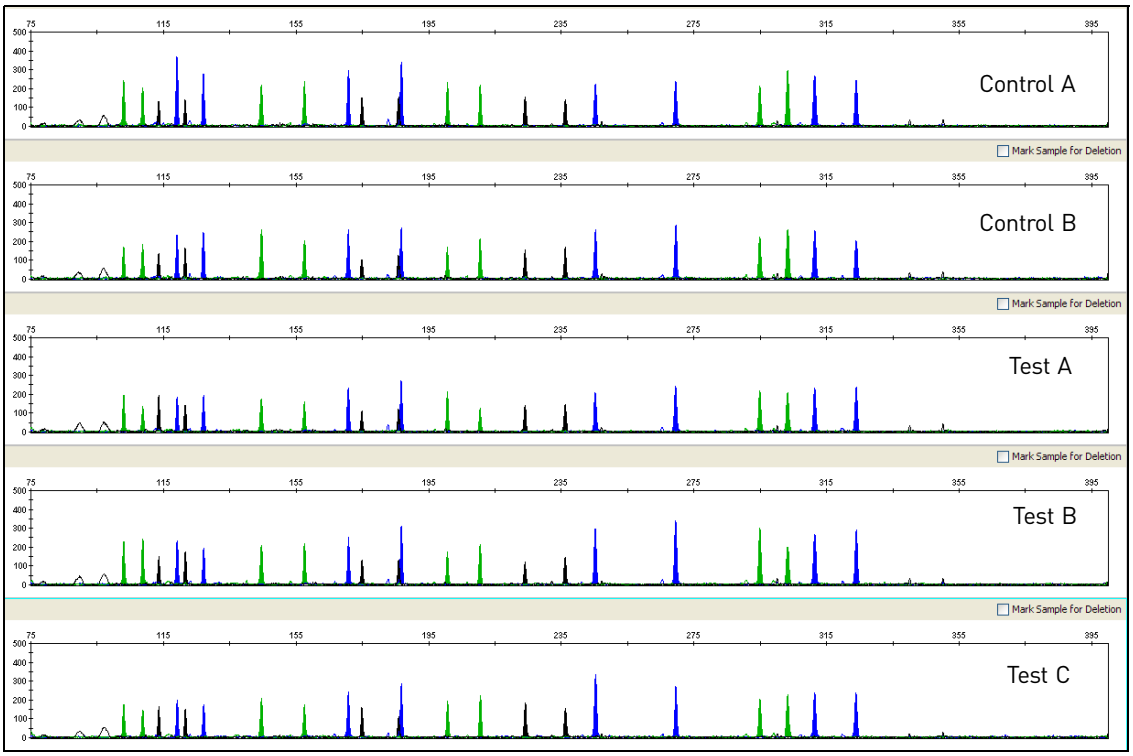


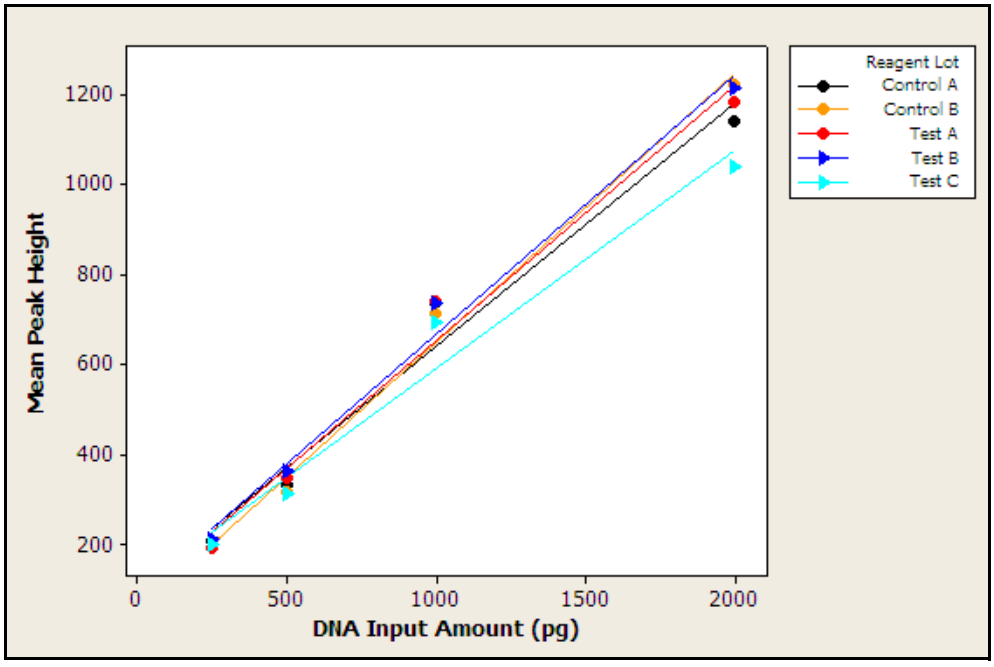
Figure 21 Sensitivity study: representative electropherograms for Sample 2 amplified using 250 pg input DNA (Y-scale 500 RFU)



DNA concentration and peak height

The calculated slope and R^2 values for each of the plotted curves are equivalent, showing comparable relationships between peak height and DNA input amount for the Test and Control mixes (Figure 22).

Figure 22 Sensitivity study: linear regression plot of combined mean peak height for three genomic DNA samples



Allelic dropout

No allelic dropout was observed for any of the Test or Control mixes.

Genotype concordance

Genotypes for Test and Control mixes were 100% concordant (Table 7).

Table 7 Sensitivity study: genotype concordance

DNA Input Amount	Reagent Mix	Genotype Concordance
250 pg	Test A	100%
	Test B	100%
	Test C	100%
	Control A	100%
	Control B	100%
500 pg	Test A	100%
	Test B	100%
	Test C	100%
	Control A	100%
	Control B	100%
1 ng	Test A	100%
	Test B	100%
	Test C	100%
	Control A	100%
	Control B	100%
2 ng	Test A	100%
	Test B	100%
	Test C	100%
	Control A	100%
	Control B	100%

Inhibition study

An inhibition series of 2 ng control DNA 007 consisting of uninhibited control, humic acid at a final concentration of 15.2 ng/µL, and hematin at a final concentration of 40 µM in replicates of five were amplified using each of Test and Control mixes. The amount of each inhibitor tested was titrated to cause an approximate 50% reduction in overall peak height of the samples. Results were evaluated for mean peak height, minimum peak height, intracolor balance, and levels of allelic dropout.

Mean peak height, minimum mean peak height, and intracolor balance

No significant difference in mean peak height, mean minimum peak height and intracolor balance was observed for any Test or Control mixes tested on uninhibited Control DNA 007 and Control DNA 007 inhibited with hematin or humic acid. (Figure 23, 24, and 25).

Figure 23 Inhibition study: mean peak height

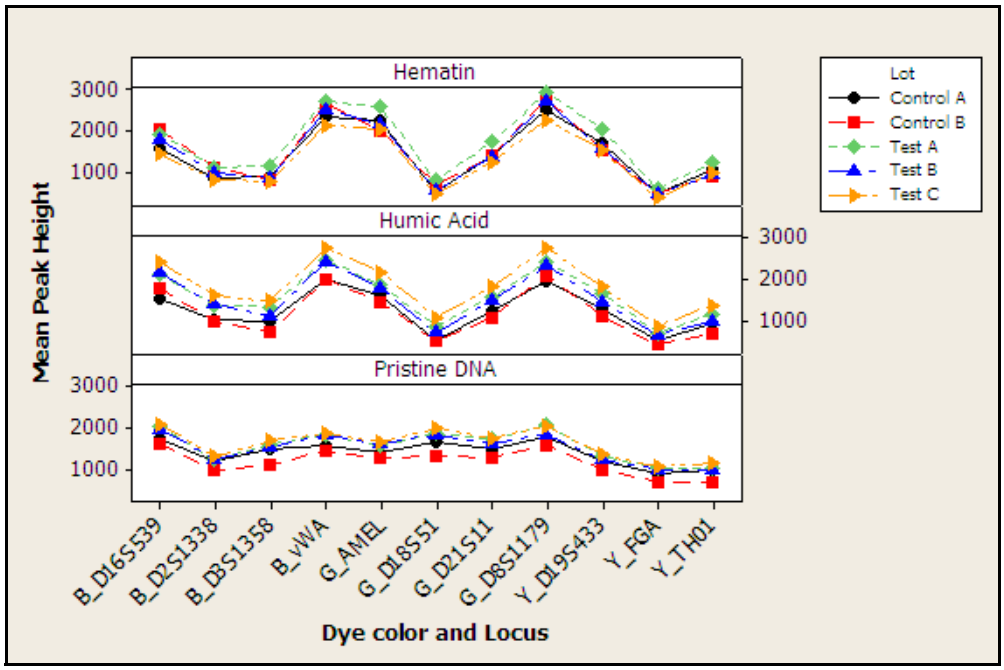


Figure 24 Inhibition study: minimum peak height

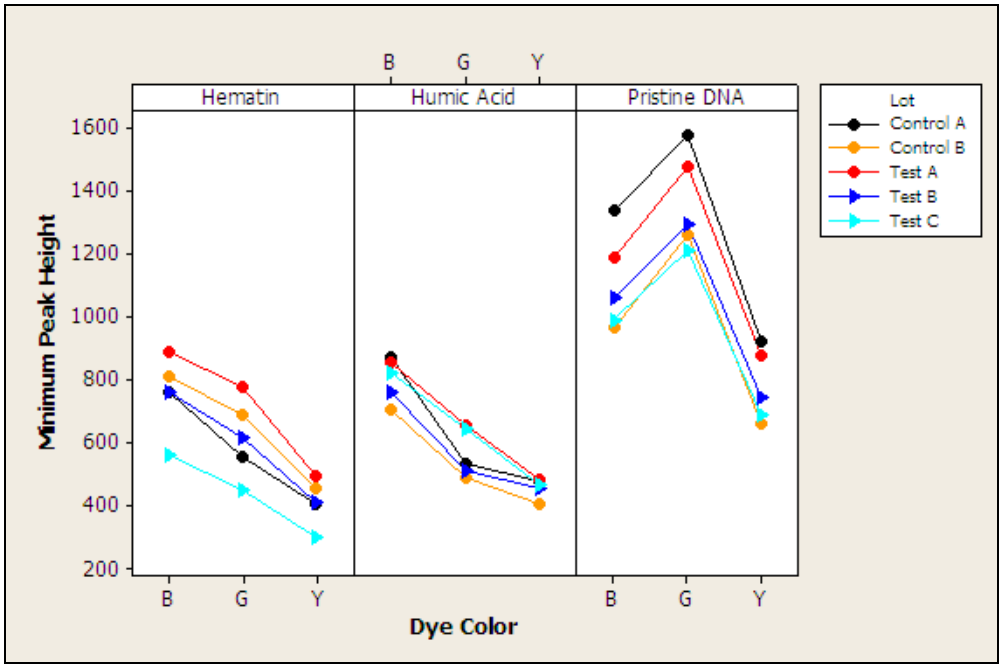
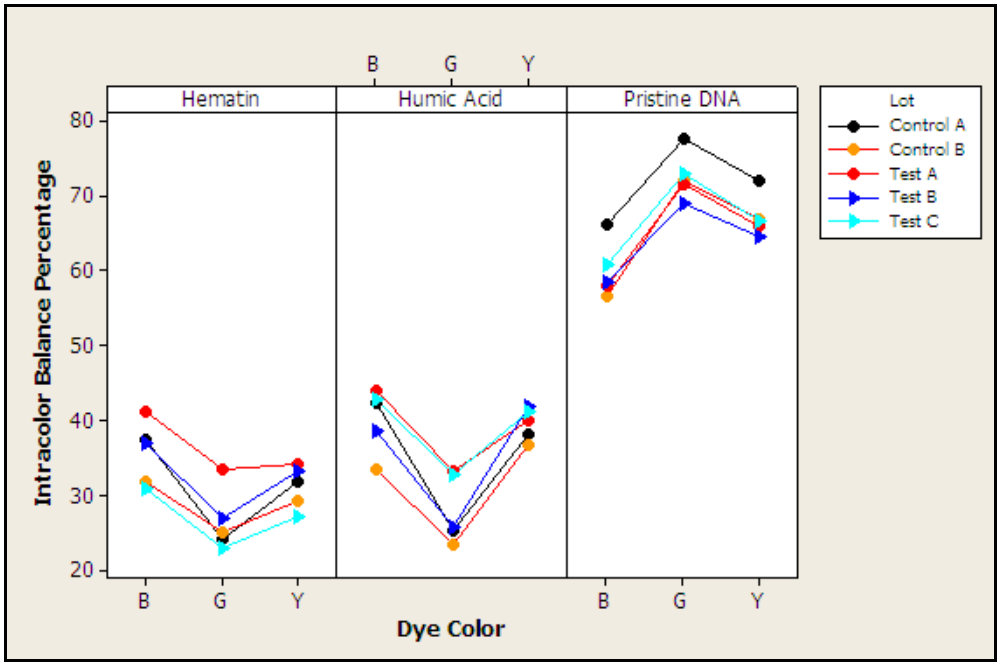


Figure 25 Inhibition study: intracolor balance



Representative electropherograms from the inhibition study are shown in Figure 26, Figure 27, and Figure 28.

Figure 26 Inhibition study: representative electropherograms using uninhibited Control DNA 007 (Y-scale 4000 RFU)

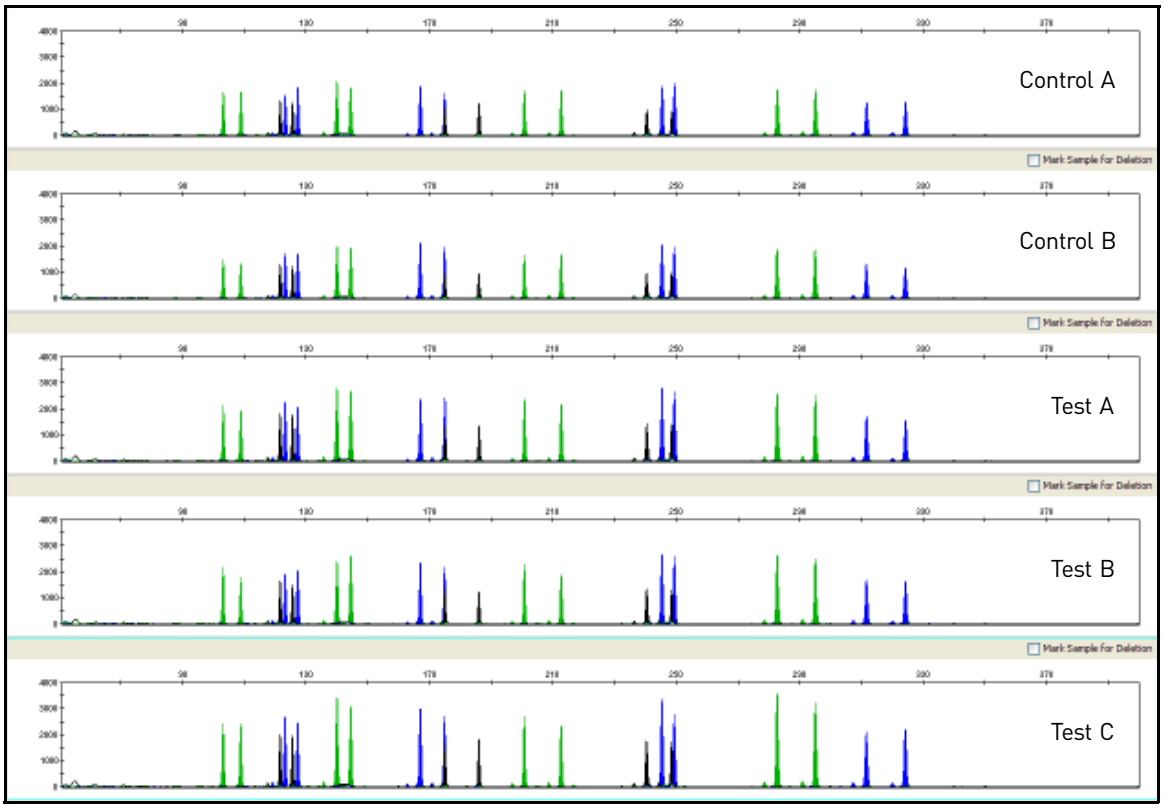


Figure 27 Inhibition study: representative electropherograms using Control DNA 007 inhibited with 40 µM Hematin (Y-scale 4000 RFU)

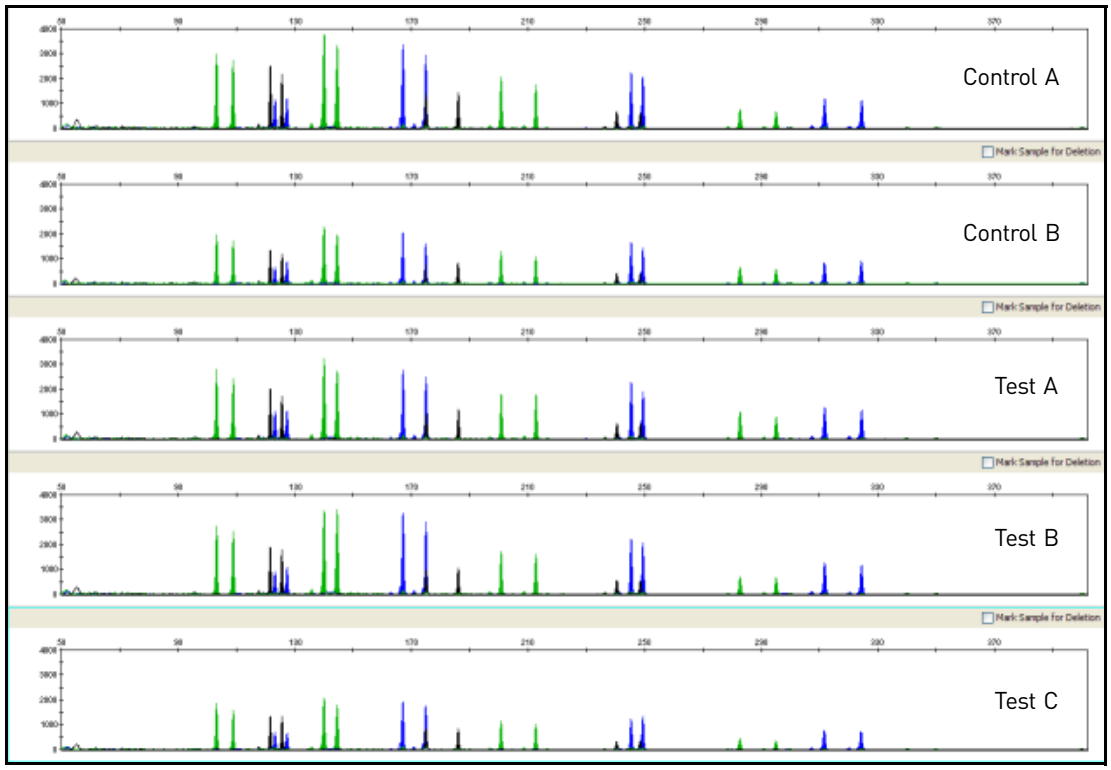
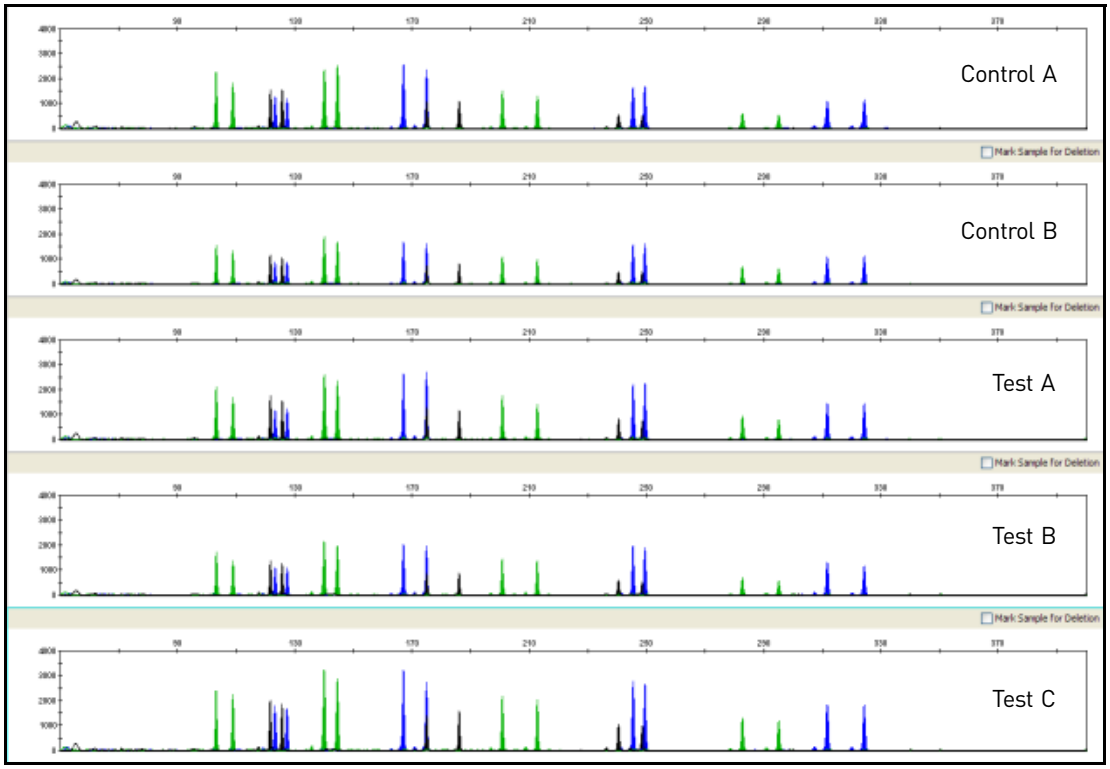


Figure 28 Inhibition study: representative electropherograms using Control DNA 007 inhibited with15.25 ng/µL Humic Acid (Y-scale 4000 RFU)



Allelic dropout

No allelic dropout events were seen for any Test or Control mixes tested on uninhibited Control DNA 007 and Control DNA 007 inhibited with hematin or humic acid.

Conclusions

Laboratories can expect to obtain equivalent quality profiles across a wide range of forensic samples when using the SGM Plus™ Kit containing the AmpliTaq Gold™ enzyme and 10× PCR Buffer II manufactured by Life Technologies as compared to the original SGM Plus™ Kit containing AmpliTaq Gold™ enzyme and 10× PCR Buffer II manufactured by Roche Molecular Systems.

A Troubleshooting

Follow the actions recommended in this appendix to troubleshoot problems that occur during analysis.

Table 8 Troubleshooting

Observation	Possible causes	Recommended actions
Faint or no signal from both the AmpFℓSTR™ Control DNA 007 and the DNA test samples at all loci	Incorrect volume or absence of AmpFℓSTR™ PCR Reaction Mix, SGM Plus™ Primer Set, or AmpliTaq Gold™ DNA Polymerase	Repeat amplification.
	No activation of AmpliTaq Gold™ DNA Polymerase	Repeat amplification, making sure to hold reactions initially at 95°C for 11 minutes.
	Master Mix not vortexed thoroughly before aliquoting	Vortex the Master Mix thoroughly.
	SGM Plus™ Primer Set exposed to too much light	Store the Primer Set protected from light.
	GeneAmp™ PCR System malfunction	Refer to the thermal cycler user's manual and check instrument calibration.
	Use of incorrect thermal cycling parameters	Check the protocol for correct thermal cycling 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 or a MicroAmp™ optical 96-well plate.
	MicroAmp™ Base used with tray/retainer set and tubes in GeneAmp™ 9700	Remove MicroAmp™ Base from tray/retainer set and repeat test.
	Insufficient PCR product electrokinetically injected	Prepare PCR product as described in Chapter 3, "Electrophoresis" on page 25.
	Degraded formamide	Check the storage of formamide; do not thaw and refreeze multiple times. Try Hi-Di™ Formamide.

Observation	Possible causes	Recommended actions
Positive signal from AmpFℓSTR™ Control DNA 007 but partial or no signal from DNA test samples	Quantity of test DNA sample is below assay sensitivity	Quantitate DNA and add 1.0–2.5 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 AmpFℓSTR™ 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 74.
	Mixed sample	
Some but not all loci visible on electropherogram of DNA test samples	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 AmpFℓSTR™ 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.
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, or the Veriti™ 96-Well Thermal Cycler.

B PCR Work Areas

Work area setup and lab design

Many resources are available for the appropriate design of a PCR laboratory. If you are using the AmpFℓSTR™ SGM 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
- 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 AmpFℓSTR™ Kits (and other PCR-based tests) enables amplification of minute quantities of DNA, necessitating precautions to avoid contamination of samples yet to be amplified (Kwok and Higuchi, 1989).

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

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

PCR setup work area

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

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

- Tube decapper, autoclavable
- Vortex

Amplified DNA work area

IMPORTANT! Place the thermal cyclers in the Amplified DNA Work Area.

You can use the following systems:

- GeneAmp™ PCR System 9700 with the Silver 96-Well Block
- GeneAmp™ PCR System 9700 with the Gold-plated Silver 96-Well Block

  IMPORTANT! The SGM 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 kit.

- Veriti™ 96-Well Thermal Cycler

C

Ordering Information

Equipment and materials not included

Table 9 and Table 10 list required and optional equipment and materials not supplied with the SGM Plus™ Kit. Unless otherwise noted, many of the items are available from major laboratory suppliers (MLS).

Table 9 Equipment

Equipment	Source
Applied Biosystems 3100/3100-*Avant* Genetic Analyzer	Contact your local Life Technologies sales representative
Applied Biosystems 3130/3130*xl* Genetic Analyzer	
Applied Biosystems 3500/3500xL Genetic Analyzer for Human Identification	
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 10 User-supplied materials

Item†	Source
AmpFℓSTR™ SGM Plus™ PCR Amplification Kit	4307133
3100 Analyzer materials	
96-well plate septa	4315933
Reservoir septa	4315932
3100/3130*xl* Genetic Analyzer capillary array, 36-cm	4315931
POP-4™ polymer for 3100/3100-*Avant* Genetic Analyzers	4316355
3100/3100-*Avant* Genetic Analyzer Autosampler Plate Kit, 96-well	4316471
GeneScan™ 500 ROX™ Size Standard	401734
Running Buffer, 10X	402824
Hi-Di™ Formamide	4311320
DS-32 Matrix Standard Kit (Dye Set F)	4345831
MicroAmp™ Optical 96-well reaction plate	N8010560

Item†	Source
250-µL glass syringe (array-fill syringe)	4304470
5.0-mL glass syringe (polymer-reserve syringe)	628-3731
For a complete list of parts and accessories for the 3100/3100-*Avant* instrument, refer to Appendix B of the *3100 Genetic Analyzer and 3100-Avant Genetic Analyzer User Reference Guide* (Pub. no. 4335393).	
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
3100/3100-*Avant* Genetic Analyzer Autosampler Plate Kit, 96-well	4316471
GeneScan™ 500 ROX™ Size Standard	401734
Running Buffer, 10X	402824
DS-32 Matrix Standard Kit (Dye Set F)	4345831
MicroAmp™ Optical 96-well reaction plate	N8010560
Hi-Di™ Formamide	4311320
For a complete list of parts and accessories for the 3130/3130*xl* instrument, refer to Appendix A of the *Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide* (Pub. no. 4352716).	
3500/3500xL Analyzer materials	
Anode buffer container (ABC)	4393927
Cathode buffer container (CBC)	4408256
POP-4™ polymer (960 samples) for 3500/3500xL Genetic Analyzers	4393710
POP-4™ polymer (384 samples) for 3500/3500xL Genetic Analyzers	4393715
Conditioning reagent	4393718
8-Capillary array, 36 cm for 3500 Genetic Analyzers	4404683
24-Capillary array, 36 cm for 3500xL Genetic Analyzers	4404687
96-well retainer & base set (Standard) 3500/3500xL Genetic Analyzers	4410228
8-Tube retainer & base set (Standard) for 3500/3500xL Genetic Analyzers	4410231
8-Strip Septa for 3500/3500xL Genetic Analyzers	4410701
96-Well Septa for 3500/3500xL Genetic Analyzers	4412614
Septa Cathode Buffer Container, 3500 series	4410715
GeneScan™ 500 ROX™ Size Standard	401734
For a complete list of parts and accessories for the 3500/3500xL instrument, refer to the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide* (Pub. no. 4401661)	
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 ROX™ Size Standard	401734

Item†	Source
Running Buffer, 10X	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-32 Matrix Standard Kit (Dye Set F) for the 310 Genetic Analyzer	4312131
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 *310 Genetic Analyzer User Guide* (Pub. no. 4317588).	
PCR Amplification	
MicroAmp™ 96-well tray	N8010541
MicroAmp™ reaction tube with cap, 0.2-mL	N8010540
MicroAmp™ 8-tube strip, 0.2-mL	N8010580
MicroAmp™ 8-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 Safety Data Sheet (SDS) of any chemical not distributed by Life Technologies, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

D

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of 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.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the 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.

Specific chemical handling

CAS	Chemical	Phrase
26628-22-8	Sodium Azide	Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

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:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **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**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

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Documentation and Support

Related documentation

Document title	Pub. no.
AmpFℓSTR™ SGM Plus™ PCR Amplification Kit: Human Identification: Application Note	040302
3100/3100-Avant Data Collection v2.0 User Guide	4347102
3100/3100-Avant Genetic Analyzers Using Data Collection Software v2.0 User Bulletin	4350218
3100 Genetic Analyzer User Manual (Data Collection v1.1)	4315834
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
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Applied Biosystems 3500/3500xL Genetic Analyzer User Bulletin: Solutions to issues related to software, data, hardware, and consumables **Note:** Additional user bulletins may be available at **www.lifetechnologies.com**	4445098
Applied Biosystems 3730/3730xl Genetic Analyzer Getting Started Guide	4359476
GeneAmp™ PCR System 9700 Base Module User's Manual	N805-0200
Veriti™ 96-Well Thermal Cycler AmpFℓSTR™ *Kit Validation User Bulletin*	4440754
Quantifiler™ Kits: Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Y Human Male DNA Quantification Kit User's Manual	4344790
PrepFiler™ Forensic DNA Extraction Kit User Guide	4390932
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
GeneMapper™ ID-X Software Version 1.1 (Mixture Analysis) Getting Started Guide	4396773

Document title	Pub. no.
GeneMapper™ ID-X Software Version 1.1 (Mixture Analysis) Quick Reference Guide	4402094
GeneMapper™ ID-X Software Version 1.2 Reference Guide	4426481
GeneMapper™ ID-X Software Version 1.2 Quick Reference Guide	4426482

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