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AmpFℓSTR® NGM™

PCR Amplification Kit

User's Guide

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User's Guide

Applied Biosystems

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Use of the AmpFℓSTR® NGM™ PCR Amplification Kit is covered by US patent claims and patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product solely in forensic and paternity testing, including reporting results of purchaser's activities for a fee or other commercial consideration, and also for the purchaser's own internal research. No right under any other patent claim is conveyed expressly, by implication, or by estoppel. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

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Contents

Preface

Safety information

Note: For general safety information, see this Preface and Appendix B, "Safety" on page 119. When a hazard symbol and hazard type appear by an instrument hazard, see the "Safety" Appendix for the complete alert. For all chemicals, read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Safety alert words

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

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

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

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

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

MSDSs

The MSDSs for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining MSDSs, see "Obtaining MSDSs" on page 121.

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

Overview 1

This chapter covers:

Product overview

Purpose

The AmpFℓSTR® NGM™ PCR Amplification Kit is a short tandem repeat (STR) multiplex assay that amplifies 14 tetranucleotide repeat loci and one trinucleotide repeat locus, D22S1045. The kit simultaneously coamplifies the 10 loci contained in the AmpFℓSTR® SGM Plus® kit (D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01, and FGA) together with two highly polymorphic STR loci (D1S1656 and D12S391), 3 "mini" STR loci (D10S1248, D22S1045 and D2S441), and the gender determination locus Amelogenin. The AmpFℓSTR® NGM™ Kit delivers a 16-locus multiplex with a greater power of discrimination, better sensitivity, and improved robustness than earlier generation kits. The kit uses modified PCR cycling conditions for enhanced sensitivity, a new buffer formulation to improve performance with inhibited samples, more loci concentrated in the low molecular-weight region of the profile to improve performance on degraded samples, and an improved process for synthesis and purification of the amplification primers to deliver a much cleaner electrophoretic background.

Product description

The AmpFℓSTR® NGM™ Kit contains all the necessary reagents for the amplification of human genomic DNA.

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

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

About the primers

The AmpFℓSTR® NGM™ Kit employs the latest improvements in primer synthesis and purification techniques to minimize the presence of dye-labeled artifacts. These improvements result in a much cleaner electropherogram background that enhances the assay's signal-to-noise ratio and simplifies the interpretation of results.

Loci amplified by the kit

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

Table 1 AmpFℓSTR® NGM™ Kit loci and alleles

Locus designation	Chromosome location	Alleles included in AmpFℓSTR® NGM™ Kit Allelic Ladder	Dye label	Control DNA 007
D10S1248	10q26.3	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18	6-FAM™	12, 15
vWA	12p13.31	11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24	6-FAM™	14, 16
D16S539	16q24.1	5, 8, 9, 10, 11, 12,13, 14, 15	6-FAM™	9, 10
D2S1338	2q35	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28	6-FAM™	20, 23
Amelogenin	X: p22.1-22.3 Y: p11.2	X, Y	VIC®	X, Y
D8S1179	8q24.13	8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	VIC®	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	VIC®	28, 31
D18S51	18q21.33	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	VIC®	12, 15
D22S1045	22q12.3	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	NED™	11, 16
D19S433	19q12	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	NED™	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	NED™	24, 26
D2S441	2p14	9, 10, 11, 11.3, 12, 13, 14, 15, 16	PET®	14, 15
D3S1358	3p21.31	12, 13, 14, 15, 16, 17, 18, 19	PET®	15, 16
D1S1656	1q42.2	9, 10, 11, 12, 13, 14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18.3, 19.3, 20.3	PET®	13, 16
D12S391	12p13.2	14, 15, 16, 17, 18, 19, 19.3, 20, 21, 22, 23, 24, 25, 26, 27	PET®	18, 19

Allelic ladder profile

Figure 1 shows the allelic ladder for the AmpFℓSTR® NGM™ Kit. See **"Allelic ladder requirements" on page 28** for information on ensuring accurate genotyping.

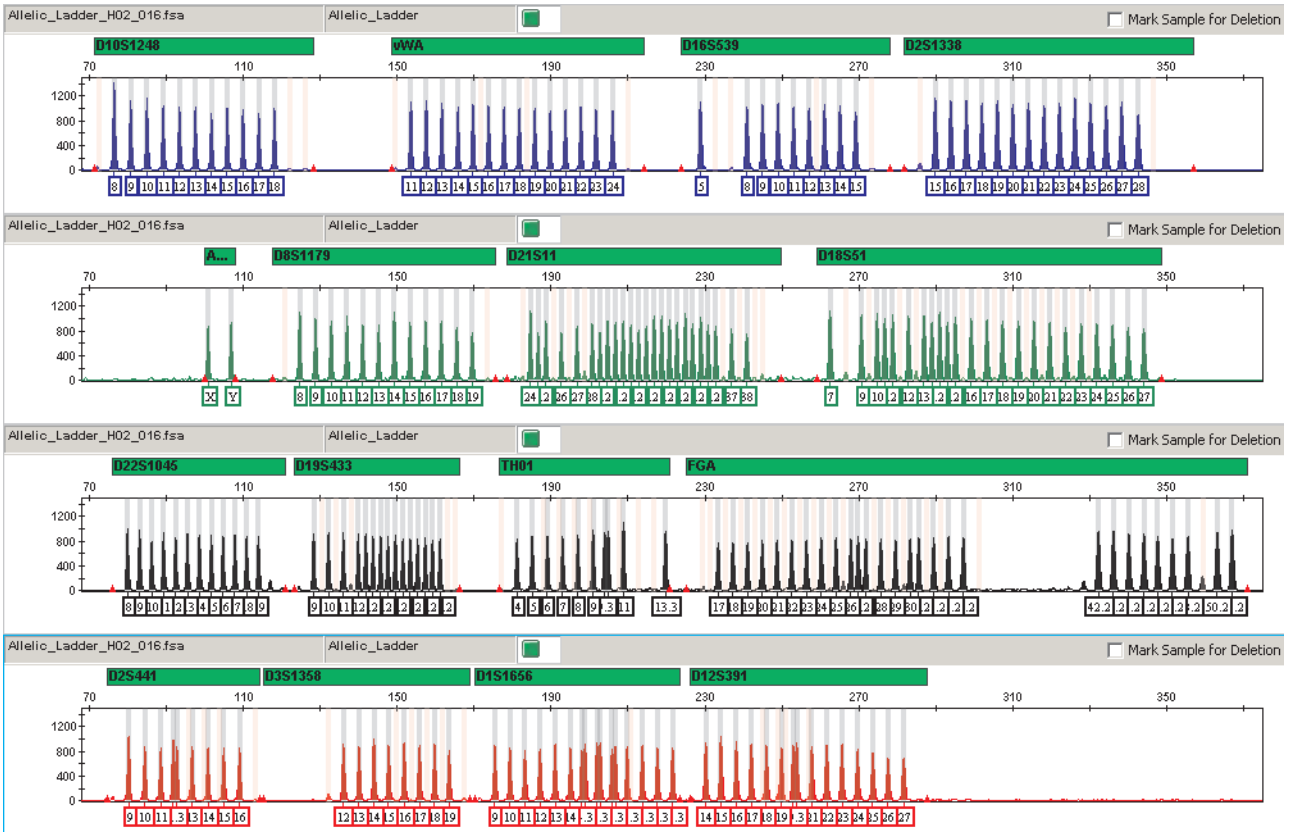


Figure 1 GeneMapper® *ID-X* Software plot of the AmpFℓSTR® NGM™ Kit Allelic Ladder

Control DNA 007 profile

Figure 2 shows amplification of Control DNA 007 using the AmpF*ℓ*STR® NGM™ Kit.

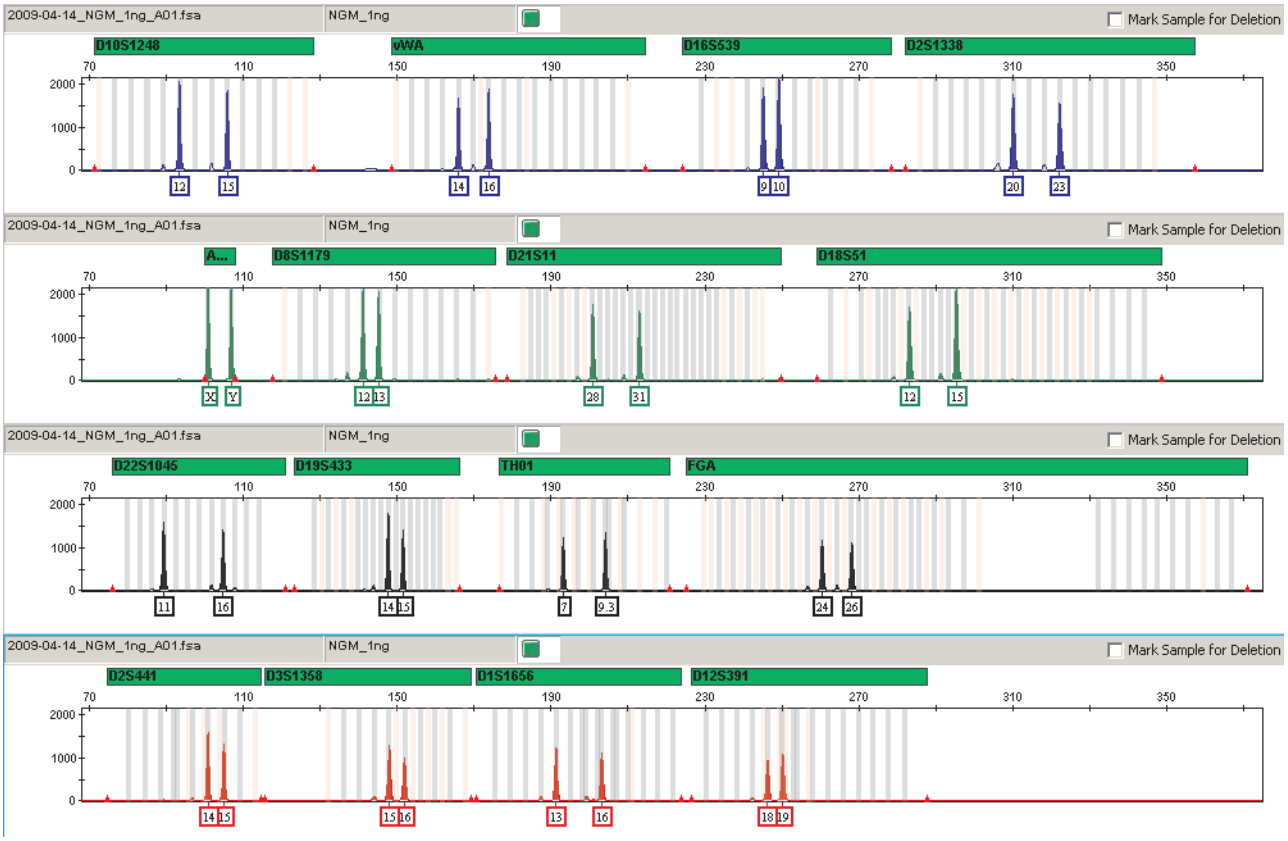


Figure 2 1 ng of Control DNA 007 amplified with the AmpF*ℓ*STR® NGM™ Kit and analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer

Workflow overview

Extract DNA

PrepFiler™ Forensic DNA Extraction Kit

Quantify DNA

Quantifiler® Duo DNA Quantification Kit

Perform PCR

AmpFℓSTR® NGM™ PCR Amplification Kit

GeneAmp® PCR System 9700 Thermal Cycler*

* Amplification should be performed on silver- or gold-plated silver 96-well blocks only.

Perform Electrophoresis

ABI PRISM® 310 Genetic Analyzer

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

Applied Biosystems 3130/3130*xl* Genetic Analyzer

Analyze Data

GeneMapper® *ID* or GeneMapper® *ID-X* Software

Instrument and software overview

This section provides information about the Data Collection Software versions required to run the AmpFℓSTR® NGM™ 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
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 and Windows 2000	3.0	

‡ Applied Biosystems conducted validation studies for the AmpFℓSTR® NGM™ Kit using this configuration.

About multicomponent analysis

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

Multicomponent analysis is the process that separates the five different fluorescent dye colors into distinct spectral components. The four dyes used in the AmpFℓSTR® NGM™ PCR Amplification Kit to label samples are 6-FAM™, VIC®, NED™, and PET® dyes. The fifth dye, LIZ®, is used to label the GeneScan™ 500 LIZ® Size Standard.

How multicomponent analysis works

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

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

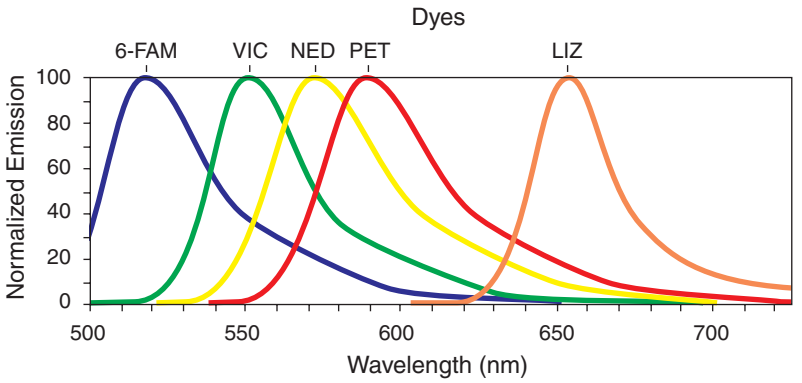


Figure 3 Emission spectra of the five dyes used in the AmpFℓSTR® NGM™ Kit

Materials and equipment

Kit contents and storage

The AmpFℓSTR® NGM™ PCR Amplification Kit contains materials sufficient to perform 200 (PN 4415020) or 1000 (PN4415021) amplifications at 25-µL reaction volumes.

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

Table 3 Kit Contents and Storage

Component	Description	200× Volume	1000× Volume	Storage
AmpFℓSTR® NGM Primer Set	Contains forward and reverse primers to amplify human DNA targets.	1 tube, 1.0 mL	1 bottle, 5.0 mL	-15 to -25 °C on receipt, 2 to 8 °C after initial use
AmpFℓSTR® NGM Master Mix	Contains enzyme, salts, dNTPs, carrier protein, and 0.05% sodium azide.	2 tubes, 1.0 mL each	1 bottle, 10.0 mL	-15 to -25 °C on receipt, 2 to 8 °C after initial use
AmpFℓSTR® NGM Allelic Ladder	Contains amplified alleles. See Table 1 on page 3 for a list of alleles included in the allelic ladder.	1 tube, 50.0 µL	1 tube, 75.0 µL	-15 to -5 °C on receipt, 2 to 8 °C after initial use
AmpFℓSTR® Control DNA 007	Contains 0.10 ng/µL human male 007 DNA in 0.02% sodium azide and buffer‡. See Table 1 on page 3 for profile.	1 tube, 0.3 mL	1 tube, 0.3 mL	2 to 8 °C

‡ The AmpFℓSTR® Control DNA 007 is included at a concentration appropriate to its intended use as an amplification control (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 laboratories may expect to see variation from the labelled concentration when quantitating aliquots of the AmpFℓSTR® Control DNA 007.

Standards for samples

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

- **Control DNA 007** – A positive control for evaluating the efficiency of the amplification step and STR genotyping using the AmpFℓSTR® NGM™ Allelic Ladder.
- **GeneScan™ 500 LIZ® Size Standard** – Standard used for obtaining sizing results. It contains 16 single-stranded labeled fragments of: 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 nucleotides. This standard, which has been evaluated as an internal lane size standard, yields precise sizing results for AmpFℓSTR® NGM™ Kit PCR products. Order the GeneScan 500 LIZ Size Standard (PN 4322682) separately.
- **AmpFℓSTR® NGM™ Allelic Ladder** – Allelic ladder developed by Applied Biosystems for accurate characterization of the alleles amplified by the AmpFℓSTR® NGM™ Kit. The AmpFℓSTR® NGM™ Allelic Ladder contains most of the alleles reported for the 15 autosomal loci. Refer to Table 1 on page 3 for a list of the alleles included in the AmpFℓSTR® NGM™ Allelic Ladder.

Equipment and materials not included

Tables 4 and 5 list required and optional equipment and materials not supplied with the AmpFℓSTR® NGM™ Kit. Unless otherwise noted, many of the items are available from major laboratory suppliers (MLS).

Table 4 Equipment

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

Table 5 User-supplied materials‡

Item	Source
AmpFℓSTR® NGM™ PCR Amplification Kit (200x/1000x)	4415020/4415021
3100/3100-*Avant* Analyzer materials	
96-Well Plate Septa	4315933
Reservoir Septa	4315932
3100/3100-*Avant* Genetic Analyzer Capillary Array, 36-cm	4333464
POP-4™ Polymer for 3100/3100-*Avant* Genetic Analyzers	4316355
3100/3100-*Avant* Genetic Analyzer Autosampler Plate Kit, 96-well	4316471
GeneScan™ 500 LIZ® Size Standard	4322682
Running Buffer, 10×	402824
DS-33 Matrix Standard Kit (Dye Set G5)	4345833
MicroAmp® Optical 96-Well Reaction Plate	N8010560
250-µL Glass Syringe (array-fill syringe)	4304470
5.0-mL Glass Syringe (polymer-reserve syringe)	628-3731

For a complete list of parts and accessories for the 3100 instrument, refer to Appendix B of the *ABI PRISM® 3100 Genetic Analyzer and 3100-Avant Genetic Analyzer User Reference Guide* (PN 4335393).

Table 5 User-supplied materials‡

Item	Source
3130/3130*xl* Analyzer materials	
96-Well Plate Septa	4315933
Reservoir Septa	4315932
3100/3130*xl* Genetic Analyzer Capillary Array, 36-cm	4315931
POP-4™ Polymer for 3130/3130*xl* Genetic Analyzers	4352755
3130/3130*xl* Genetic Analyzer Autosampler Plate Kit, 96-well	4316471
GeneScan™ 500 LIZ® Size Standard	4322682
Running Buffer, 10×	402824
DS-33 Matrix Standard Kit (Dye Set G5)	4345833
MicroAmp® Optical 96-Well Reaction Plate	N8010560
For a complete list of parts and accessories for the 3130*xl* instrument, refer to Appendix A of the *Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide* (PN 4352716).	
310 Analyzer materials	
310 DNA Analyzer Capillary Array, 47-cm	402839
0.5 mL Sample Tray	5572
96-Well Tray Adaptor (for 9700 thermal cycler trays)	4305051
GeneScan™ 500 LIZ® Size Standard	4322682
Running Buffer, 10×	4335643
Genetic Analyzer Septa Retainer Clips for 96-Tube Sample Tray	402866
Genetic Analysis Sample Tubes (0.5-mL)	401957
Septa for 0.5-mL Sample Tubes	401956
DS-33 Matrix Standard Set (6-FAM™, VIC®, NED™, PET®, and LIZ® dyes) for ABI PRISM® 310/377 systems	4318159
MicroAmp® 8-Tube Strip, 0.2-mL	N8010580
MicroAmp® 96-Well Base (holds 0.2-mL reaction tubes)	N8010531
MicroAmp® 96-Well Full Plate Cover	N8010550
MicroAmp® 96-Well Tray/Retainer Set	403081
POP-4™ Polymer for the 310 Genetic Analyzer	402838
For a complete list of parts and accessories for the 310 instrument, refer to Appendix B of the *ABI PRISM® 310 Genetic Analyzer User Guide* (PN 4317588).	

Table 5 User-supplied materials‡

Item	Source
PCR Amplification	
MicroAmp® 96-Well Tray	N8010541
MicroAmp® Reaction Tube with Cap, 0.2-mL	N8010540
MicroAmp® 8-Tube Strip, 0.2-mL	N8010580
MicroAmp® 8-Cap Strip	N8010535
MicroAmp® 96-Well Tray/Retainer Set	403081
MicroAmp® 96-Well Base	N8010531
MicroAmp® Clear Adhesive Film	4306311
MicroAmp® Optical Adhesive Film	4311971
MicroAmp® Optical 96-Well Reaction Plate	N8010560
Other user-supplied materials	
Hi-Di™ Formamide, 25-mL	4311320
Aerosol resistant pipette tips	MLS
Microcentrifuge tubes	MLS
Pipettors	MLS
Tape, labeling	MLS
Tube, 50-mL Falcon	MLS
Tube decapper, autoclavable	MLS
Deionized water, PCR grade	MLS
Tris-HCL, pH 8.0	MLS
EDTA, 0.5 M	MLS
Vortex	MLS

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

PCR Amplification 2

This chapter covers:

PCR work areas

Work area setup and lab design

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

- For AmpFℓSTR® NGM™ PCR Amplification Kit forensic DNA testing, refer to: National Institute of Justice Office of Law Enforcement Standards. 1998. *Forensic Laboratories: Handbook for Facility Planning, Design, Construction and Moving*. Washington, DC: National Institute of Justice. 76 pp.
- For AmpFℓSTR® NGM™ Kit parentage DNA testing, refer to: American Association of Blood Banks. 2004. *Guidance for Standards for Parentage Relationship Testing Laboratories*. 7th ed. Bethesda, Md: American Association of Blood Banks. 58 pp.

The sensitivity of the AmpFℓSTR® NGM™ Kit (and other PCR-based tests) enables amplification of minute quantities of DNA, necessitating precautions to avoid contamination of samples yet to be amplified (Kwok and Higuchi, 1989).

To prevent contamination by human DNA, be careful while handling and processing samples. Wear gloves at all times and change them frequently. Close sample tubes when not in use. Limit aerosol dispersal by handling sample tubes and reagents carefully.

Note: These laboratory design resources and guidances constitute only a sample of the precautions that need to be observed when using PCR technology. Refer to your laboratory's internal policies and procedures for additional information and references.

PCR-setup tools

IMPORTANT! These items should never leave the PCR setup work area.

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

Amplified DNA work area tools

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

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

Required user-supplied materials and reagents

Kit contents and storage

The AmpFℓSTR® NGM™ PCR Amplification Kit is available as either a 200-reaction kit or 1000-reaction kit. The number of reactions is based on a 25-µL reaction volume. See "Kit contents and storage" on page 9 for details on kit contents.

User-supplied reagents

In addition to the AmpFℓSTR® NGM™ 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 based on your 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 AmpFℓSTR® NGM™ Kit is 1.0 ng in a maximum input volume of 10 µL.

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

- Fluorescence intensity that exceeds the linear dynamic range for detection by the instrument ("off-scale" data). Off-scale data are problematic because:
  - Quantification (peak height and area) for off-scale peaks is not accurate. For example, an allele peak that is off-scale can cause the corresponding stutter peak to appear higher in relative intensity, thus increasing the calculated percent stutter.
  - Multicomponent analysis of off-scale data is not accurate, and it results in poor spectral separation ("pull-up").
- Incomplete A-nucleotide addition.

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

Methods of quantifying DNA

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

Product	Description	References
Quantifiler® Human DNA Quantification Kit (PN 4343895) *and* Quantifiler® Y Human Male DNA Quantification Kit (PN 4343906)	**Properties:** The Quantifiler® Human and Quantifiler® Y Human Male Kits are highly specific for human DNA, and they 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 that consists of an IPC template DNA (a synthetic sequence not found in nature), two primers for amplifying the IPC template DNA, and one TaqMan® MGB probe labeled with VIC® dye for detecting the amplified IPC DNA.	*Quantifiler® Human DNA Quantification Kits User's Manual* (PN 4344790)
Quantifiler® Duo DNA Quantification Kit (PN 4387746)	**Properties:** The Quantifiler® Duo Kit is highly specific for human DNA and combines the detection of both total human and male DNA in one PCR reaction.The kit detects single-stranded and degraded DNA. **How it works:** The Quantifiler® Duo DNA Quantification Kit consists of target-specific and internal control 5' nuclease assays. The Quantifiler® Duo kit combines two human-specific assays in one PCR reaction (for total human DNA and human male DNA). The two human DNA specific assays each consist of two PCR primers and a TaqMan® probe. The TaqMan® probes for the human DNA and human male DNA assays are labeled with VIC® and FAM™ dyes, respectively. In addition, the kit contains an internal PCR control (IPC) assay similar in principle to that used in the other Quantifiler kits, but labeled with NED™ dye.	*Quantifiler® Duo DNA Quantification Kit User's Manual* (PN 4391294)

Prepare the amplification kit reactions

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

DNA sample	Volume per reaction (µL)
AmpFℓSTR® NGM™ Master Mix	10.0
AmpFℓSTR® NGM™ Primer Set	5.0

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

2. Prepare reagents. Thaw the AmpFℓSTR® NGM™ Master Mix and the AmpFℓSTR® NGM™ Primer Set, then vortex the tubes for 3 seconds and centrifuge them briefly before opening.

IMPORTANT! Thawing is required only during first use of the kit. After first use, reagents are stored at 2 to 8 °C and, therefore, do not require subsequent thawing. Do not refreeze the reagents.

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

4. Vortex the reaction mix for 3 seconds, then centrifuge briefly.

5. Dispense 15 µL of reaction mix into each reaction well of a MicroAmp® Optical 96-Well Reaction Plate or each MicroAmp® tube.

6. Prepare the DNA samples:

DNA sample	To prepare...
Negative control	Add 10 µL of low TE buffer (10mM Tris, 0.1mM EDTA, pH 8.0).
Test sample	Dilute a portion of the test DNA sample with low TE buffer so that 1.0 ng of total DNA is in a final volume of 10 µL. Add 10 µL of the diluted sample to the reaction mix.
Positive control	Add 10 µL of 007 control DNA (0.1 ng/µL) to provide 1.0 ng of total DNA in the positive control reaction.

The final reaction should be 25 µL.

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

8. Centrifuge the tubes or plate at 3000 rpm for about 20 seconds in a tabletop centrifuge (with plate holders if using 96-well plates) to remove bubbles.

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

Note: The AmpFℓSTR® NGM™ 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 the performance of the AmpFℓSTR® NGM™ Kit.

Perform PCR

WARNING! PHYSICAL INJURY HAZARD. Thermal cycler.

1. Program the thermal cycling conditions.

IMPORTANT! When using the GeneAmp PCR System 9700 with either 96-well silver or gold-plated silver block, select the **9600 Emulation Mode**.

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

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

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

3. Start the run.

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

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

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

Amplification using bloodstained FTA® cards

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

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

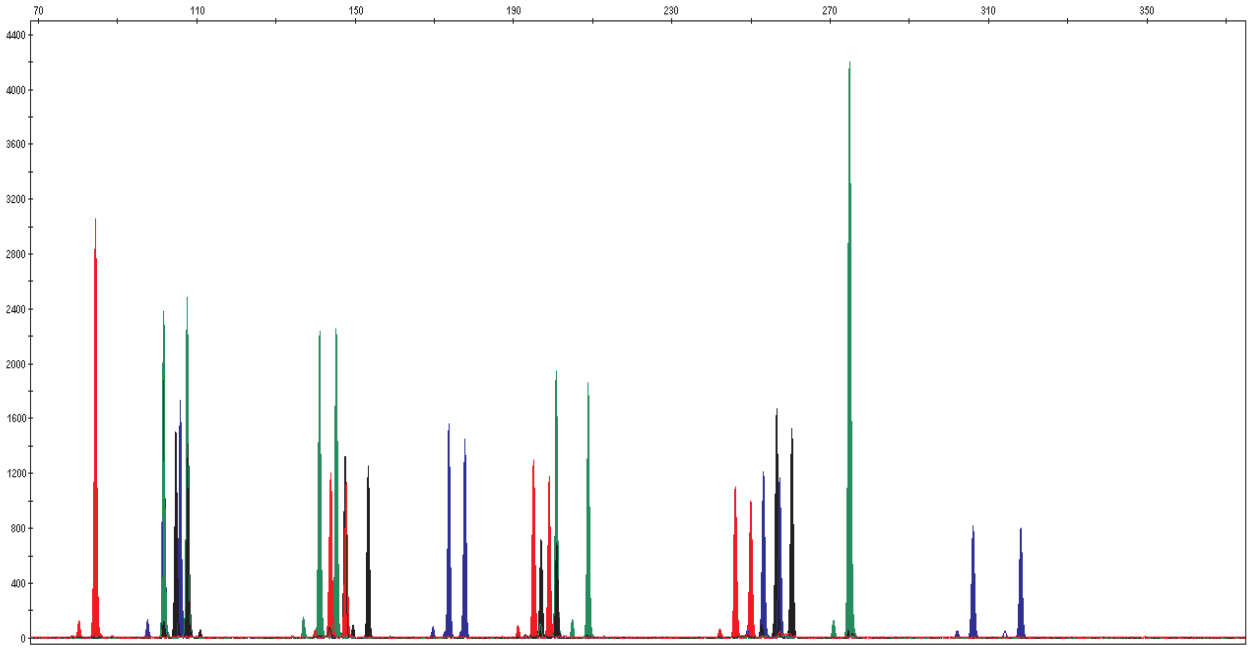


Figure 4 AmpFℓSTR® NGM™ PCR Amplification Kit results from a 1.2-mm FTA bloodstain disc (24-cycle amplification), analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer

Electrophoresis 3

This chapter covers:

Allelic ladder requirements

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

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

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

  - Applied Biosystems 3130*xl* or ABI PRISM® 3100 systems – One ladder per injection; one injection = 16 samples (15 samples + 1 allelic ladder)
  - Applied Biosystems 3130 or ABI PRISM® 3100-*Avant* – One ladder for every 4 injections; one injection = 4 samples

When genotyping, it is critical to use an allelic ladder run under the same conditions as the samples because:

- Size values obtained for the same sample can differ between instrument platforms because of different polymer matrices and electrophoretic conditions.
- Variation in laboratory temperature can affect migration speed (see IMPORTANT above). These variations can result in sizing variations between both single and multiple capillary runs, with a greater size variation between those samples injected in multiple capillary runs, than between those samples injected in a single capillary run.

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

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

Reagents and parts

Table 5 on page 10 lists the required materials not supplied with the AmpFℓSTR® NGM™ PCR Amplification Kit.

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

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

The following table lists Data Collection Software and the run modules that can be used to analyze AmpFℓSTR® NGM™ 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
3.0‡ (3130/3130*xl* Analyzer)	Windows XP	• HIDFragmentAnalysis36_POP4_1 Injection conditions: – 3130 = 3 kV/5 sec – 3130*xl* = 3 kV/10 sec • Dye Set G5	*Applied Biosystems 3130/3130xl Genetic Analyzers Using Data Collection Software v3.0, Protocols for Processing AmpFℓSTR PCR Amplification Kit PCR Products User Bulletin* (PN 4363787)
2.0 (3100 Analyzer)	Windows 2000	• HIDFragmentAnalysis36_POP4_1 Injection condition: 3kV/10 sec • Dye Set G5	*ABI PRISM® 3100/3100-Avant Genetic Analyzers Using Data Collection Software v2.0, Protocols for Processing AmpFℓSTR PCR Amplification Kit PCR Products User Bulletin* (PN 4350218)
1.1 (3100 Analyzer)	Windows NT®	• GeneScan36vb_DyeSetG5Module Injection condition: 3kV/10 sec • GS500Analysis.gsp	*ABI PRISM® 3100/3100-Avant Genetic Analyzers Protocols for Processing AmpFℓSTR PCR Amplification Kit PCR Products User Bulletin* (PN 4332345)
1.0 (3100-*Avant* Analyzer)	Windows NT®	• GeneScan36Avb_DyeSetG5Module Injection condition: 3 kV/5sec • GS500Analysis.gsp	*ABI PRISM® 3100/3100-Avant Genetic Analyzers Protocols for Processing AmpFℓSTR PCR Amplification Kit PCR Products User Bulletin* (PN 4332345)

‡ Applied Biosystems conducted validation studies for the AmpFℓSTR® NGM™ Kit using this configuration.

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 GeneScan™ 500 LIZ® Internal Size Standard needed to prepare the samples, using the table below.

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

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

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

2. Pipette the required volumes of components into an appropriately sized polypropylene tube.
3. Vortex the tube, then centrifuge briefly.
4. Into each well of a MicroAmp® Optical 96-Well Reaction Plate, add:
   - 9 µL of the formamide:size standard mixture
   - 1 µL of PCR product or allelic ladder

   Note: For blank wells, add 10 µL of Hi-Di™ Formamide.
5. Seal the reaction plate with appropriate septa, then 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 onto the autosampler.
9. Ensure that a plate record is completed and link the plate record to the plate.
10. Start the electrophoresis run.

Section 3.2 310 Instrument

Set up the 310 instrument for electrophoresis

Reagents and parts

Table 5 on page 10 lists the required materials not supplied with the AmpFℓSTR® NGM™ PCR Amplification Kit.

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

310 instrument requirements

The following table lists Data Collection Software and the run modules that can be used to analyze AmpFℓSTR® NGM™ 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) G5 v2.md5 Injection condition: 15 kV/5 sec	ABI PRISM® *310 Genetic Analyzer User's Manual (Windows)* (PN 4317588) ABI PRISM® *310 Protocols for Processing* AmpFℓSTR *PCR Amplification Kit Products with Microsoft Windows NT Operating System: User Bulletin* (PN 4341742)

‡ Applied Biosystems conducted concordance studies for the AmpFℓSTR® NGM™ Kit using this configuration.

Prepare samples for electrophoresis on the 310 instrument

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

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

Reagent	Volume per reaction (µL)
GeneScan 500 LIZ Size Standard	0.75
Hi-Di Formamide	24.25

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 10 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.

Data Analysis 4

This chapter covers:

Section 4.1 GeneMapper® *ID* Software

Before you start

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.

Note: Refer to **"Instrument and software overview" on page 7** for a list of compatible instruments.

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 that you 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, verify the sample result according to your laboratory's protocol.

If you are using GeneMapper *ID-X* Software to perform Human Identification (HID) analysis with AmpFℓSTR kits, go to "Set up GeneMapper® ID-X Software for data analysis" on page 54 or refer to the *GeneMapper® ID-X Software Version 1.0 Human Identification Analysis Getting Started Guide* (PN 4375574).

Set up GeneMapper® *ID* Software for data analysis

Workflow

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

- Import panels and bins into the Panel Manager, as explained in "Import panels and bins" on page 40.
- Import an analysis method, as explained in **"Import a HID analysis method" on page 43**.
- Import a size standard, as explained in **"Import a HID size standard" on page 48**.
- Define custom views of analysis tables.

  Refer to Chapter 1 of the *GeneMapper® ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial* (PN 4335523) for more information.
- Define custom views of plots.

  Refer to Chapter 1 of the *GeneMapper® ID Software Versions 3.1 and 3.2 Human Identification Analysis Tutorial* (PN 4335523) for more information.

Import panels and bins

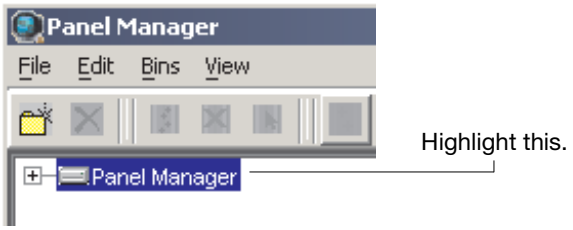
To import the AmpFℓSTR® NGM™ Kit panel and bin set from the Applied Biosystems web site into the GeneMapper® *ID* Software v3.2.1 database:

1. Download and open the file containing panels and bins:
   a. From the Support menu of **www.appliedbiosystems.com**, select **Support ▸ Software Downloads, Patches & Updates ▸** GeneMapper® *ID* Software **v 3.2 ▸ Updates & Patches**, and download the file **NGM Analysis Files GMID**.
   b. Unzip the file.
2. Start the GeneMapper® *ID* Software, then log in with the appropriate user name and password.

   IMPORTANT! For logon instructions, refer to page 2-7 of the *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide* (PN 4338775).
3. Select **Tools ▸ Panel Manager**.

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

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

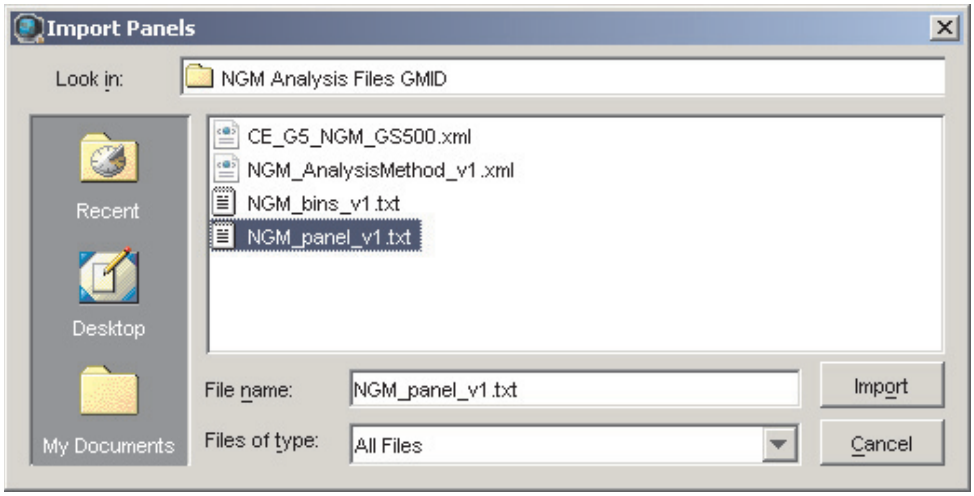


   b. Select **File ▸ Import Panels** to open the Import Panels dialog box.

   c. Navigate to, then open the **NGM Analysis Files GMIDX** folder that you unzipped in step 1 on page 40.

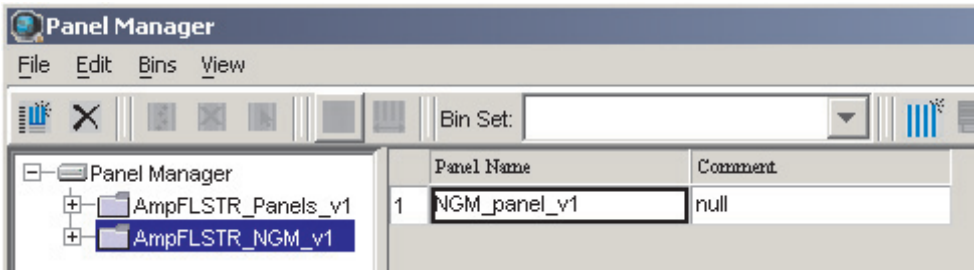
5. Select **NGM_panel_v1**, then click **Import**.

Note: Importing this file creates a new folder in the navigation pane of the Panel Manager, AmpFLSTR_NGM_v1. This folder contains the panel and associated markers.



6. Import **NGM_bins_v1**:

   a. Select the **AmpFLSTR_NGM_v1** folder in the navigation pane.

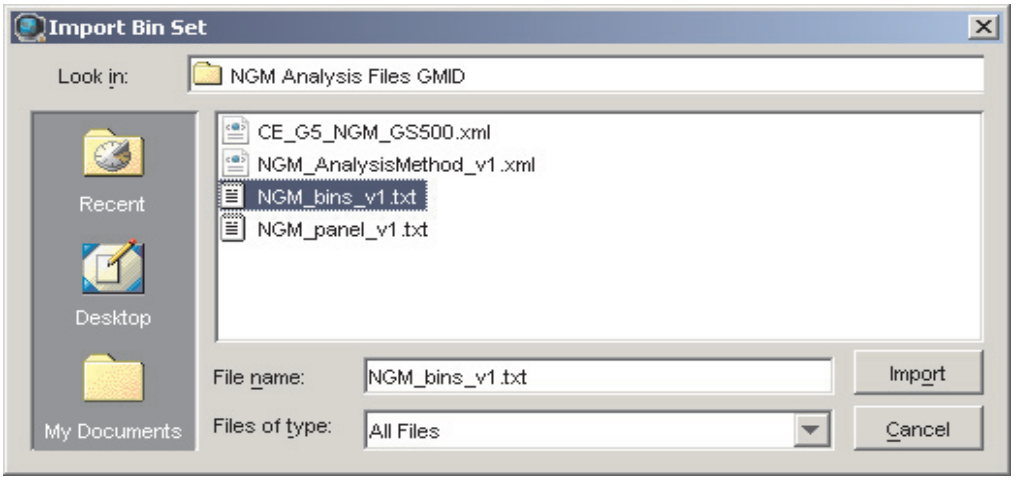


   b. Select **File ▸ Import Bin Set** to open the Import Bin Set dialog box.

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

d. Select **NGM_bins_v1**, then click **Import**.

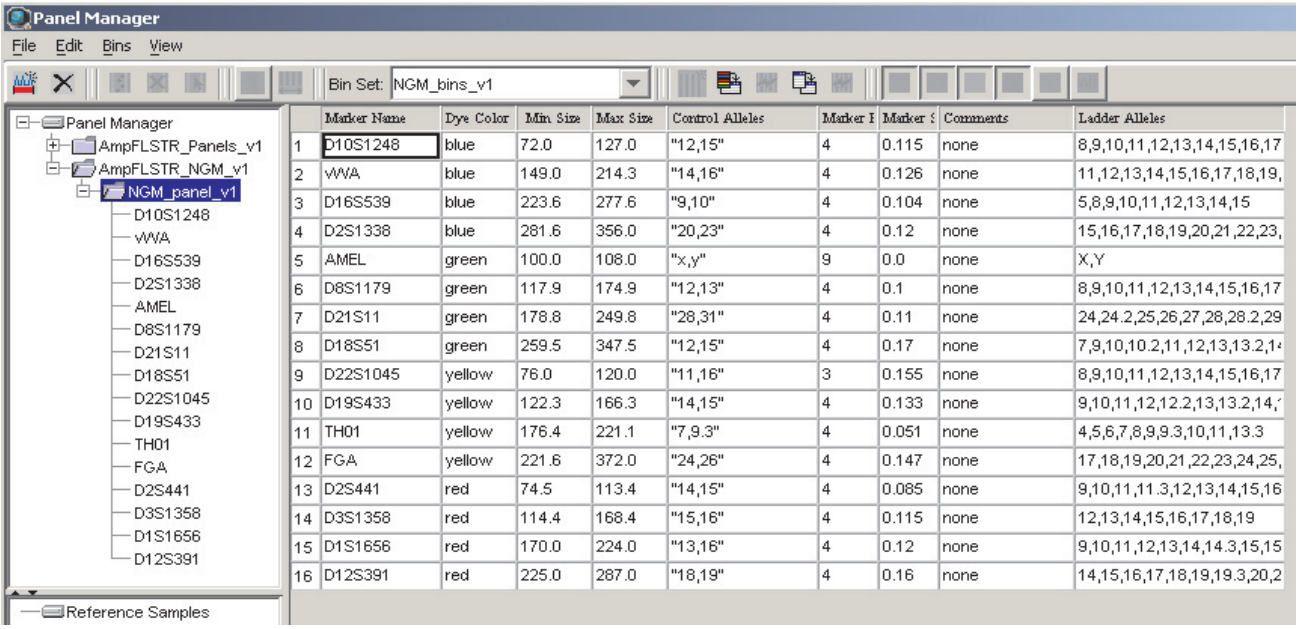
Note: Importing this file associates the bin set with the panels in the NGM_panel_v1 folder.



7. View the imported panels in the navigation pane:

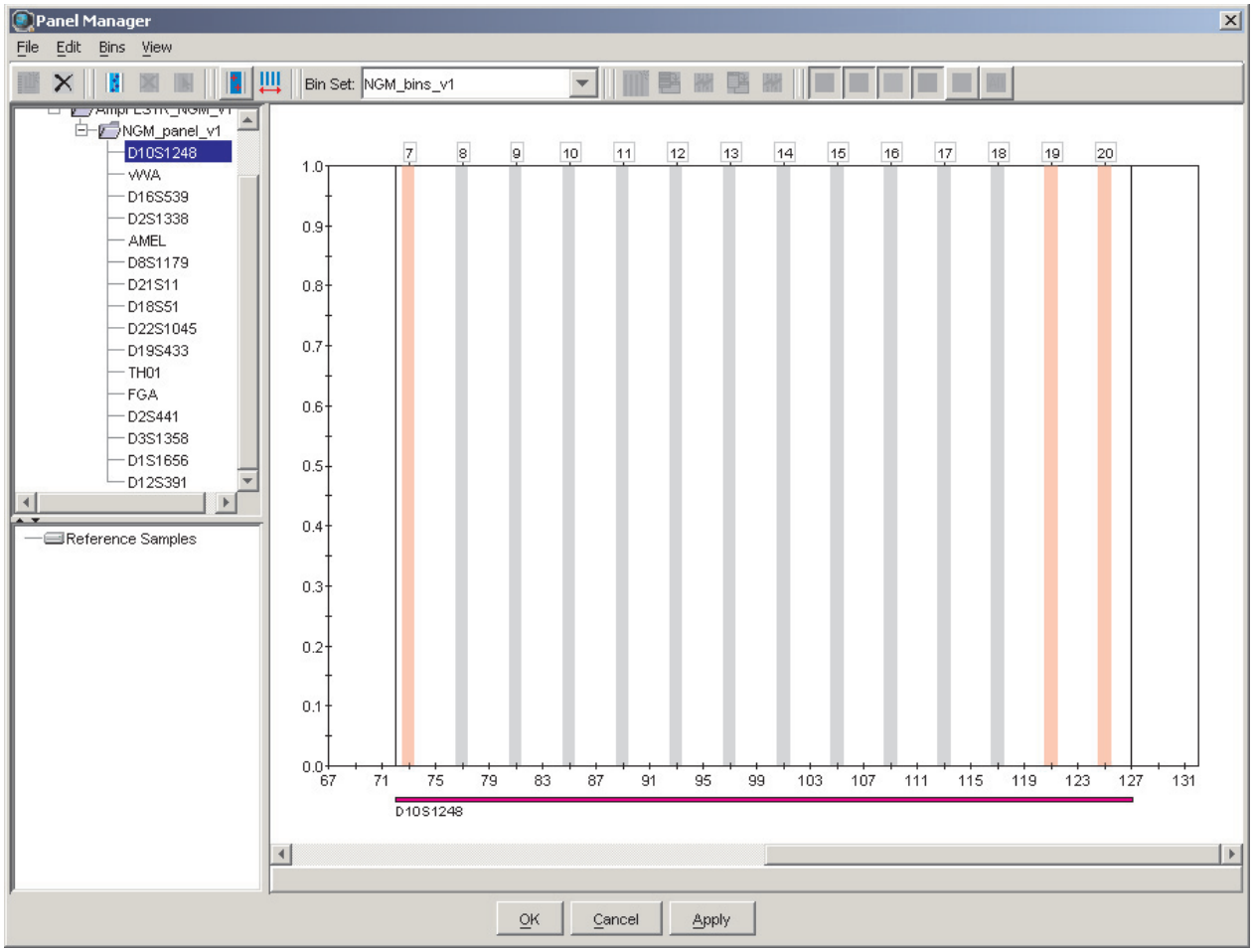
   a. Double-click the **AmpFLSTR_NGM_v1** folder to view the **NGM_panel_v1** folder.

   b. Double-click the **NGM_panel_v1** folder to display the panel information in the right pane.



	Marker Name	Dye Color	Min Size	Max Size	Control Alleles	Marker I	Marker S	Comments	Ladder Alleles
1	D10S1248	blue	72.0	127.0	"12,15"	4	0.115	none	8,9,10,11,12,13,14,15,16,17
2	vWA	blue	149.0	214.3	"14,16"	4	0.126	none	11,12,13,14,15,16,17,18,19,
3	D16S539	blue	223.6	277.6	"9,10"	4	0.104	none	5,8,9,10,11,12,13,14,15
4	D2S1338	blue	281.6	356.0	"20,23"	4	0.12	none	15,16,17,18,19,20,21,22,23,
5	AMEL	green	100.0	108.0	"x,y"	9	0.0	none	X,Y
6	D8S1179	green	117.9	174.9	"12,13"	4	0.1	none	8,9,10,11,12,13,14,15,16,17
7	D21S11	green	178.8	249.8	"28,31"	4	0.11	none	24,24.2,25,26,27,28,28.2,29
8	D18S51	green	259.5	347.5	"12,15"	4	0.17	none	7,9,10,10.2,11,12,13,13.2,1
9	D22S1045	yellow	76.0	120.0	"11,16"	3	0.155	none	8,9,10,11,12,13,14,15,16,17
10	D19S433	yellow	122.3	166.3	"14,15"	4	0.133	none	9,10,11,12,12.2,13,13.2,14,
11	TH01	yellow	176.4	221.1	"7,9.3"	4	0.051	none	4,5,6,7,8,9,9.3,10,11,13.3
12	FGA	yellow	221.6	372.0	"24,26"	4	0.147	none	17,18,19,20,21,22,23,24,25,
13	D2S441	red	74.5	113.4	"14,15"	4	0.085	none	9,10,11,11.3,12,13,14,15,16
14	D3S1358	red	114.4	168.4	"15,16"	4	0.115	none	12,13,14,15,16,17,18,19
15	D1S1656	red	170.0	224.0	"13,16"	4	0.12	none	9,10,11,12,13,14,14.3,15,15
16	D12S391	red	225.0	287.0	"18,19"	4	0.16	none	14,15,16,17,18,19,19.3,20,2

8. Select **D10S1248** to display the Bin view for the marker in the right pane.



9. Click **Apply**, then **OK** to add the AmpFℓSTR® NGM™ Kit panel and bin set to the GeneMapper® *ID* Software database.

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

Import a HID analysis method

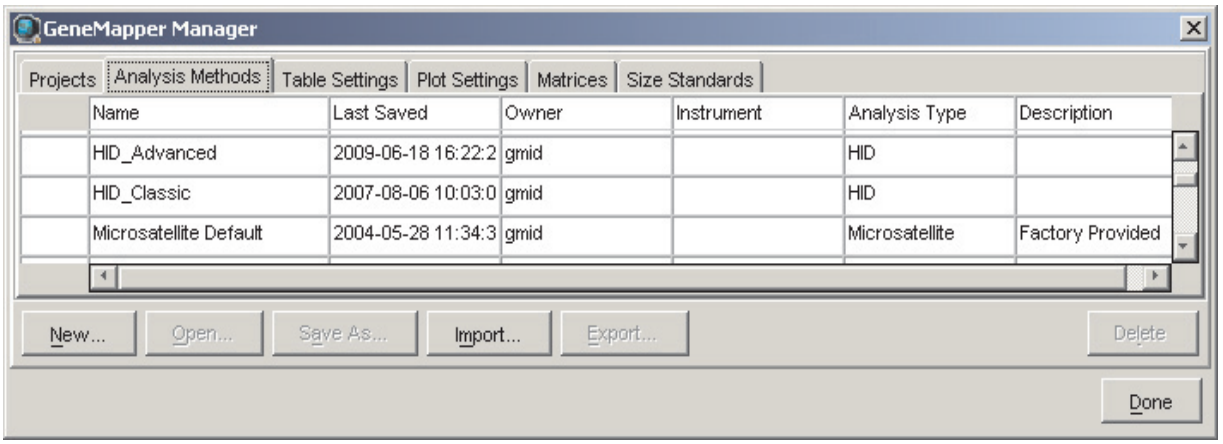
The HID Advanced analysis method for the AmpFℓSTR® NGM™ Kit uses the NGM_bins_v1 file described in step 6 on page 41.

Use the following procedure to import the analysis method from the folder that you downloaded from the Applied Biosystems web site into the GeneMapper® *ID* Software database. Refer to step 1 on page 40 for downloading instructions.

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

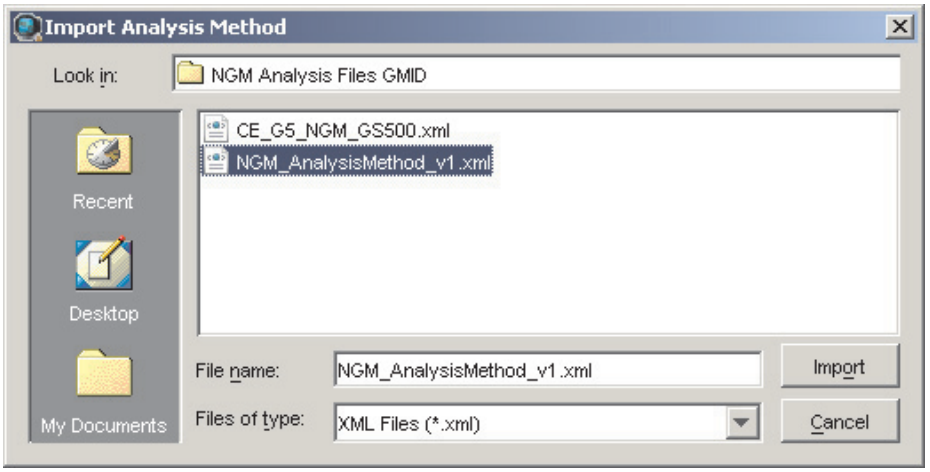
2. Import an analysis method for HID_Advanced:

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



   b. Navigate to, then open the **NGM Analysis Files GMID** folder.

3. Select **NGM_AnalysisMethod_v1**, then click **Import** to import the NGM_AnalysisMethod_v1 into the GeneMapper® *ID* Software database.



View HID Analysis Method Editor tab settings

To view the settings for NGM_AnalysisMethod_v1, select the **Analysis Methods** tab, then select **NGM_AnalysisMethod_v1** in the Name column and click **Open**.

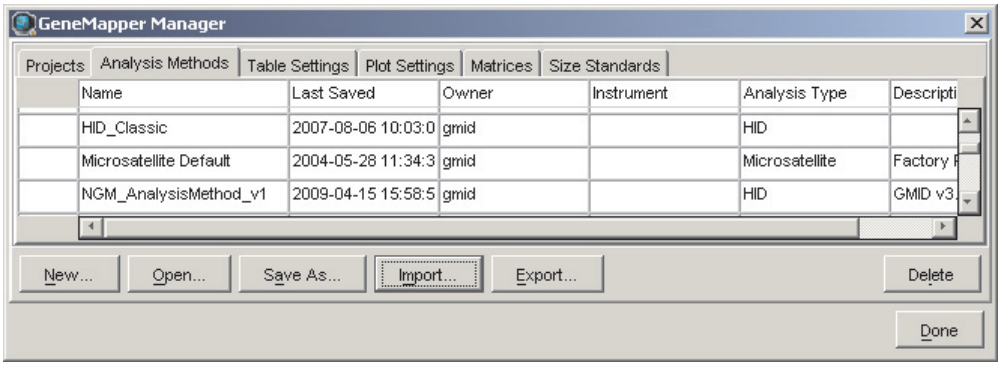


Figure 5 Analysis Method Editor - HID: General tab settings

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

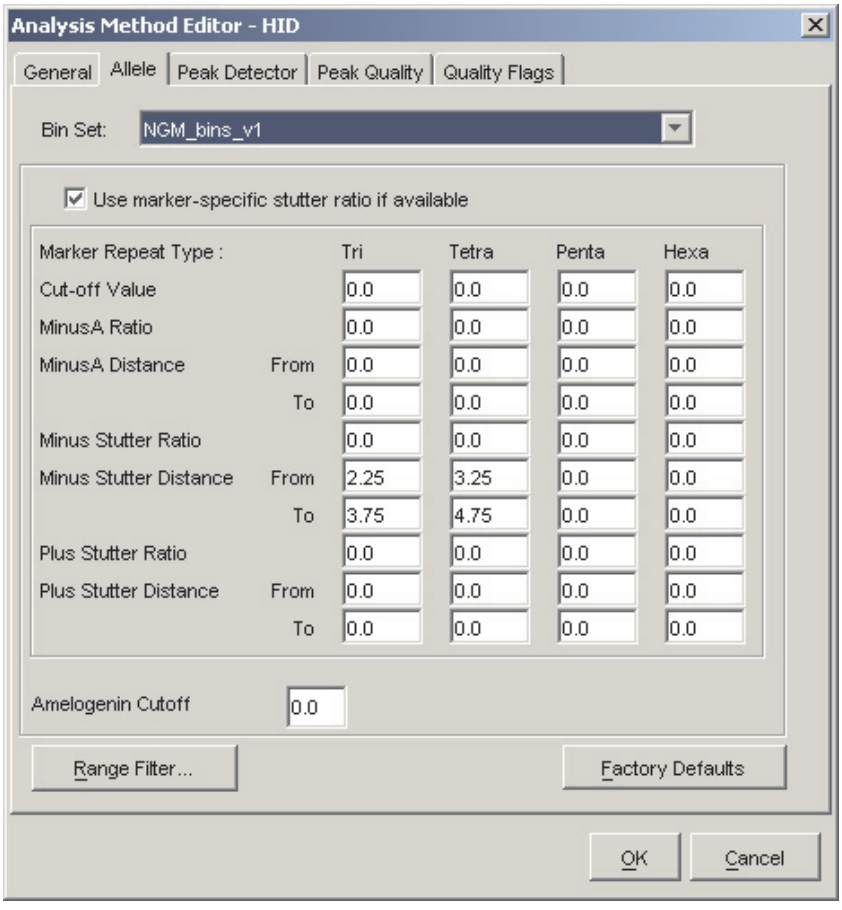


Figure 6 Analysis Method Editor - HID: Allele tab settings

- GeneMapper® *ID* Software v3.2.1 allows you to specify four types of marker repeat motifs: tri, tetra, penta, and hexa. You can enter parameter values for each type of repeat in the appropriate column.
- The "Use marker-specific stutter ratio if available" check box is selected by default. Consequently, the software applies the stutter ratio filters supplied in the NGM_panel_v1 file.

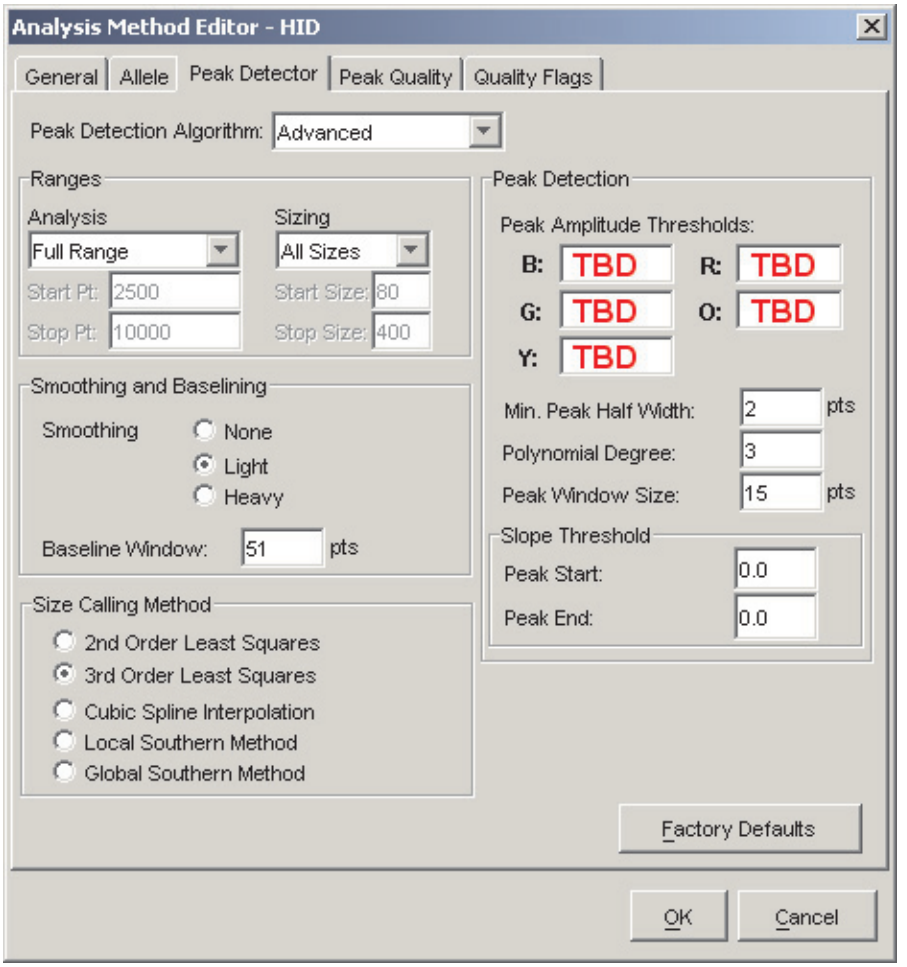


Figure 7 Analysis Method Editor - HID: Peak Detector tab settings

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

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

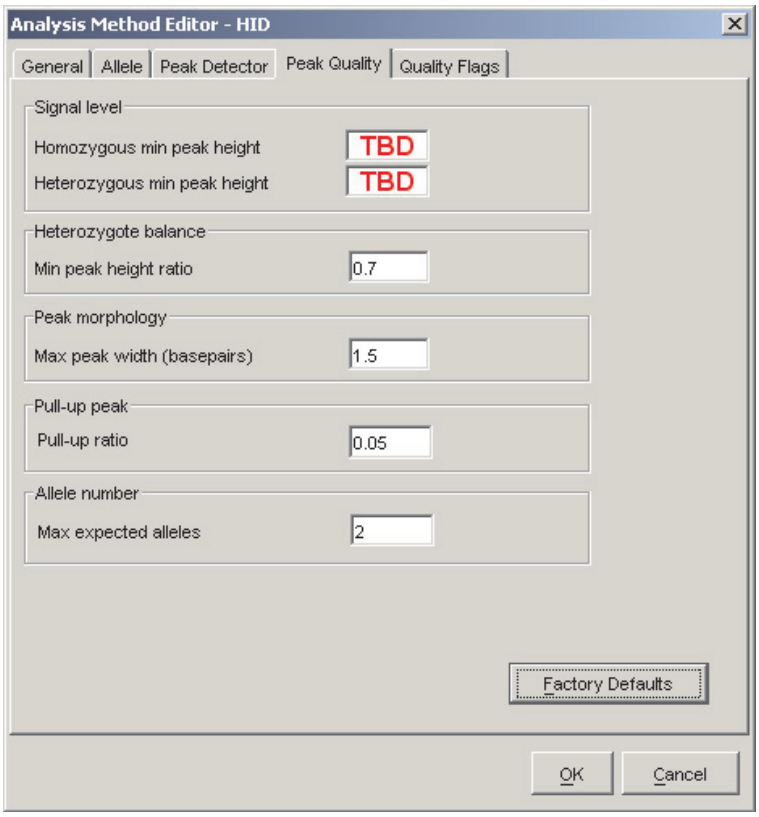


Figure 8 Analysis Method Editor - HID: Peak Quality tab settings

IMPORTANT! Laboratories need to perform the appropriate internal validation studies to determine the minimum heterozygous and homozygous minimum peak height thresholds and the minimum peak height ratio threshold that allow for reliable interpretation of AmpFℓSTR® NGM™ Kit data.

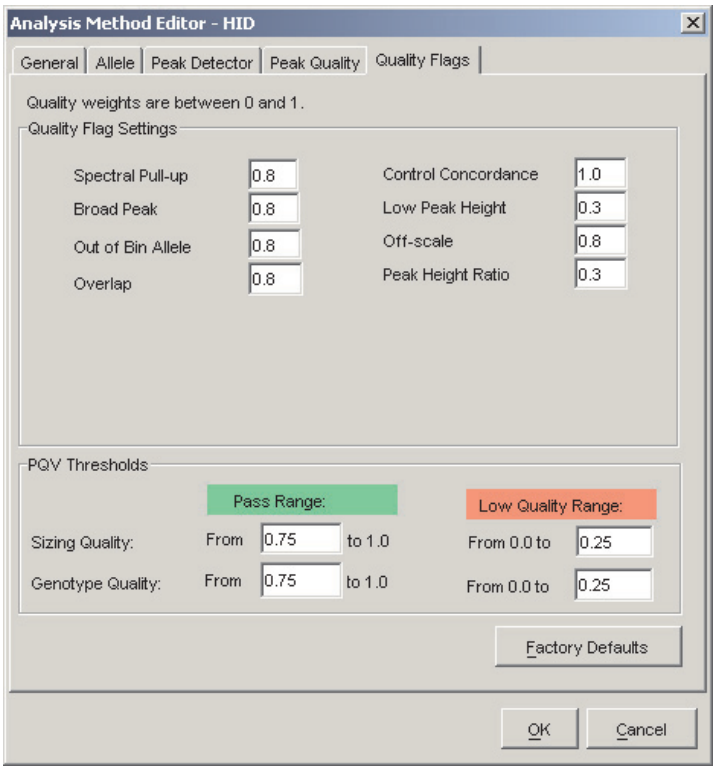


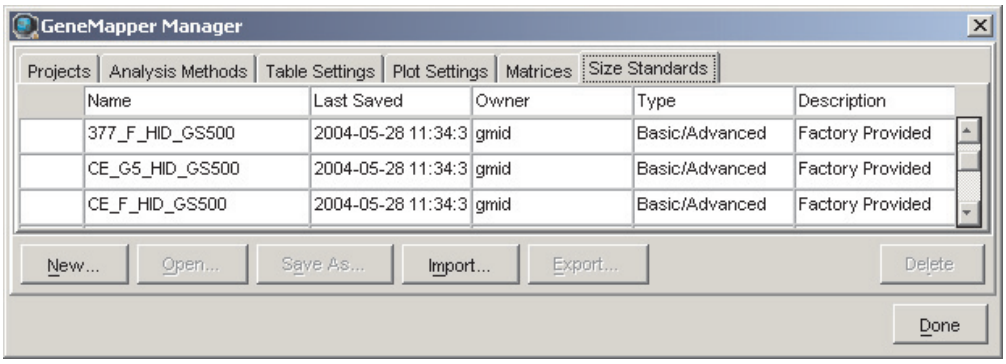
Figure 9 Analysis Method Editor - HID: Quality Flags tab settings

Import a HID size standard

The size standard for the AmpFℓSTR® NGM™ PCR Amplification Kit uses the following GS500 peaks in its sizing algorithm: 75, 100, 139, 150, 160, 200, 300, 350, 400, and 450.

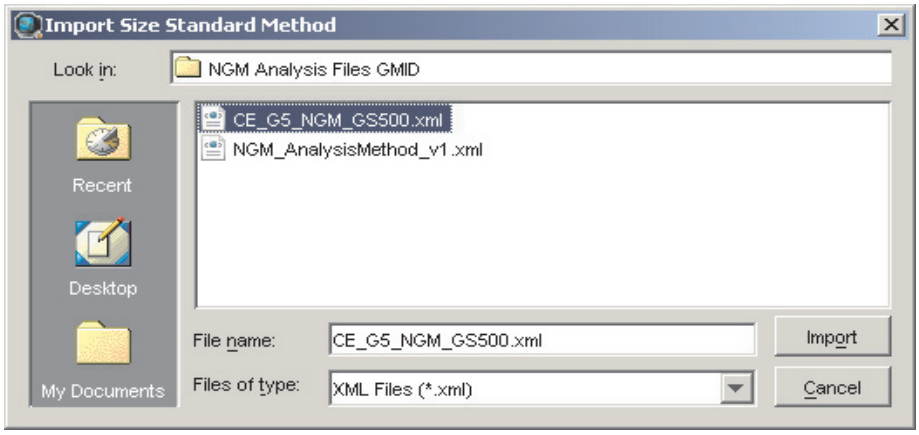
Use the following procedure to import the size standard for the AmpFℓSTR® NGM™ Kit from the folder that you downloaded from the Applied Biosystems web site, into the GeneMapper® *ID* Software database. Refer to step 1 on page 40 for downloading instructions.

1. Select **Tools ▸ GeneMapper Manager** to open the GeneMapper Manager.
2. Import a size standard:
    a. Select the **Size Standards** tab, then click **Import**.



b. Navigate to, then open the **NGM Analysis Files GMID** folder.

3. Select **CE_G5_NGM_GS500**, then click **Import** to import the CE_G5_NGM_GS500 size standard into the GeneMapper® *ID* Software database.



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

Analyze a project

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

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

Parameter	Settings
Sample Type	Select the sample type.
Analysis Method	NGM_AnalysisMethod_v1
Panel	NGM_panel_v1
Size Standard	CE_G5_NGM_GS500

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

- CE_G5_NGM_GS500 (size standard fragments defined in the AmpFℓSTR® NGM™ Kit): 75, 100, 139, 150, 160, 200, 300, 350, 400, and 450. For additional information about size standards, refer to the GeneMapper® *ID* Software *Version 3.1 Human Identification Analysis User Guide* (PN 4338775), Appendix D.
- CE_G5_NGM_GS500: Neither the 250-nt nor the 340-nt peak is included in the size standard definition. These peaks can be used as an indicator of precision within a run.

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

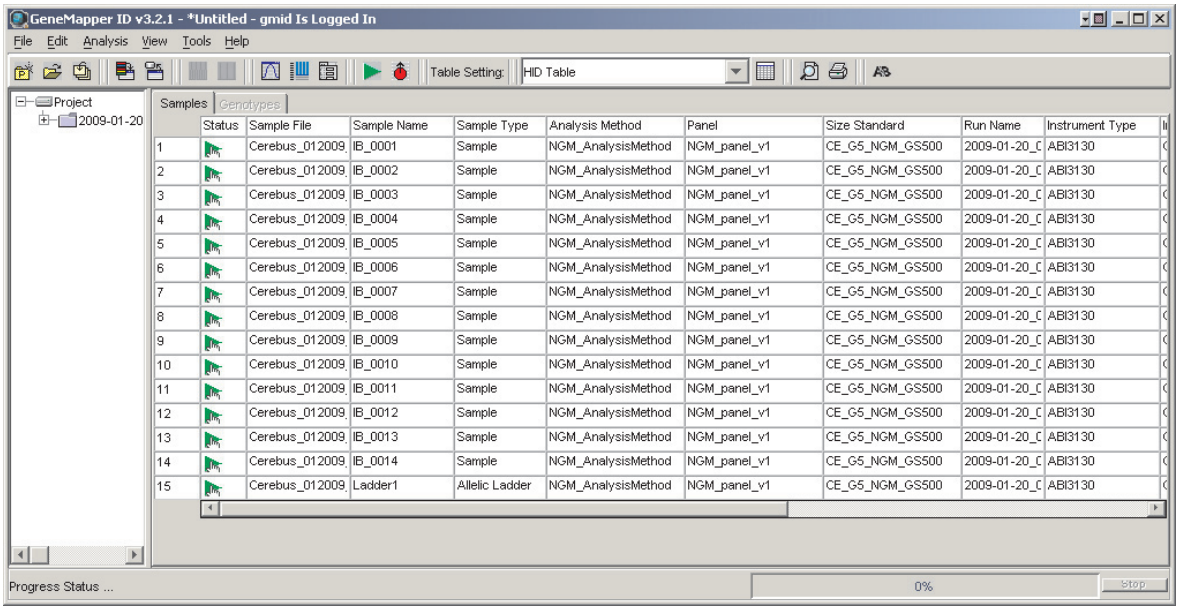


Figure 10 Project Window before analysis

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

Section 4.2 GeneMapper® *ID-X* Software

Before you start

GeneMapper® *ID-X* Software is an automated genotyping software for forensic casework, databasing, and paternity data analysis. After electrophoresis, the Data Collection Software stores information for each sample in a .fsa file. Using GeneMapper® *ID-X* Software v1.0.1 or higher, you can then analyze and interpret the data from the .fsa files.

Note: Refer to **"Instrument and software overview" on page 7** for a list of compatible instruments.

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 that you 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, verify the sample result according to your laboratory's protocol.

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

Workflow

To analyze sample (.fsa) files using GeneMapper® *ID-X* Software v1.0.1 or higher for the first time:

- Import panels, bins, and marker stutter into the Panel Manager, as explained in **"Import panels, bins, and marker stutter" on page 54**.
- Import an analysis method, as explained in **"Import an analysis method" on page 59**.
- Import a size standard, as explained in **"Import a HID size standard" on page 63**.
- Define custom views of analysis tables.

  Refer to Chapter 1 of the *GeneMapper® ID-X Software Version 1.0 Getting Started Guide* (PN 4375574) for more information.
- Define custom views of plots.

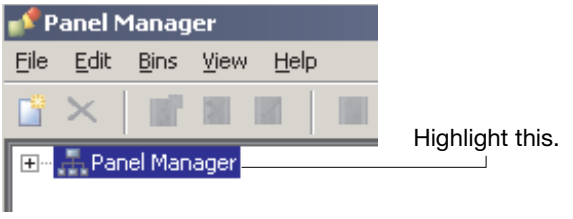
  Refer to Chapter 1 of the *GeneMapper® ID-X Software Version 1.0 Getting Started Guide* (PN 4375574) for more information.

Import panels, bins, and marker stutter

To import the AmpFℓSTR® NGM™ Kit panels, bin sets, and marker stutter from the Applied Biosystems web site into the GeneMapper® *ID-X* Software v1.0.1 or higher database:

1. Download and open the file containing panels, bins, and marker stutter:
   a. From the Support menu of **www.appliedbiosystems.com**, select **Support ▸ Software Downloads, Patches & Updates ▸** GeneMapper® *ID* Software **v 3.2 ▸ Updates & Patches**, and download the file **NGM 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 page 22 of the *GeneMapper® ID-X Software Version 1.0 Getting Started Guide* (PN 4375574).
3. Select **Tools ▸ Panel Manager**.
4. Find, then open the folder containing the panels, bins, and marker stutter:
   a. Select **Panel Manager** in the navigation pane.

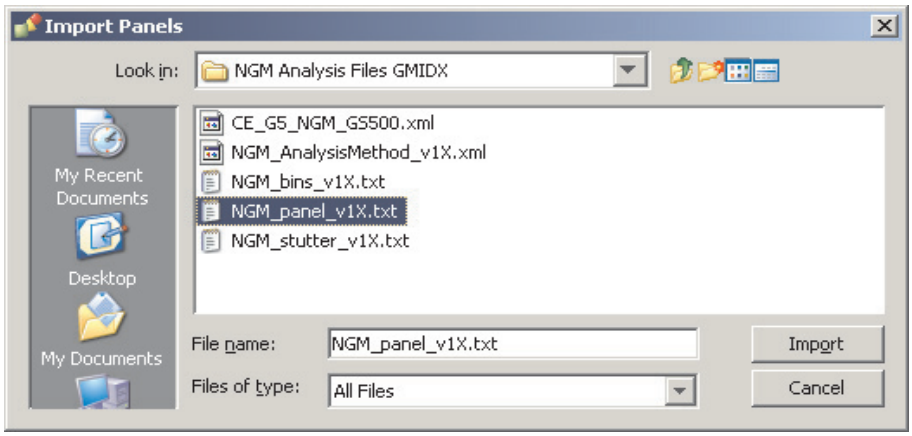


b. Select **File ▸ Import Panels** to open the Import Panels dialog box.

c. Navigate to, then open the **NGM Analysis Files GMIDX** folder that you unzipped in step 1 on page 54.

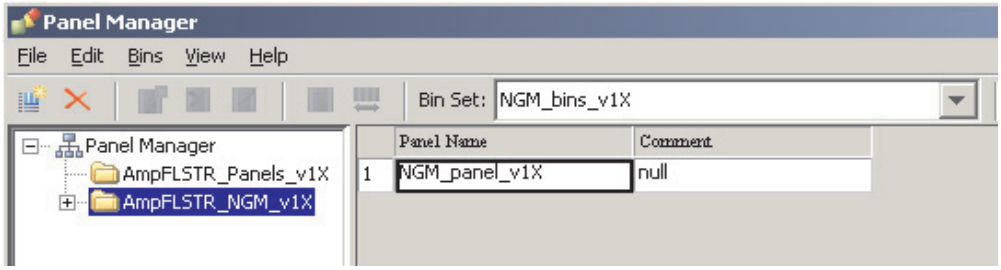
5. Select **NGM_panel_v1X**, then click **Import**.

Note: Importing this file creates a new folder in the navigation pane of the Panel Manager "AmpFLSTR_NGM_v1X". This folder contains the panel and associated markers.



6. Import NGM_bins_v1X:

a. Select the **AmpFLSTR_NGM_v1X** folder in the navigation pane.

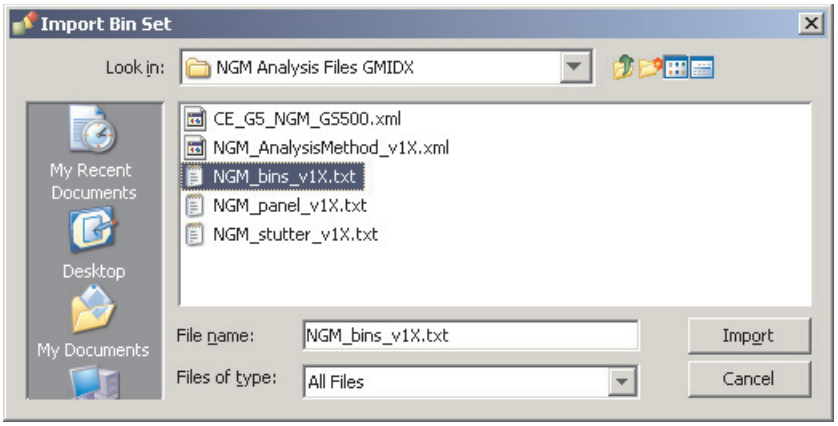


b. Select **File ▸ Import Bin Set** to open the Import Bin Set dialog box.

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

d. Select **NGM_bins_v1X**, then click **Import**.

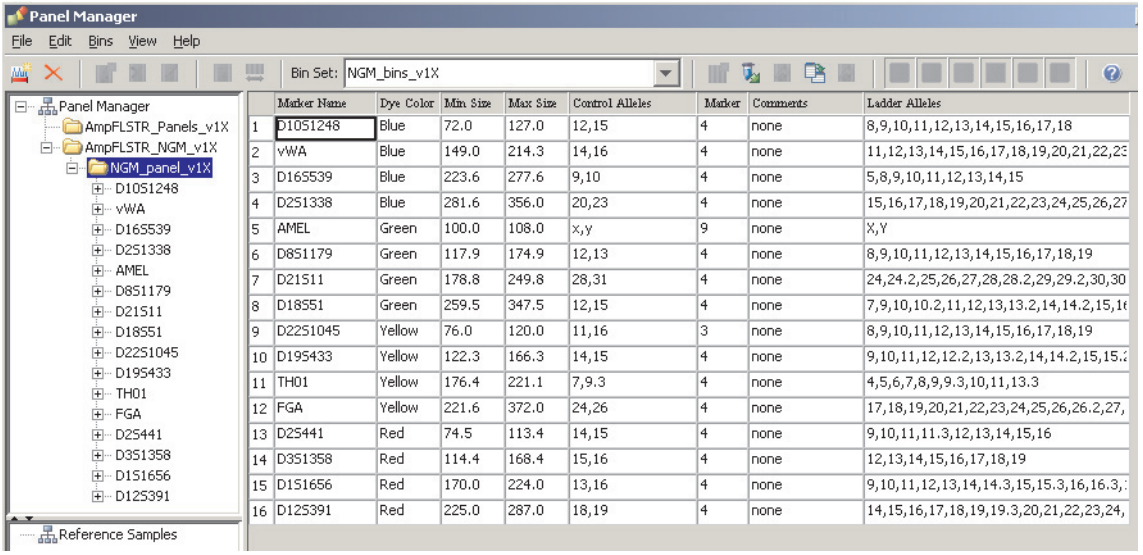
Note: Importing this file associates the bin set with the panels in the NGM_panel_v1X folder.



7. View the imported panels in the navigation pane:

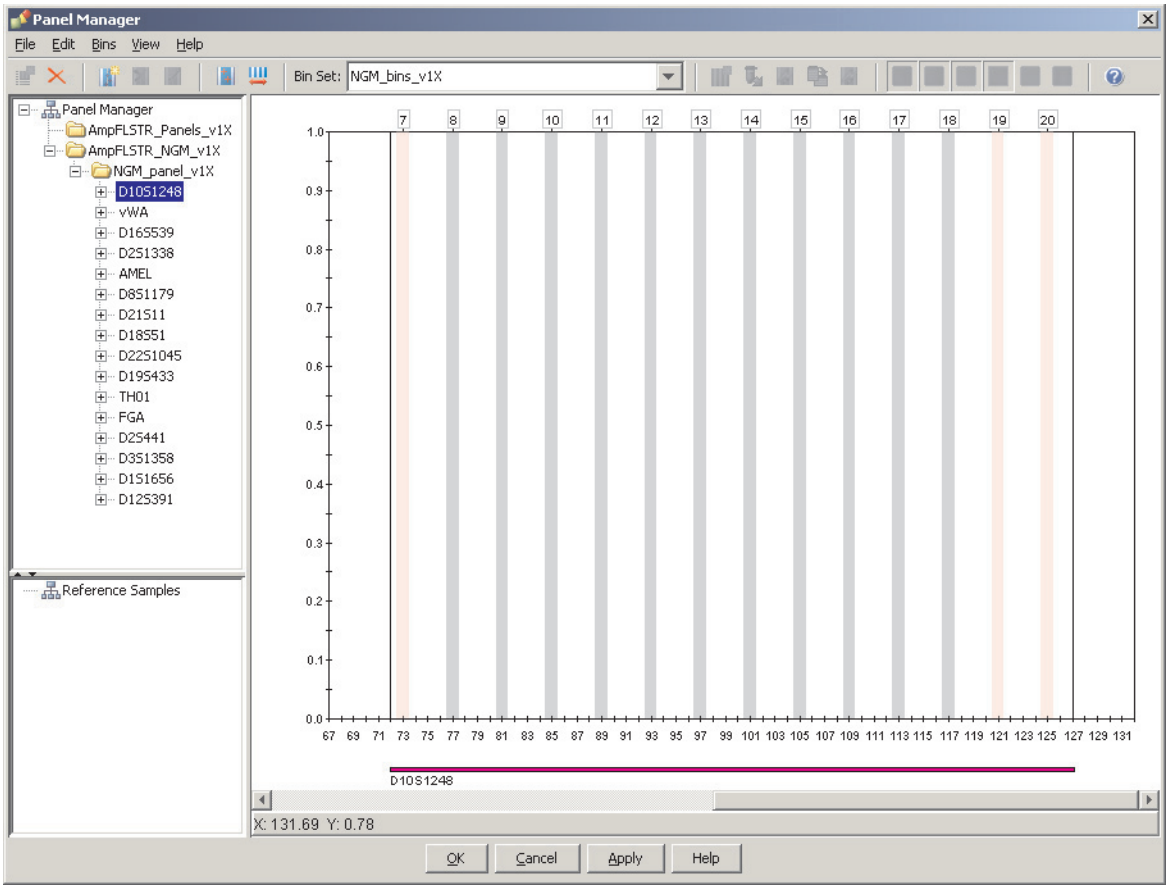
   a. Double-click the **AmpFLSTR_NGM_v1X** folder to view the NGM_panel_v1X folder.

   b. Double-click the **NGM_panel_v1X** folder to display the panel information in the right pane and the markers below it.



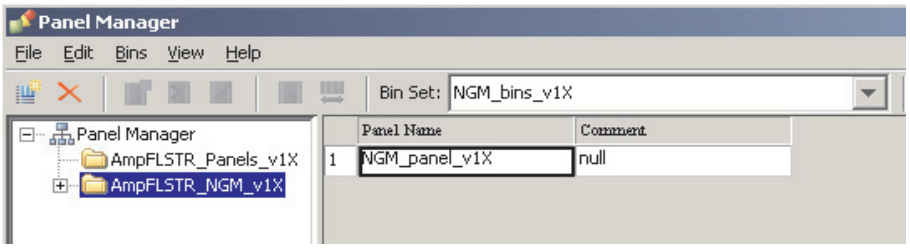
	Marker Name	Dye Color	Min Size	Max Size	Control Alleles	Marker	Comments	Ladder Alleles
1	D10S1248	Blue	72.0	127.0	12,15	4	none	8,9,10,11,12,13,14,15,16,17,18
2	vWA	Blue	149.0	214.3	14,16	4	none	11,12,13,14,15,16,17,18,19,20,21,22,23
3	D16S539	Blue	223.6	277.6	9,10	4	none	5,8,9,10,11,12,13,14,15
4	D2S1338	Blue	281.6	356.0	20,23	4	none	15,16,17,18,19,20,21,22,23,24,25,26,27
5	AMEL	Green	100.0	108.0	x,y	9	none	X,Y
6	D8S1179	Green	117.9	174.9	12,13	4	none	8,9,10,11,12,13,14,15,16,17,18,19
7	D21S11	Green	178.8	249.8	28,31	4	none	24,24.2,25,26,27,28,28.2,29,29.2,30,30
8	D18S51	Green	259.5	347.5	12,15	4	none	7,9,10,10.2,11,12,13,13.2,14,14.2,15,1
9	D22S1045	Yellow	76.0	120.0	11,16	3	none	8,9,10,11,12,13,14,15,16,17,18,19
10	D19S433	Yellow	122.3	166.3	14,15	4	none	9,10,11,12,12.2,13,13.2,14,14.2,15,15.
11	TH01	Yellow	176.4	221.1	7,9.3	4	none	4,5,6,7,8,9,9.3,10,11,13.3
12	FGA	Yellow	221.6	372.0	24,26	4	none	17,18,19,20,21,22,23,24,25,26,26.2,27,
13	D2S441	Red	74.5	113.4	14,15	4	none	9,10,11,11.3,12,13,14,15,16
14	D3S1358	Red	114.4	168.4	15,16	4	none	12,13,14,15,16,17,18,19
15	D1S1656	Red	170.0	224.0	13,16	4	none	9,10,11,12,13,14,14.3,15,15.3,16,16.3,
16	D12S391	Red	225.0	287.0	18,19	4	none	14,15,16,17,18,19,19.3,20,21,22,23,24,

8. Select **D10S1248** to display the Bin view for the marker in the right pane.



9. Import NGM_stutter_v1X:

   a. Select the **AmpFLSTR_NGM_v1X** folder in the navigation panel.

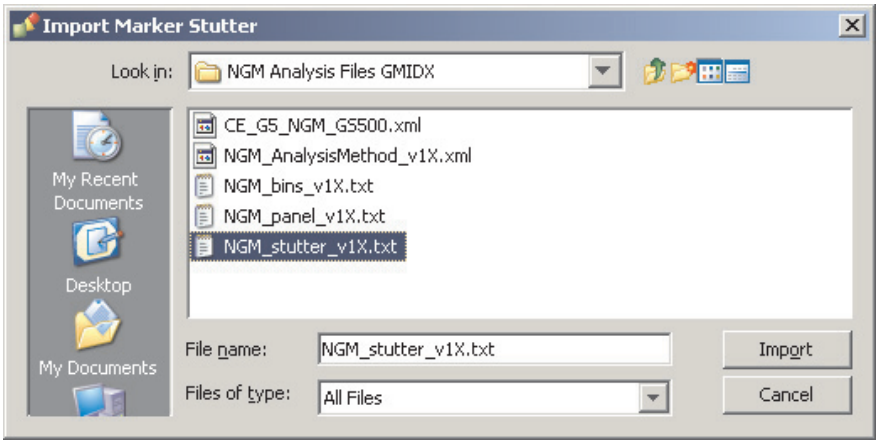


   b. Select File ▸ **Import Marker Stutter** to open the Import Marker Stutter dialog box.

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

d. Select **NGM_stutter_v1X**, then click **Import**.

Note: Importing this file associates the marker stutter ratio with the bin set in the NGM_bins_v1X folder.



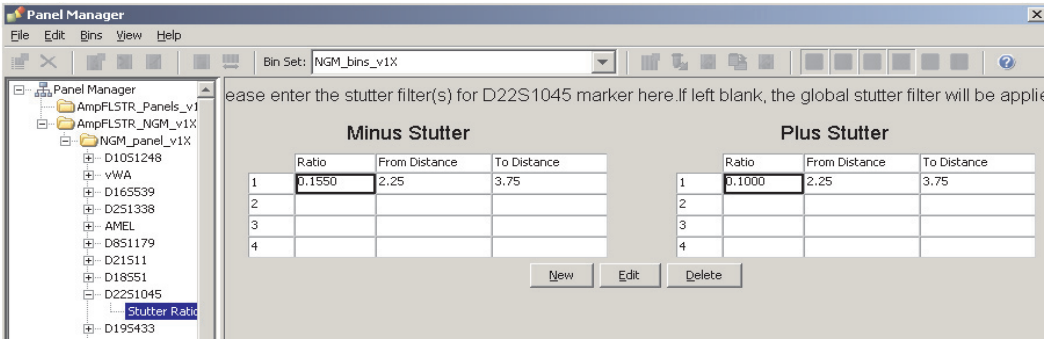
10. View the imported marker stutters in the navigation pane:

    a. Select the **NGM_panel_v1X** folder to display its list of markers in the right pane.

    b. Double-click the **NGM_panel_v1X** folder to display its list of markers below it.

    c. Double-click **D22S1045** to display the Stutter Ratio & Distance view for the marker in the right pane.

    Because D22S1045 has a trinucleotide repeat unit, it produces a higher level of plus stutter than tetranucleotide markers, and so requires the use of a plus stutter filter. The settings for the D22S1045 plus stutter filter can be seen in the table in the right pane. Other markers may not require a plus stutter filter, in which case the settings for plus stutter are left blank.



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

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

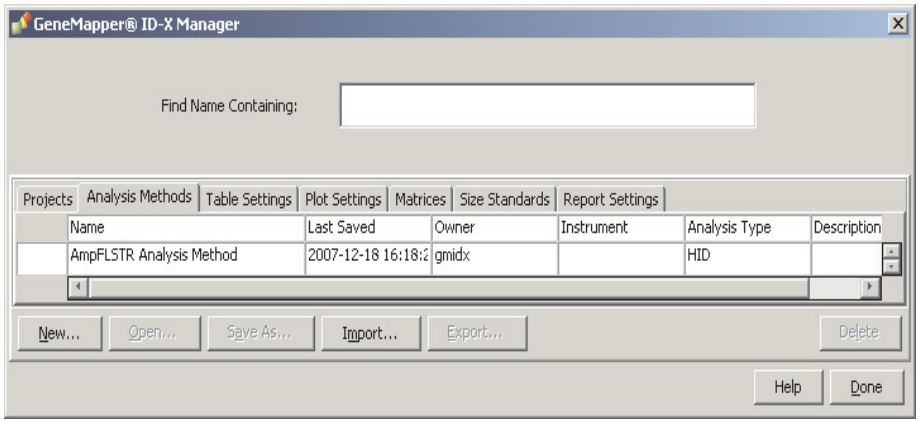
Import an analysis method

Use the following procedure to import the analysis method for the AmpFℓSTR® NGM™ Kit from the folder that you downloaded from the Applied Biosystems web site into the GeneMapper® *ID-X* Software database. Refer to step 1 on page 54 for downloading instructions.

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

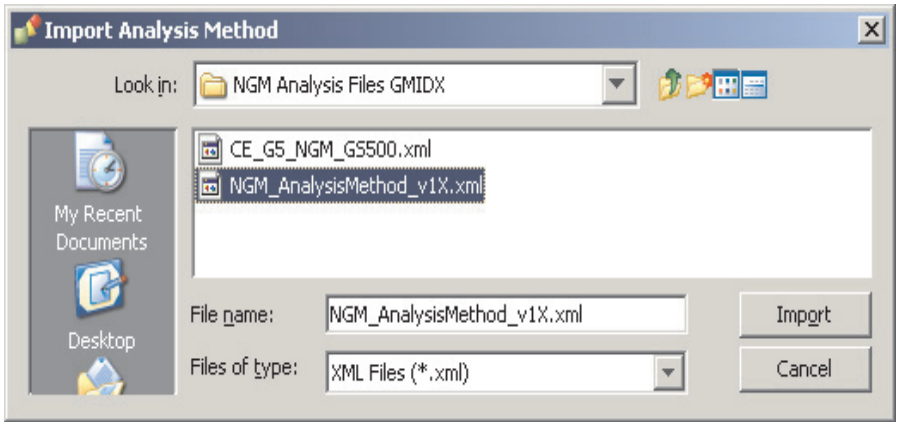
2. Import an analysis method:

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



   b. Navigate to, then open the **NGM Analysis Files GMIDX** folder.

3. Select **NGM_AnalysisMethod_v1X**, then click **Import** to import NGM_AnalysisMethod_v1X into the GeneMapper® *ID-X* Software database.



View Analysis Method Editor tab settings

To view the settings for NGM_AnalysisMethod_v1X, select the **Analysis Methods** tab, then select **NGM_AnalysisMethod_v1X** in the Name column and click **Open**.

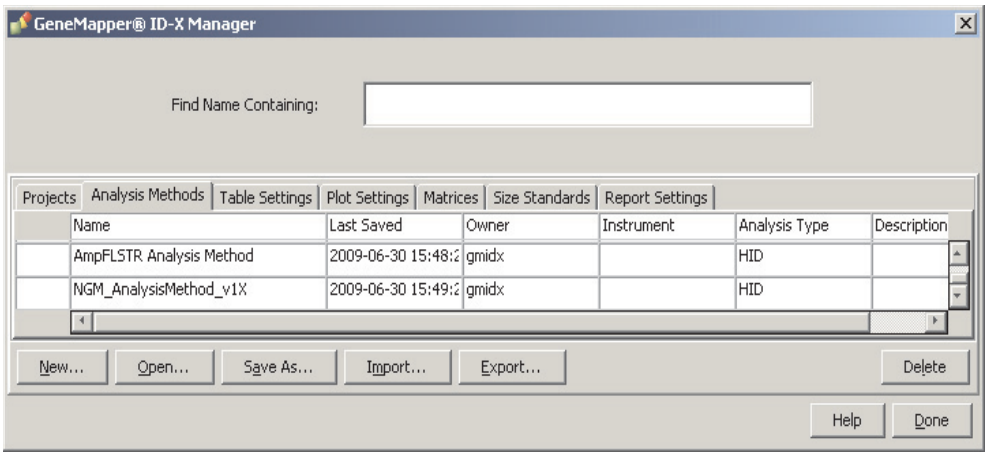


Figure 11 Analysis Method Editor: General tab settings

Figures 12 through 15 show the settings for each tab of the Analysis Method Editor.

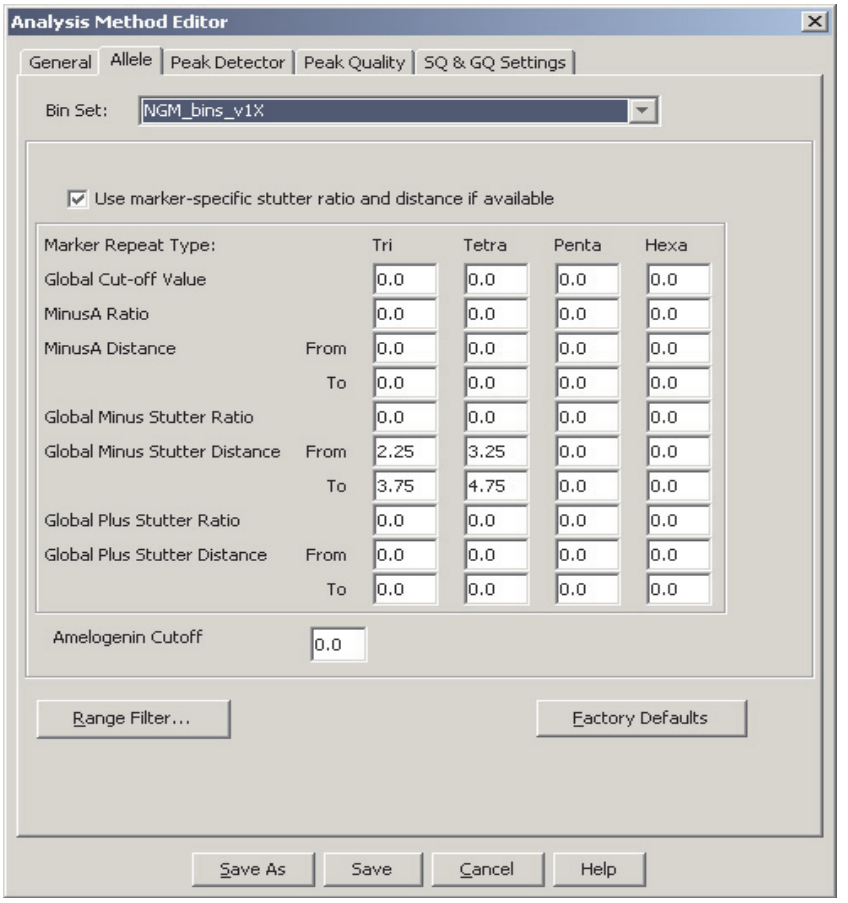


Figure 12 Analysis Method Editor: Allele tab settings

- GeneMapper® *ID-X* Software *v*1.0.1 or higher allows you to specify four types of marker repeat motifs: tri, tetra, penta, and hexa. You can enter parameter values for each type of repeat in the appropriate column.
- The "Use marker-specific stutter ratio if available" check box is selected by default. Consequently, the software applies the stutter ratio filters supplied in the NGM_stutter_v1X file.

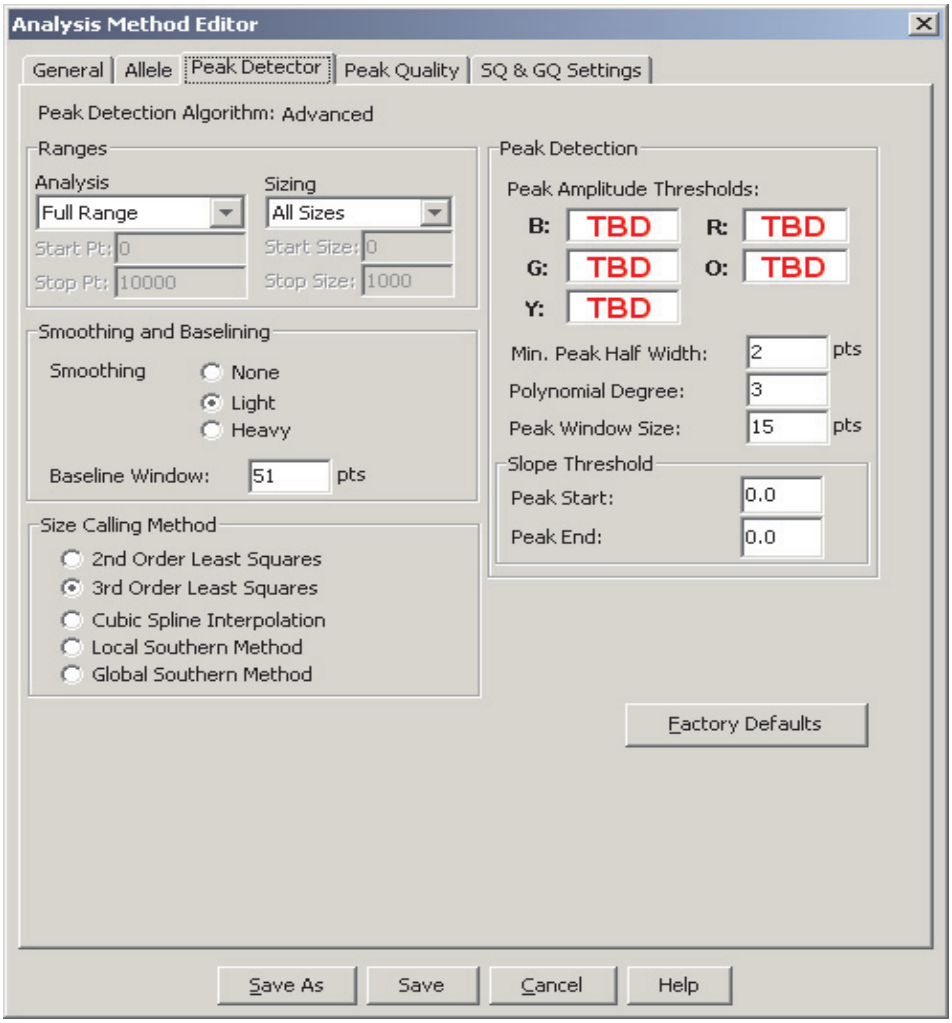


Figure 13 Analysis Method Editor: Peak Detector tab settings

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

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

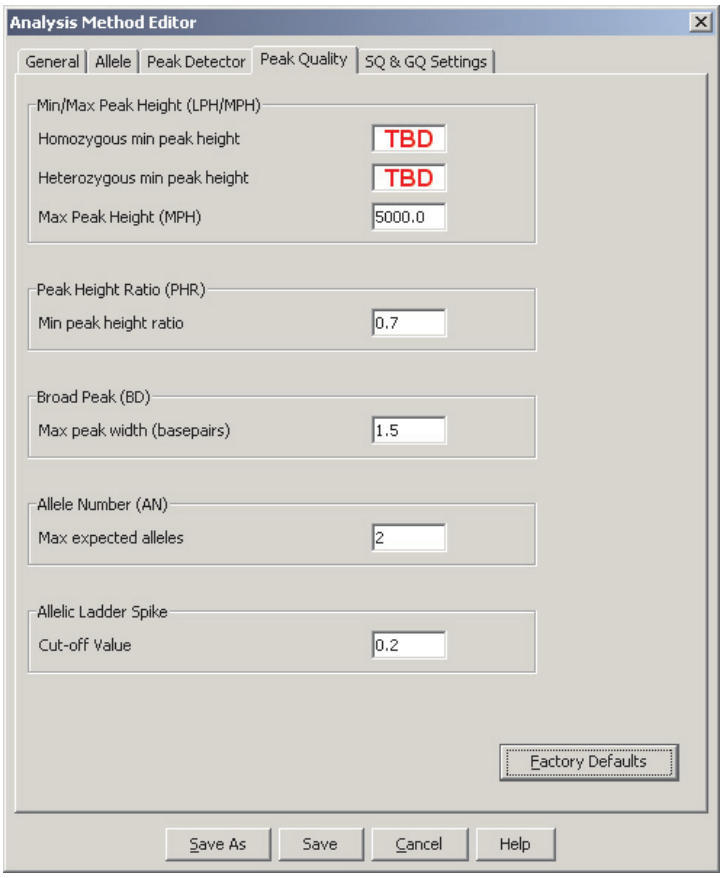


Figure 14 Analysis Method Editor: Peak Quality tab settings

IMPORTANT! Laboratories need to perform the appropriate internal validation studies to determine the minimum heterozygous and homozygous minimum peak height thresholds and the minimum peak height ratio threshold that allow for reliable interpretation of AmpFℓSTR® NGM™ Kit data.

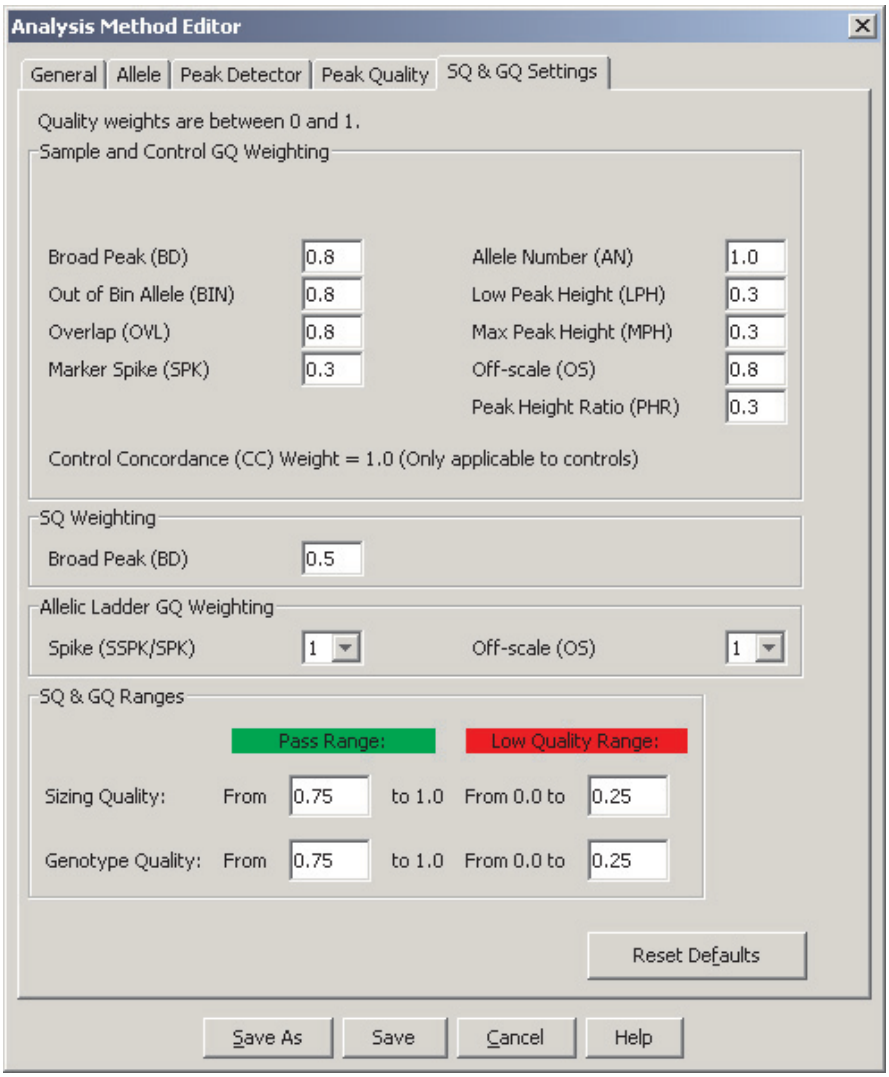


Figure 15 Analysis Method Editor: SQ and GQ tab settings

Import a HID size standard

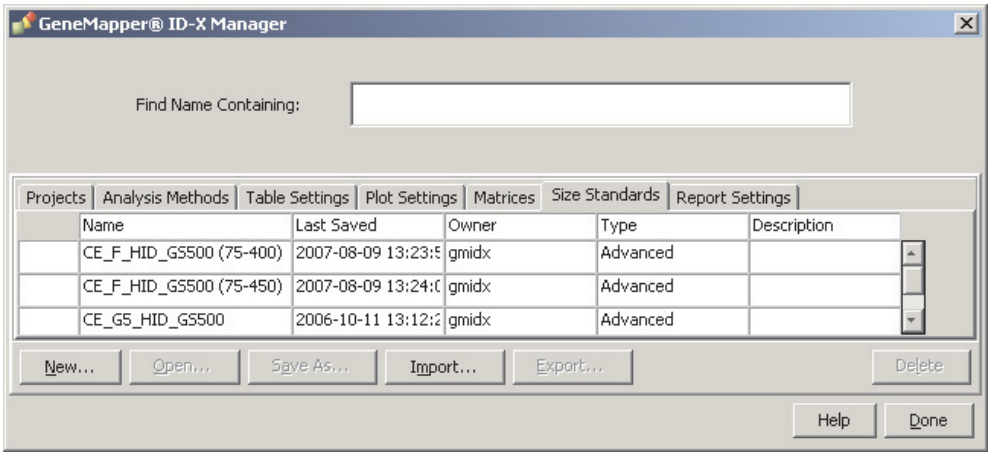
The size standard for the AmpFℓSTR® NGM™ PCR Amplification Kit uses the following GS500 peaks in its sizing algorithm: 75, 100, 139, 150, 160, 200, 300, 350, 400, and 450.

Use the following procedure to import the size standard for the AmpFℓSTR® NGM™ Kit from the folder that you downloaded from the Applied Biosystems web site, into the GeneMapper® *ID-X* Software database. Refer to step 1 on page 54 for downloading instructions.

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

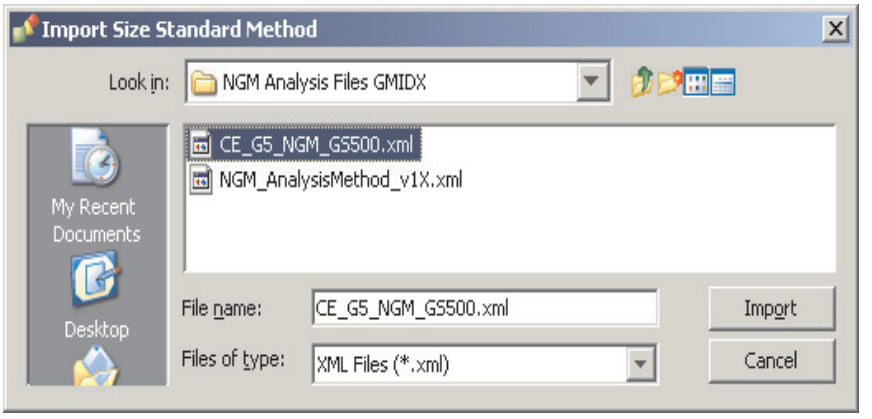
2. Import a size standard:

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



   b. Navigate to, then open the **NGM Analysis Files GMIDX** folder.

3. Select **CE_G5_NGM_GS500**, then click **Import** to import the CE_G5_NGM_GS500 analysis method into the GeneMapper® *ID-X* Software database.



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

Analyze a project

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

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

Parameter	Settings
Sample Type	Select the sample type.
Analysis Method	NGM_AnalysisMethod_v1X
Panel	NGM_panel_v1X
Size Standard	CE_G5_NGM_GS500

   - Size Standard: For more information about how the Size Caller works, refer to the ABI PRISM® GeneScan® Analysis Software for the *Windows NT® Operating System Overview of the Analysis Parameters and Size Caller User Bulletin* (PN 4335617).
   - CE_G5_NGM_GS500 (size standard fragments defined in the AmpFℓSTR® NGM™ Kit): 75, 100, 139, 150, 160, 200, 300, 350, 400, and 450. For additional information about size standards, refer to the *GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide* (PN 4338775), Appendix D.
   - CE_G5_NGM_GS500: Neither the 250-nt nor the 340-nt peak is included in the size standard definition. These peaks can be used as an indicator of precision within a run.

3. Click (Analyze), enter a name for the project (in the Save Project dialog box), then click **OK** to start analysis.
   - The status bar displays the progress of analysis as a completion bar extending to the right with the percentage completed indicated.
   - The table displays the row of the sample currently being analyzed in green (or red if analysis failed for the sample).
   - The Analysis Summary tab is displayed upon completion of the analysis.

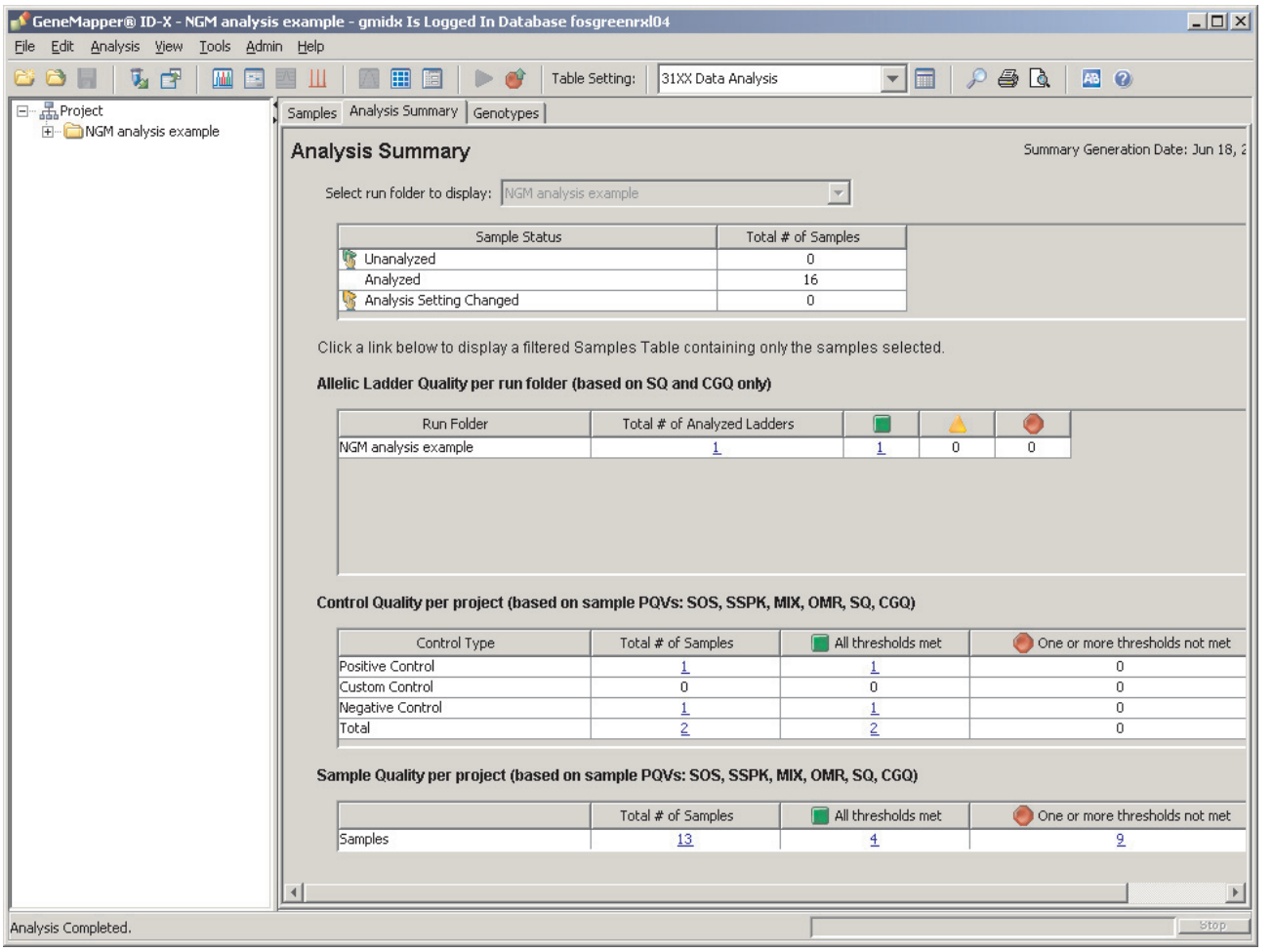


Figure 16 Analysis summary window after analysis

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 quick set-up instructions, refer to the *GeneMapper® ID-X Software Version 1.0 Getting Started Guide* (PN 4375574).
- For details about *GeneMapper® ID-X Software* features, allele filters, peak detection algorithms, and project editing, refer to:
  - *GeneMapper® ID-X Software Version 1.0 Getting Started Guide* (PN 4375574)
  - *GeneMapper® ID-X Software Version 1.0 Quick Reference Guide* (PN 4375670)
  - *GeneMapper® ID-X Software Version 1.0 Reference Guide* (PN 4375671)
  - *GeneMapper® ID-X Software Version 1.1 (Mixture Analysis Tool) Getting Started Guide* (PN 4396773)
  - *GeneMapper® ID-X Software Version 1.1 (Mixture Analysis Tool) Quick Reference Guide* (PN 4402094)

Experiments and Results 5

This chapter covers:

Overview

Experiments using the AmpFℓSTR® NGM™ Kit

This chapter provides results of the developmental validation experiments performed by Applied Biosystems using the AmpFℓSTR® NGM™ PCR Amplification Kit.

Importance of validation

Validation of a DNA typing procedure for human identification applications is an evaluation of the procedure's efficiency, reliability, and performance characteristics. By challenging the procedure with samples commonly encountered in forensic and parentage laboratories, the validation process uncovers attributes and limitations that 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

Experiments to evaluate the performance of the AmpFℓSTR® NGM™ PCR Amplification Kit were performed at Applied Biosystems. The experiments were performed according to the revised guidelines from the Scientific Working Group on DNA Analysis Methods (SWGDAM, July 10, 2003). Based on these guidelines, Applied Biosystems conducted experiments that comply with guidelines 1.0 and 2.0 and its associated subsections. This DNA methodology is not novel. (Moretti *et al.,* 2001; Frank *et al.,* 2001; Wallin *et al.,* 2002; and Holt *et al.,* 2000).

This chapter discusses many of the experiments performed by Applied Biosystems and provides examples of results obtained. Applied Biosystems chose conditions that produced optimum PCR product yield and that met reproducible performance standards. It is the opinion of Applied Biosystems that while these experiments are not exhaustive, they are appropriate for a manufacturer of STR kits intended for forensic and/or parentage testing use.

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

Developmental validation

SWGDAM guideline 1.2.1

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

SWGDAM guideline 2.10.1

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

PCR components

Applied Biosystems examined the concentration of each component of the AmpFℓSTR® NGM™ Kit and established that the concentration of each component was within the range where data indicated that the amplification met the required performance criteria for specificity, sensitivity, and reproducibility. For example, 1ng of Control DNA 007 was amplified in the presence of varying concentrations of magnesium chloride, and the results were analyzed on an Applied Biosystems 3130*xl* Genetic Analyzer. Results are shown in Figure 17. The performance of the multiplex is most robust within ±20% of the optimal magnesium chloride concentration.

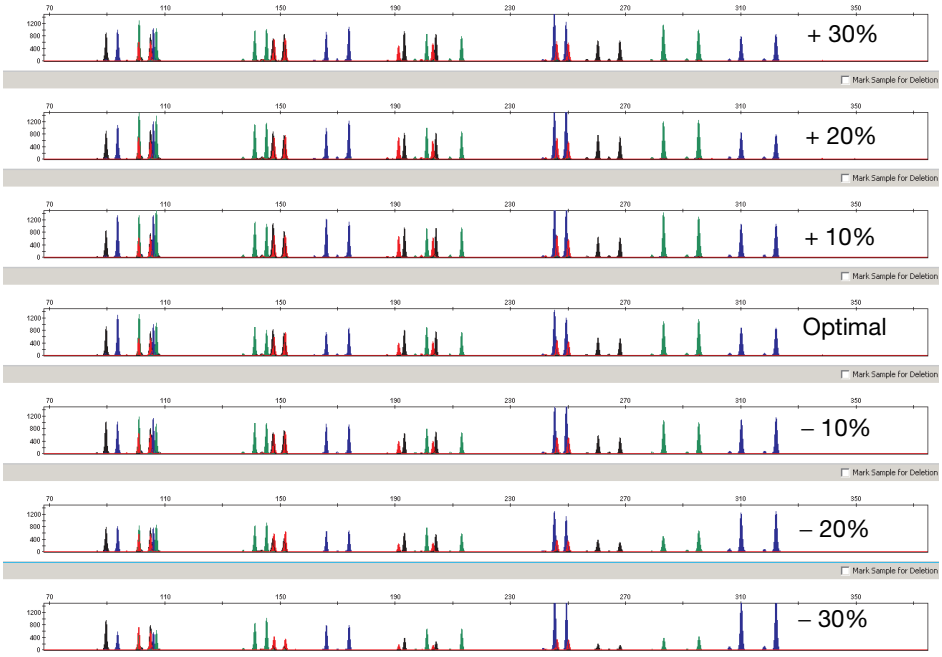


Figure 17 1.0 ng of control DNA 007 amplified with the AmpFℓSTR® NGM™ Kit in the presence of varying concentrations of magnesium chloride and analyzed on an Applied Biosystems 3130*xl* Genetic Analyzer.

Thermal cycler parameters

Thermal cycling parameters were optimized using a Design of Experiments (DOE) approach that attempts to identify the combination of temperatures and hold times that produce the best assay performance. Optimal assay performance was determined through evaluation of assay sensitivity, peak-height balance and resistance to PCR inhibitors.

For example, annealing temperatures of 55, 57, 59, 61, and 63 °C were tested using a Silver 96-Well GeneAmp® PCR System 9700 (Figure 18). The PCR products were analyzed using an Applied Biosystems 3130*xl* Genetic Analyzer.

Of the tested annealing temperatures, 55 to 61 °C produced robust profiles. At 63 °C the yield of the majority of loci was significantly reduced. The optimal combination of specificity, sensitivity, and resistance to PCR inhibition was observed at 59 °C. Thermal cycler temperature is critical to assay performance; therefore, routine, regularly scheduled thermal cycler calibration is strongly recommended.

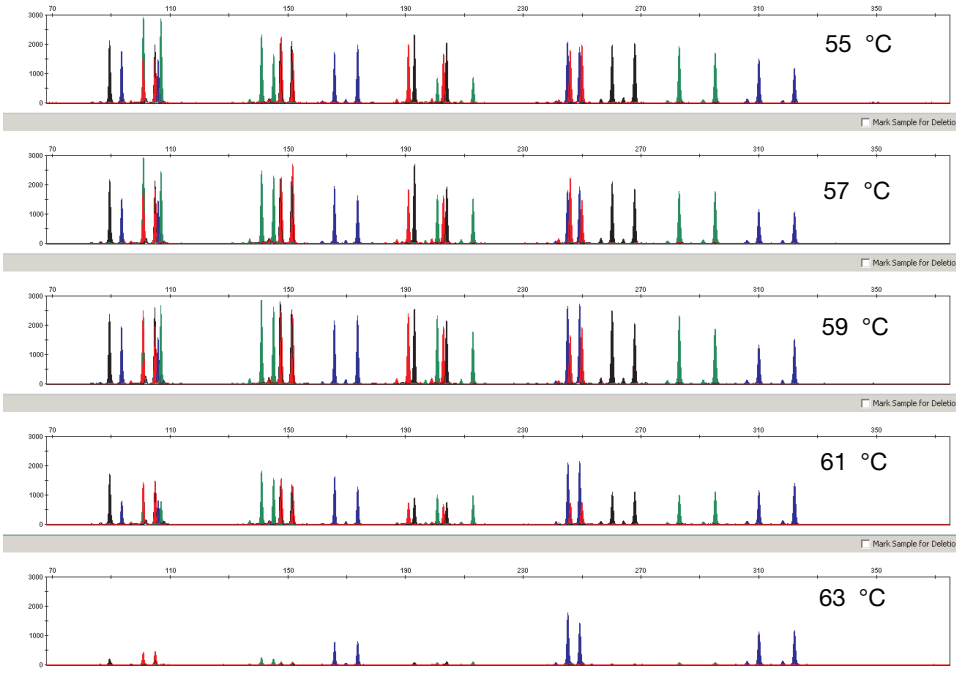


Figure 18 Electropherograms obtained from amplification of 1.0 ng of control DNA 007 at annealing temperatures of 55 °C, 57 °C, 59 °C, 61 °C, and 63 °C, analyzed on an Applied Biosystems 3130*xl* Genetic Analyzer, (Y-axis scale 0 to 3,000 RFU).

PCR cycle number

AmpFℓSTR® NGM™ Kit reactions were amplified for 27, 28, 29, 30, and 31 cycles on the Silver 96-Well GeneAmp® PCR System 9700 using 1.0 ng of each of three DNA samples. As expected, the amount of PCR product increased with the number of cycles. A full profile was generated for all numbers of thermal cycles (27-31) and off-scale data were collected for several allele peaks at 30 and 31 cycles (Figure 19).

Optimal sensitivity was produced by 29 cycles when the amplified products were analyzed on Applied Biosystems 3130*xl* Genetic Analyzers. None of the cycle numbers tested produced nonspecific peaks.

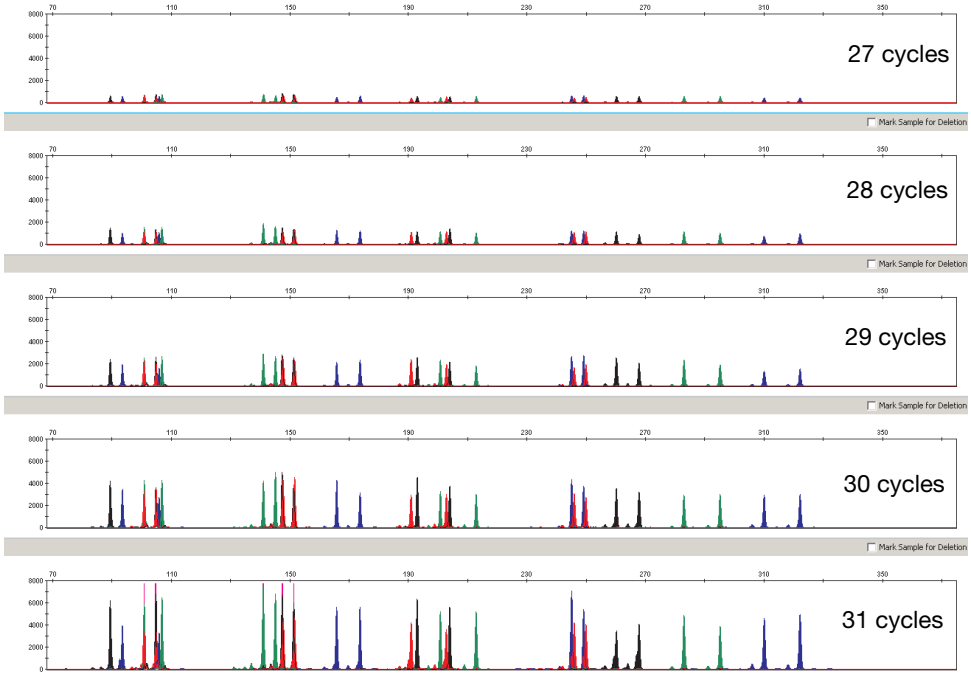


Figure 19 Representative AmpFℓSTR® NGM™ Kit profiles obtained from amplification of 1.0 ng DNA template using 27, 28, 29, 30, and 31 cycles, analyzed on an Applied Biosystems 3130*xl* Genetic Analyzer, (Y-axis scale 0 to 8,000 RFU)

Accuracy, precision, and reproducibility

SWGDAM guideline 2.9

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

Accuracy

Laser-induced fluorescence detection of length polymorphism at short tandem repeat loci is not a novel methodology (Holt *et al.,* 2000; and Wallin *et al.,* 2002). However, accuracy and reproducibility of AmpFℓSTR® NGM™ Kit profiles have been determined from various sample types. Figure 20 shows the size differences that are typically observed between sample alleles and allelic ladder alleles on the Applied Biosystems 3130*xl* Genetic Analyzer with POP-4™ polymer. The x-axis in Figure 20 represents the nominal nucleotide sizes for the AmpFℓSTR NGM™ Allelic Ladder. The dashed lines parallel to the x-axis represent the ±0.25-nt windows. The y-axis represents the deviation of each sample allele size from the corresponding allelic ladder allele size. All sample alleles are within ±0.5 nt from a corresponding allele in the allelic ladder.

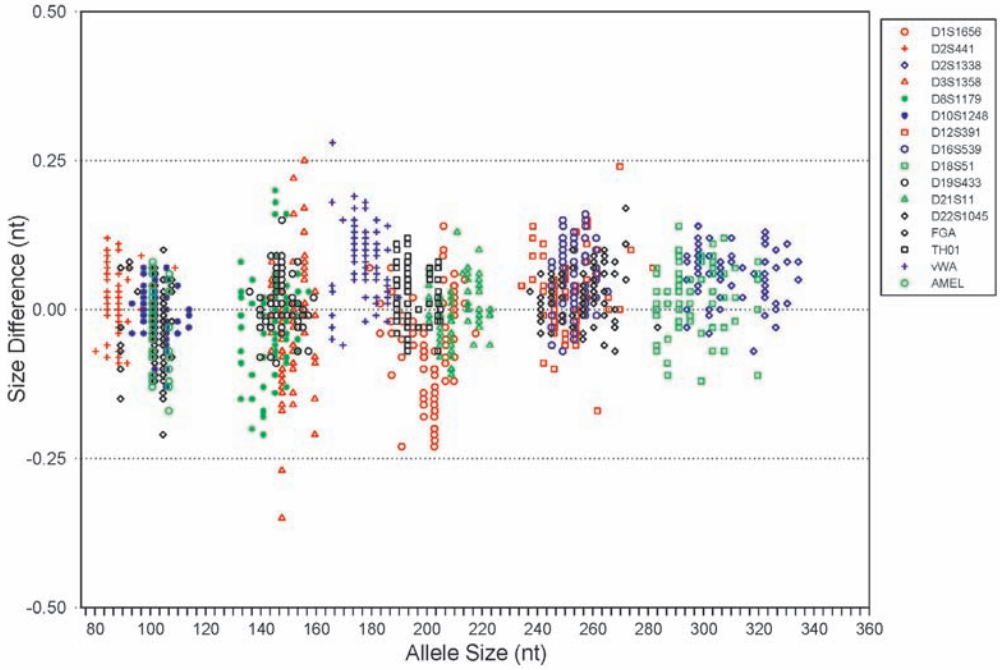


Figure 20 Allele Size vs. Allelic Ladder Sizing for 42 samples analyzed on an Applied Biosystems 3130*xl* Genetic Analyzer. Size and ladder sizing for the AmpFℓSTR NGM™ Kit were calculated using the GeneScan™ 500 LIZ® Size Standard.

Precision and size windows

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

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

or

- An allele that does correspond to an allelic ladder allele, but whose size is just outside a window because of measurement error

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

Table 6 on page 80 shows typical precision results obtained from five runs (16 capillaries/run) of the AmpFℓSTR® NGM™ Allelic Ladder on an Applied Biosystems 3130*xl* Genetic Analyzer (36-cm capillary and POP-4™ polymer), using the GeneScan™ 500 LIZ® Size Standard. The results were obtained within a set of injections on a single capillary array.

Sample alleles may occasionally size outside of the ±0.5-nt window for a respective allelic ladder allele because of measurement error. The frequency of such an occurrence is lowest in detection systems having the smallest standard deviations in sizing. Figure 20 on page 78 illustrates the tight clustering of allele sizes obtained on the Applied Biosystems 3130*xl* Genetic Analyzer, where the standard deviation in sizing is typically less than 0.15 nt. The instance of a sample allele sizing outside the ±0.5-nt window because of measurement error is relatively rare when the standard deviation in sizing is approximately 0.15 nt or less (Smith, 1995).

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

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

Maximum sizing precision is obtained within the same set of capillary injections. Cross-platform sizing differences occur due to a number of factors including type and concentration of polymer, run temperature, and electrophoresis conditions. Variations in sizing can also occur between runs on the same instrument and between runs on different instruments of the same platform type because of these factors.

Applied Biosystems strongly recommends that the allele sizes be compared to the sizes obtained for known alleles in the AmpFℓSTR® NGM™ Allelic Ladder from the same run and then be converted to genotypes (as described in **"Before you start"** on pages 39 and 53). See Table 6 for the results of five runs of the AmpFℓSTR® NGM™ Allelic Ladder on an Applied Biosystems 3130*xl* Genetic Analyzer. For more information on precision and genotyping, see Lazaruk *et al.,* 1998 and Mansfield *et al.*, 1998.

In Table 6, the mean sizes for all the alleles in each run (16 capillaries) were calculated. The mean range shown in the table represents the lowest- and highest-mean size values obtained across all five runs. Similarly, the standard deviation for the allele sizing was calculated for all the alleles in each run. The standard deviation range shown in Table 6 represents the lowest and highest standard deviation values obtained across all five runs.

Table 6 Precision results of five runs (16 capillaries/run) of the AmpFℓSTR® NGM™ Allelic Ladder

Allele	Mean	Standard Dev.
Amelogenin		
X	100.54 - 100.68	0.052 - 0.064
Y	106.21 - 106.34	0.045 - 0.061
D10S1248		
8	76.64 - 76.67	0.031 - 0.045
9	80.85 - 80.9	0.029 - 0.04
10	85.03 - 85.09	0.024 - 0.045
11	89.19 - 89.25	0.032 - 0.043
12	93.31 - 93.37	0.027 - 0.046
13	97.4 - 97.48	0.035 - 0.049
14	101.41 - 101.48	0.029 - 0.053
15	105.32 - 105.4	0.03 - 0.05
16	109.24 - 109.31	0.035 - 0.046
17	113.18 - 113.24	0.032 - 0.048
18	117.14 - 117.21	0.025 - 0.044
D12S391		
14	229.97 - 230.12	0.042 - 0.056
15	233.9 - 234.05	0.035 - 0.057
16	237.87 - 238.01	0.036 - 0.063
17	241.75 - 241.91	0.046 - 0.051
18	245.68 - 245.83	0.042 - 0.056
19	249.59 - 249.73	0.038 - 0.062
19.3	252.59 - 252.75	0.034 - 0.054
20	253.54 - 253.68	0.034 - 0.058
21	257.45 - 257.58	0.04 - 0.052

Allele *(continued)*	**Mean**	**Standard Dev.**
22	261.3 - 261.47	0.037 - 0.05
23	265.27 - 265.43	0.039 - 0.063
24	269.29 - 269.44	0.039 - 0.05
25	273.28 - 273.42	0.034 - 0.059
26	277.22 - 277.38	0.045 - 0.053
27	281.31 - 281.45	0.049 - 0.066
D16S539		
5	228.68 - 228.78	0.04 - 0.05
8	240.7 - 240.83	0.04 - 0.052
9	244.72 - 244.83	0.05 - 0.055
10	248.73 - 248.84	0.048 - 0.059
11	252.73 - 252.87	0.046 - 0.063
12	256.78 - 256.89	0.04 - 0.055
13	260.8 - 260.93	0.046 - 0.061
14	264.82 - 264.95	0.037 - 0.056
15	268.86 - 268.99	0.04 - 0.054
D18S51		
7	262.24 - 262.34	0.04 - 0.068
9	270.36 - 270.49	0.046 - 0.062
10	274.43 - 274.56	0.04 - 0.063
10.2	276.45 - 276.56	0.05 - 0.065
11	278.5 - 278.62	0.048 - 0.069
12	282.56 - 282.71	0.059 - 0.075
13	286.64 - 286.78	0.038 - 0.073
13.2	288.67 - 288.79	0.057 - 0.073
14	290.76 - 290.88	0.046 - 0.069
14.2	292.75 - 292.9	0.061 - 0.075
15	294.83 - 294.98	0.055 - 0.082
16	298.95 - 299.08	0.056 - 0.077
17	303.1 - 303.23	0.053 - 0.088
18	307.24 - 307.39	0.06 - 0.079
19	311.39 - 311.52	0.066 - 0.086
20	315.53 - 315.65	0.056 - 0.087
21	319.66 - 319.8	0.062 - 0.077
22	323.86 - 323.99	0.063 - 0.082
23	327.92 - 328.04	0.061 - 0.076
24	332.03 - 332.16	0.058 - 0.079
25	336.16 - 336.27	0.056 - 0.085
26	340.27 - 340.39	0.072 - 0.079

Allele *(continued)*	**Mean**	**Standard Dev.**
27	344.39 - 344.5	0.057 - 0.077
D19S433		
9	127.1 - 127.2	0.027 - 0.05
10	130.98 - 131.06	0.033 - 0.05
11	134.88 - 134.96	0.032 - 0.052
12	138.83 - 138.9	0.023 - 0.045
12.2	140.96 - 141.03	0.027 - 0.043
13	143.04 - 143.13	0.03 - 0.049
13.2	145.21 - 145.28	0.029 - 0.041
14	147.28 - 147.37	0.026 - 0.051
14.2	149.44 - 149.52	0.03 - 0.047
15	151.48 - 151.57	0.028 - 0.04
15.2	153.54 - 153.63	0.036 - 0.047
16	155.53 - 155.64	0.032 - 0.048
16.2	157.58 - 157.68	0.039 - 0.051
17	159.55 - 159.67	0.04 - 0.056
17.2	161.54 - 161.66	0.039 - 0.047
D1S1656		
9	175.77 - 175.96	0.046 - 0.09
10	179.67 - 179.85	0.047 - 0.08
11	183.53 - 183.71	0.054 - 0.08
12	187.39 - 187.57	0.056 - 0.078
13	191.24 - 191.4	0.04 - 0.069
14	195.13 - 195.29	0.04 - 0.062
14.3	198.01 - 198.16	0.04 - 0.061
15	198.87 - 199.02	0.045 - 0.07
15.3	201.86 - 202.01	0.046 - 0.062
16	202.72 - 202.88	0.034 - 0.06
16.3	205.76 - 205.91	0.05 - 0.06
17	206.63 - 206.79	0.044 - 0.063
17.3	209.66 - 209.82	0.048 - 0.064
18.3	213.56 - 213.73	0.047 - 0.061
19.3	217.48 - 217.64	0.044 - 0.073
20.3	221.4 - 221.56	0.042 - 0.064
D21S11		
24	185.02 - 185.14	0.042 - 0.069
24.2	187.02 - 187.12	0.042 - 0.066
25	188.98 - 189.08	0.045 - 0.066
26	192.92 - 193.01	0.037 - 0.056

Allele *(continued)*	**Mean**	**Standard Dev.**
27	196.87 - 196.96	0.036 - 0.052
28	200.75 - 200.83	0.032 - 0.052
28.2	202.71 - 202.81	0.036 - 0.053
29	204.69 - 204.78	0.035 - 0.045
29.2	206.73 - 206.82	0.039 - 0.049
30	208.71 - 208.8	0.04 - 0.048
30.2	210.68 - 210.78	0.039 - 0.049
31	212.7 - 212.79	0.034 - 0.05
31.2	214.67 - 214.76	0.037 - 0.049
32	216.69 - 216.78	0.04 - 0.046
32.2	218.65 - 218.75	0.04 - 0.048
33	220.67 - 220.76	0.031 - 0.056
33.2	222.62 - 222.72	0.036 - 0.059
34	224.74 - 224.85	0.039 - 0.048
34.2	226.65 - 226.74	0.042 - 0.055
35	228.7 - 228.8	0.042 - 0.057
35.2	230.64 - 230.73	0.041 - 0.052
36	232.62 - 232.74	0.042 - 0.046
37	236.68 - 236.78	0.037 - 0.052
38	240.61 - 240.71	0.04 - 0.052
	D22S1045	
8	79.99 - 80.08	0.034 - 0.058
9	83.08 - 83.18	0.037 - 0.058
10	86.14 - 86.25	0.041 - 0.063
11	89.2 - 89.33	0.043 - 0.065
12	92.26 - 92.37	0.047 - 0.067
13	95.29 - 95.43	0.045 - 0.069
14	98.33 - 98.46	0.044 - 0.07
15	101.3 - 101.42	0.054 - 0.066
16	104.2 - 104.32	0.043 - 0.064
17	107.13 - 107.24	0.054 - 0.068
18	110.06 - 110.17	0.052 - 0.069
19	113.02 - 113.13	0.046 - 0.059
	D2S1338	
15	289.59 - 289.7	0.038 - 0.054
16	293.68 - 293.78	0.036 - 0.053
17	297.73 - 297.82	0.042 - 0.055
18	301.81 - 301.9	0.046 - 0.064
19	305.9 - 306	0.043 - 0.06

Allele *(continued)*	**Mean**	**Standard Dev.**
20	310.03 - 310.11	0.047 - 0.057
21	314.12 - 314.22	0.042 - 0.062
22	318.22 - 318.31	0.034 - 0.06
23	322.3 - 322.39	0.044 - 0.062
24	326.38 - 326.46	0.044 - 0.058
25	330.46 - 330.55	0.038 - 0.06
26	334.54 - 334.61	0.047 - 0.056
27	338.63 - 338.71	0.046 - 0.057
28	342.95 - 343.05	0.037 - 0.061
D2S441		
9	80.29 - 80.38	0.028 - 0.054
10	84.41 - 84.5	0.033 - 0.057
11	88.51 - 88.62	0.03 - 0.062
12	91.66 - 91.76	0.046 - 0.067
13	92.58 - 92.69	0.045 - 0.062
14	96.49 - 96.62	0.036 - 0.069
15	100.51 - 100.63	0.045 - 0.067
16	104.4 - 104.53	0.038 - 0.056
D3S1358		
12	134.88 - 134.99	0.043 - 0.064
13	138.98 - 139.12	0.042 - 0.064
14	143.23 - 143.38	0.035 - 0.051
15	147.4 - 147.55	0.047 - 0.057
16	151.78 - 151.91	0.042 - 0.063
17	155.99 - 156.16	0.051 - 0.074
18	160.04 - 160.21	0.063 - 0.075
19	163.88 - 164.06	0.064 - 0.079
D8S1179		
8	123.54 - 123.64	0.04 - 0.068
9	127.61 - 127.71	0.039 - 0.071
10	131.7 - 131.81	0.045 - 0.064
11	135.86 - 135.95	0.045 - 0.07
12	140.1 - 140.2	0.046 - 0.056
13	144.67 - 144.77	0.043 - 0.058
14	149.13 - 149.23	0.046 - 0.07
15	153.45 - 153.57	0.053 - 0.069
16	157.7 - 157.85	0.054 - 0.073
17	161.86 - 162	0.056 - 0.086
18	165.94 - 166.07	0.054 - 0.085

Allele *(continued)*	**Mean**	**Standard Dev.**
19	169.99 - 170.15	0.054 - 0.096
FGA		
17	233.22 - 233.34	0.032 - 0.044
18	237.01 - 237.13	0.036 - 0.047
19	240.8 - 240.93	0.04 - 0.053
20	244.61 - 244.74	0.04 - 0.049
21	248.43 - 248.55	0.038 - 0.045
22	252.24 - 252.35	0.034 - 0.057
23	256.09 - 256.19	0.028 - 0.047
24	259.9 - 260.01	0.041 - 0.051
25	263.74 - 263.86	0.035 - 0.049
26	267.59 - 267.7	0.035 - 0.055
26.2	269.46 - 269.57	0.029 - 0.045
27	271.5 - 271.62	0.037 - 0.054
28	275.35 - 275.47	0.036 - 0.046
29	279.2 - 279.31	0.03 - 0.051
30	282.97 - 283.07	0.036 - 0.053
30.2	285.24 - 285.36	0.035 - 0.053
31.2	289.12 - 289.23	0.028 - 0.065
32.2	292.98 - 293.09	0.042 - 0.054
33.2	296.88 - 296.97	0.044 - 0.051
42.2	332.47 - 332.54	0.03 - 0.062
43.2	336.37 - 336.45	0.031 - 0.055
44.2	340.44 - 340.51	0.034 - 0.055
45.2	344.38 - 344.45	0.033 - 0.055
46.2	348.02 - 348.09	0.036 - 0.044
47.2	351.82 - 351.85	0.036 - 0.05
48.2	355.71 - 355.78	0.031 - 0.052
50.2	363.15 - 363.2	0.032 - 0.045
51.2	366.92 - 366.96	0.034 - 0.052
TH01		
4	181.53 - 181.67	0.044 - 0.065
5	185.44 - 185.58	0.039 - 0.063
6	189.32 - 189.46	0.037 - 0.066
7	193.2 - 193.33	0.028 - 0.058
8	197.05 - 197.17	0.032 - 0.053
9	200.93 - 201.03	0.036 - 0.047
9.3	203.93 - 204.03	0.037 - 0.052
10	204.85 - 204.95	0.031 - 0.051

Allele *(continued)*	Mean	Standard Dev.
11	208.78 - 208.9	0.036 - 0.048
13.3	219.64 - 219.75	0.038 - 0.047
vWA		
11	153.74 - 153.84	0.041 - 0.065
12	157.91 - 158.03	0.038 - 0.067
13	162.04 - 162.18	0.039 - 0.069
14	166.28 - 166.42	0.054 - 0.078
15	170.22 - 170.34	0.047 - 0.081
16	174.22 - 174.36	0.043 - 0.087
17	178.23 - 178.36	0.039 - 0.074
18	182.17 - 182.29	0.044 - 0.074
19	186.13 - 186.25	0.044 - 0.075
20	190.06 - 190.2	0.039 - 0.067
21	193.95 - 194.08	0.039 - 0.058
22	197.85 - 197.97	0.04 - 0.057
23	201.69 - 201.79	0.032 - 0.051
24	206.01 - 206.12	0.032 - 0.063

Extra peaks in the electropherogram

Causes of extra peaks

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

Stutter products

Stutter is a well-characterized PCR artifact that refers to the appearance of a minor peak one repeat unit smaller (or less frequently, one repeat larger) than the major STR product (Butler, 2005; Mulero *et al.,* 2006). Sequence analysis of stutter products at tetranucleotide STR loci has revealed that the stutter product is missing a single tetranucleotide core repeat unit relative to the main allele (Walsh *et al.,* 1996).

The proportion of the stutter product relative to the main allele (percent stutter) is measured by dividing the height of the stutter peak by the height of the main allele peak. Peak heights were measured for amplified samples (n = 996) at the loci used in the AmpFℓSTR® NGM™ Kit. All data were generated on the Applied Biosystems 3130*xl* Genetic Analyzer.

Some conclusions from these measurements and observations are:

- For each AmpFℓSTR® NGM™ Kit locus, the percent stutter generally increases with allele length, as shown in Figures 21 to 25 on pages 87 to 89.

- Smaller alleles display a lower level of stutter relative to the longer alleles within each locus.
- Each allele within a locus displays a percent stutter that is consistent.
- Stutter filter sets in GeneMapper® *ID* and GeneMapper® *ID-X* Software, calculated as the mean stutter for the locus plus three standard deviations (n = 996), are shown in Table 7 on page 90. Peaks in the stutter position that are above *the highest observed percent stutter* are not filtered. Peaks in the stutter position that have not been filtered and remain labeled can be further evaluated. For evaluation of mixed samples, see Figure 36 on page 108.
- The measurement of percent stutter for allele peaks that are off-scale may be unusually high due to artificial truncation of the main allele peak.

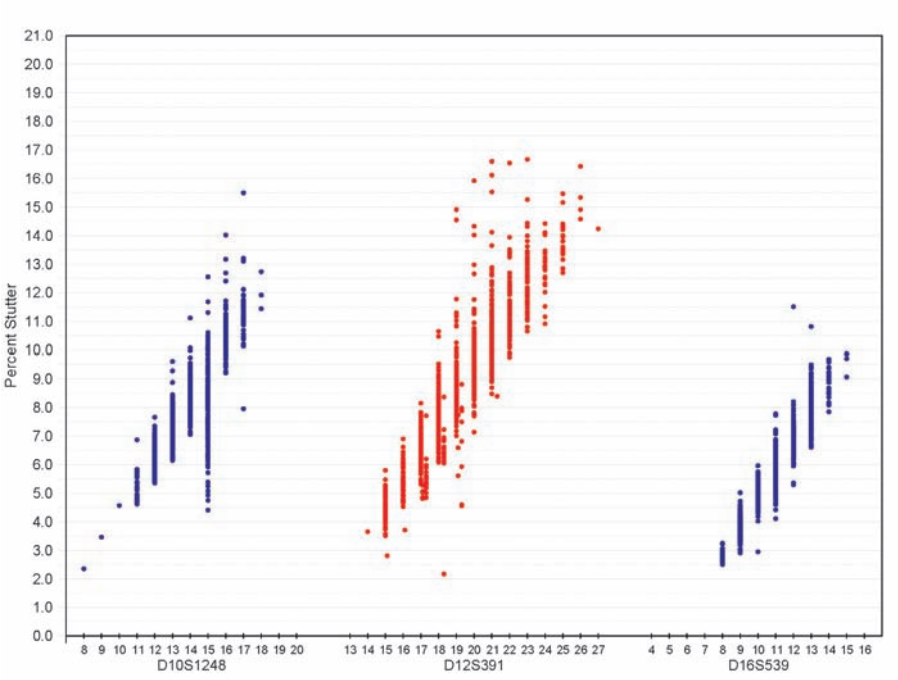


Figure 21 Stutter percentages for D10S1248, D12S391 and D16S539 loci (Blue and red colors indicate loci labeled with FAM and PET dyes, respectively).

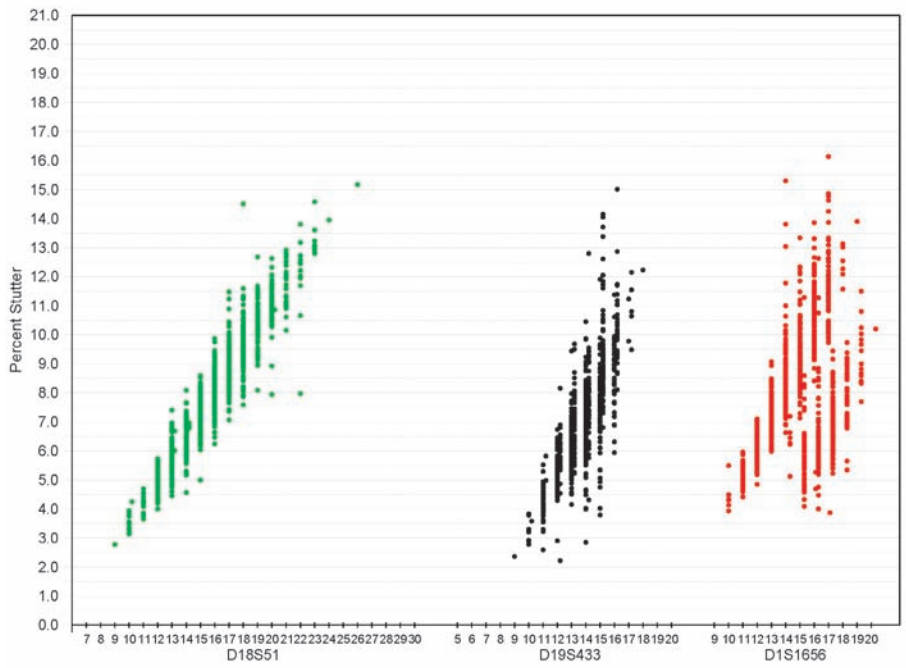


Figure 22 Stutter percentages for the D18S51, D19S433 and D1S1656 loci (Green, black and red colors indicate loci labeled with VIC, NED and PET dyes, respectively).

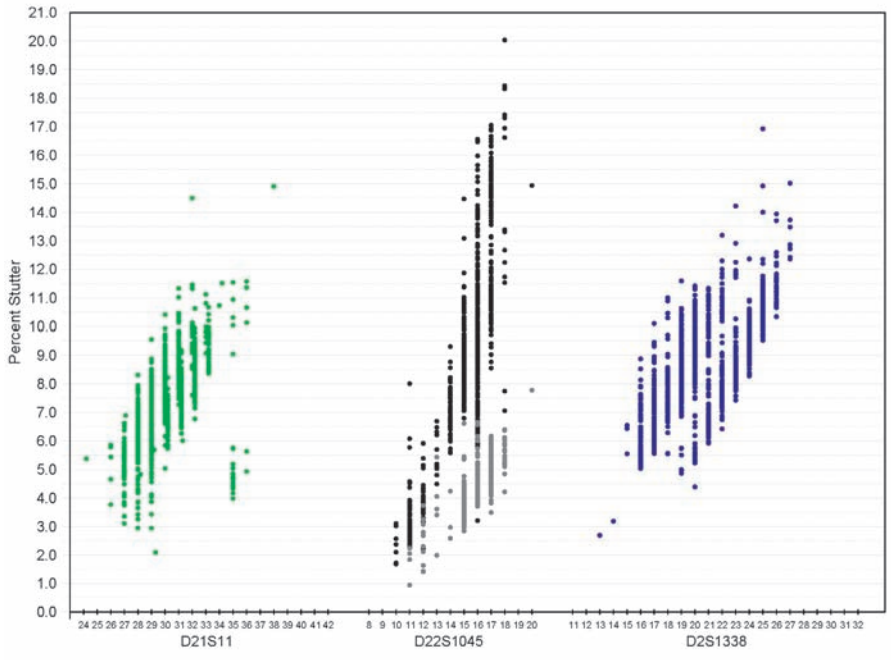


Figure 23 Stutter percentages for the D21S11, D22S1045 and D2S1338 loci (Green, black/gray, and blue colors indicate loci labeled with VIC, NED and FAM dyes, respectively. Black and gray data points associated with the D22S1045 locus indicate minus- and plus-stutter, respectively).

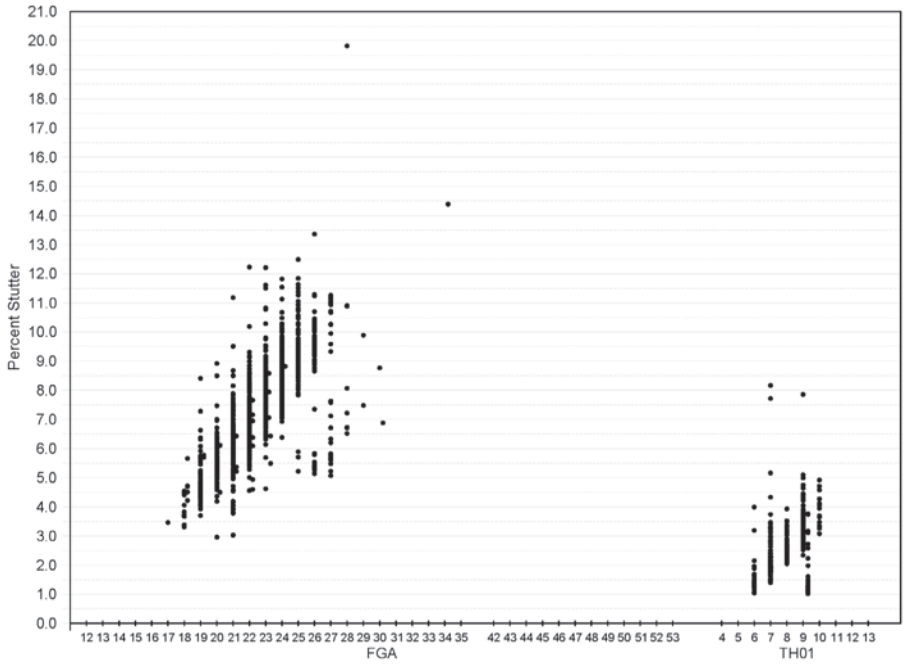


Figure 24 Stutter percentages for the FGA and TH01 loci (Black data points indicate loci labeled with NED dye).

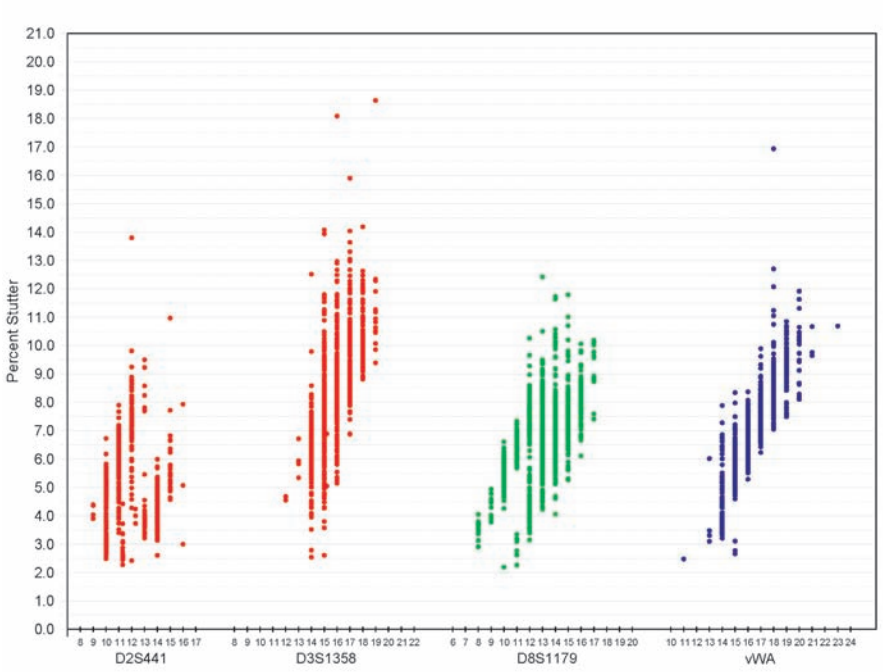


Figure 25 Stutter percentages for the D2S441, D3S1358, D8S1179, and vWA loci (Red, green, and blue colors indicate loci labeled with and PET, VIC, and FAM dyes, respectively).

Table 7 Marker-specific stutter filter percentages for AmpFℓSTR® NGM™ Kit loci

Locus‡	% Stutter
D10S1248	12.89
vWA	11.82
D16S539	10.57
D2S1338	13.55
D8S1179	10.82
D21S11	11.40
D18S51	13.89
D22S1045	17.99
D19S433	11.06
TH01	5.26
FGA	12.61
D2S441	9.47
D3S1358	13.77
D1S1656	14.16
D12S391	15.84

‡ These percentages are used as stutter filters in GeneMapper® *ID* v3.2.1 NGM_panels_v1 and GeneMapper® *ID-X* NGM_stutter_v1X.

Addition of 3´ A nucleotide

Many DNA polymerases can catalyze the addition of a single nucleotide (predominantly adenosine) to the 3´ ends of double-stranded PCR products (Clark, 1988; Magnuson *et al.,* 1996). This nontemplate addition results in a PCR product that is one nucleotide longer than the actual target sequence. The PCR product with the extra nucleotide is referred to as the "+A" form.

The efficiency of +A addition is related to the particular sequence of the DNA at the 3´ end of the PCR product. The AmpFℓSTR® NGM™ Kit includes two main design features that promote maximum +A addition:

- The primer sequences have been optimized to encourage +A addition.
- The new, highly robust PCR chemistry allows complete +A addition with a short final incubation at 60 °C for 10 minutes.

This final extension step gives the DNA polymerase additional time to complete +A addition to all double-stranded PCR products. See Figure 26 on page 91 for examples of incomplete and normal +A addition. Final extension incubation for longer than the recommended 10 minutes may result in double +A addition, in which *two* non-template adenosine residues are added to the PCR product. Double +A addition can cause a peak morphology problem with "shoulders" on the right side of main allele peaks, and is therefore to be avoided.

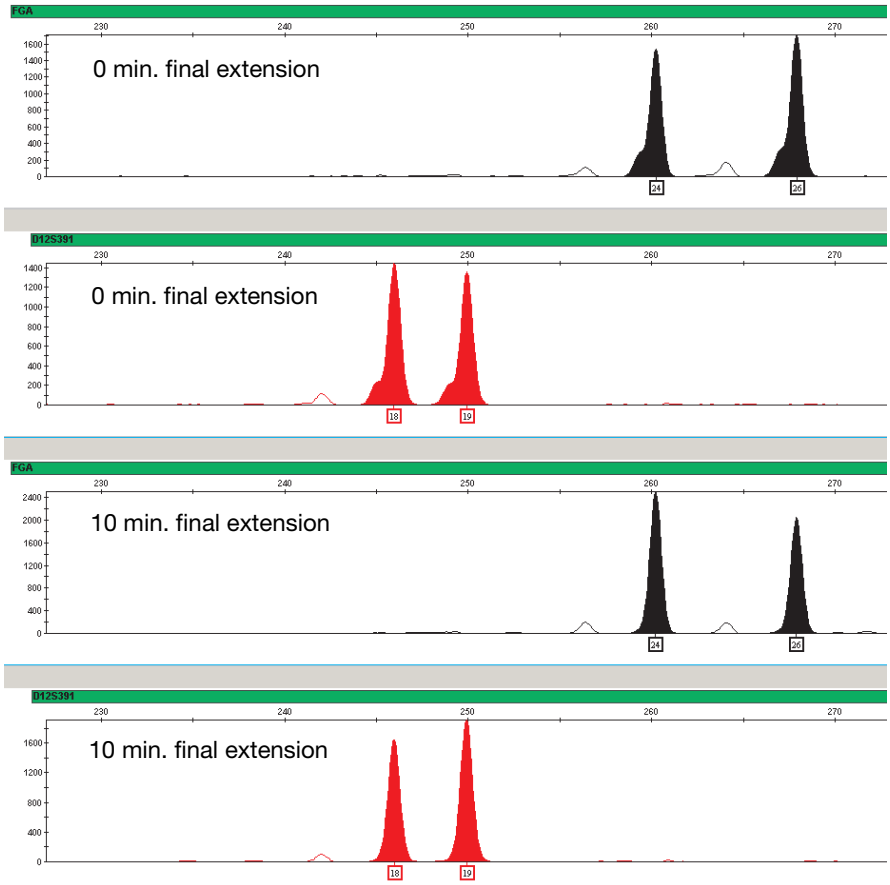


Figure 26 Omitting the final extension step results in shoulders on main allele peaks due to incomplete A nucleotide addition. Data are from an ABI PRISM® 3130*xl* Genetic Analyzer using the AmpFℓSTR® NGM™ Kit.

Due to improved PCR buffer chemistry, the lack of +A addition is generally less an issue with the AmpFℓSTR® NGM™ Kit than with earlier generation kits. However, "shouldering" of allele peaks may still be observed if the amount of input DNA is greater than that recommended by the NGM™ kit protocol. Amplification of excess input DNA may also result in offscale data.

Artifacts

Artifacts and anomalies are seen in all molecular biological systems. Artifacts are typically reproducible while anomalies are non-reproducible, intermittent occurrences that are not observed consistently in a system (for example, spikes and baseline noise). Due to improvements in PCR primer manufacturing processes, the incidence of artifacts has been greatly reduced in the AmpFℓSTR® NGM™ Kit. NGM Kit electropherograms are essentially free of reproducible dye artifacts within the Kit's read region of 68-407 nt. Figure 27 on page 93 shows the very low baseline level fluorescence of a typical negative control PCR using the AmpFℓSTR® NGM™ Kit.

Most STR loci produce minus-stutter peaks as a byproduct of PCR amplification. A process of "slippage" has been proposed as a molecular mechanism for stutter, where the Taq DNA polymerase enzyme "slips" on the template DNA during replication and produces a minority PCR product that is shorter than the template strand, usually by one repeat unit. The stutter process may also occur in the opposite direction to produce amplicon DNA that is usually one repeat unit longer than the template strand, termed plus-stutter. While plus-stutter is normally much less significant than minus-stutter in STR loci with tetranucleotide repeats, the incidence of plus-stutter may be more significant in trinucleotide repeat-containing loci. The D22S1045 locus in the NGM assay kit is a trinucleotide repeat locus, and shows an elevated level of plus-stutter. For example, Figure 29 on page 94 is an electropherogram of the D22S1045 locus showing plus stutter. GeneMapper® *ID* and GeneMapper® *ID-X* analysis parameter files supplied for use with the NGM kit contain a plus-stutter filter to prevent these peaks from being called in normal profiles.

Figure 28 on page 93 shows an example of a non-standard (minus 2-nt) stutter that may be observed in certain STR loci such as D1S1656 with more complex nucleotide sequences that include regions of dinucleotide TG repeats. Genotyping may result in the detection of these artifacts as off-ladder (OL) alleles.

It is important to consider possible noise and artifacts when interpreting data from the AmpFℓSTR® NGM™ Kit on the Applied Biosystems 3130/3130*xl*, ABI PRISM® 3100/3100-Avant, and ABI PRISM® 310 Genetic Analyzers. Note that a high degree of magnification is used in the sample electropherograms shown in Figures 27 to 29 on pages 93 to 94.

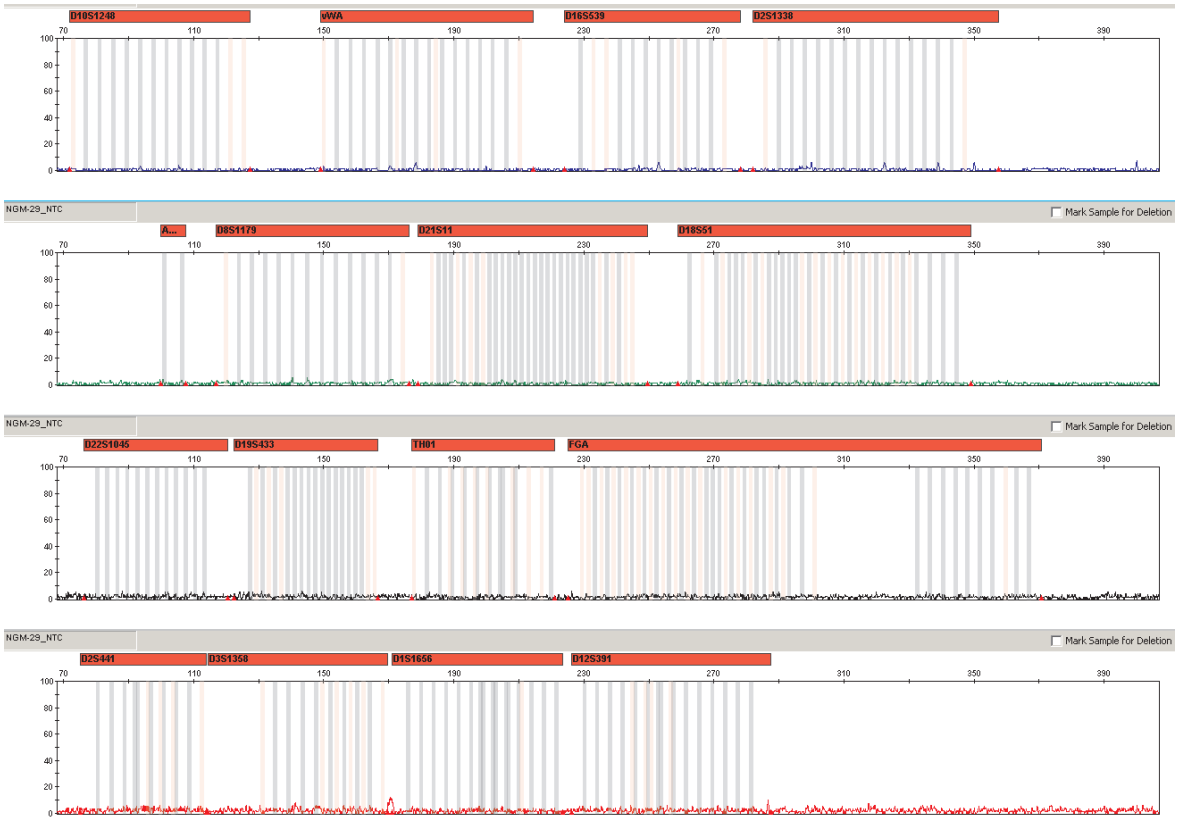


Figure 27 Examples of fluorescence background in data produced on an Applied Biosystems 3130*xl* Genetic Analyzer, (Y-axis scale 0–100 RFU).

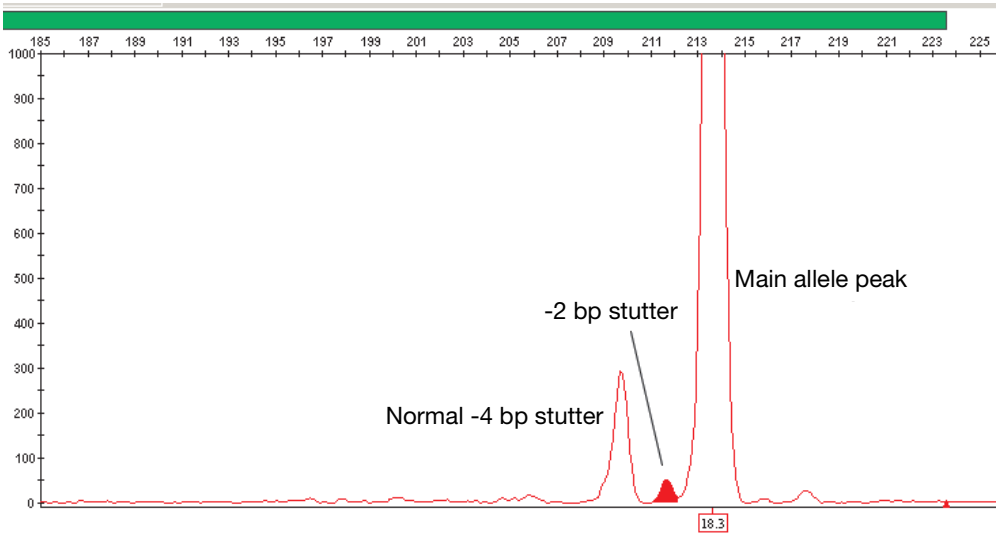


Figure 28 Example of a – 2 nt reproducible artifact at the D1S1656 locus. Data produced on an Applied Biosystems 3130*xl* Genetic Analyzer.

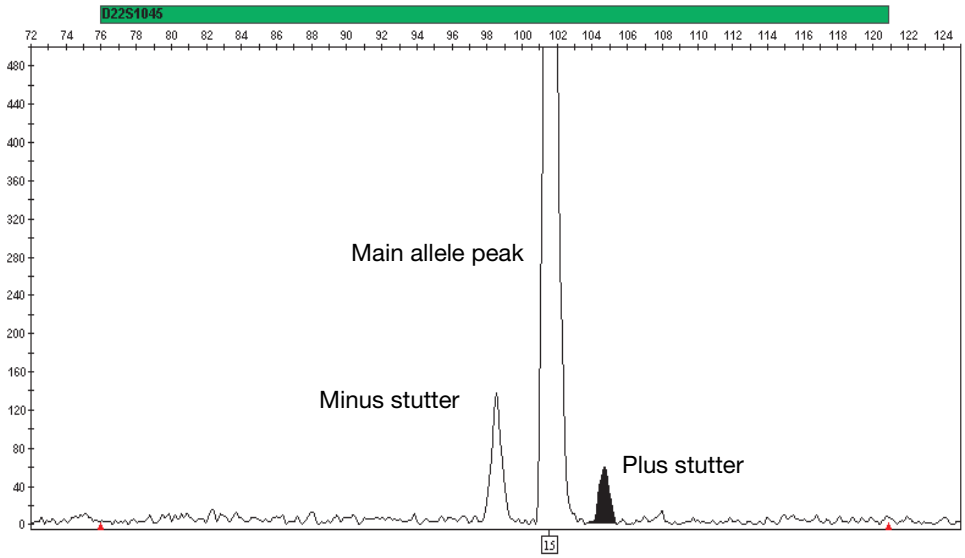


Figure 29 NGM™ Kit assay electropherogram showing plus stutter associated with the D22S1045 STR locus. Data produced on an Applied Biosystems 3130*xl* Genetic Analyzer.

Characterization of loci

SWGDAM guideline 2.1

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

This section describes basic characteristics of the 15 loci and the sex-determining marker, Amelogenin, which are amplified with the AmpFℓSTR® NGM™ Kit. Most of these loci have been extensively characterized by other laboratories.

Nature of the polymorphisms

The primers for the Amelogenin locus flank a 6-nucleotide deletion within intron 1 of the X homolog. Amplification results in 104-nt and 110-nt products from the X and Y chromosomes, respectively. (Sizes are the actual nucleotide size according to sequencing results, including 3′ A nucleotide addition, and size may not correspond exactly to allele mobility observed on capillary electrophoresis platforms.) With the sole exception of D22S1045, a trinucleotide STR, the remaining AmpFℓSTR® NGM™ Kit loci are tetranucleotide short tandem repeat (STR) loci. The length differences among alleles of a particular locus result from differences in the number of repeat units.

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

Inheritance

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

Mapping

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

Genetic linkage

Two sets of STR loci in the AmpFℓSTR® NGM™ Kit are located on the same chromosomes. vWA and D12S391 are located approximately 6.3 million bp apart on the p arm of chromosome 12, while D2S1338 and D2S441 are located approximately 150 million bp apart on opposite arms of chromosome 2. Linkage disequilibrium analysis was conducted on the genotype results from 996 individuals of three ethnic

groups (see “Population data” on page 110). STR locus genotype results from the population study were analyzed using the Linkage Disequilibrium module of GenePop software version 4.0.10 (Raymond and Rousset, 1995; Rousset, 2008). See Table 8 for results.

The relatively high probability values indicate that there is no statistically significant linkage disequilibrium found between the pairs of loci located on the same chromosome.

Table 8 GenePop LD Result (p value for pairwise analysis of loci)

Locus	Chromosome Map Position‡	Chromosome Nuclear Coordinates‡ (million bp)	African-American (N = 350)	Caucasian (N = 350)	Hispanic (N = 293)
vWA	p13.31	5.9	0.86	0.29	0.27
D12S391	p13.2	12.2			
D2S441	p14	68	0.11	0.32	0.19
D2S1338	q35	218			

‡ STR locus mapping data was obtained from the NCBI Map Viewer **http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606** or the UCSC Genome Browser (**http://genome.ucsc.edu/**). GenePop LD analysis probability results (p values) greater than 0.05 were considered to indicate that linkage disequilibrium between the loci within the population tested was not statistically significant.

Species specificity

SWGDAM Guideline 2.2

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

The AmpFℓSTR® NGM™ Kit provides the required specificity for detecting human alleles.

Nonhuman studies

Nonhuman DNA may be present in forensic casework samples. The following species were tested (in the specified amounts) using standard PCR and capillary electrophoresis conditions for the AmpFℓSTR® NGM™ Kit.

- Primates: gorilla, chimpanzee, and macaque (1.0 ng each)
- Non-primates: mouse, dog, sheep, pig, rabbit, cat, horse, hamster, rat, chicken, and cow (5.0 ng each)
- Microorganisms: *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Bacillus subtilis,* and *Lactobacillus rhamnosus* (equivalent to 10^5 copies)

Results were assessed for the presence of any amplified peaks that would indicate cross reactivity of the NGM Kit with any of these non-human species.

Figure 30 on page 98 shows example electropherogram results from the species specificity tests. The chimpanzee and gorilla DNA samples produced partial profiles within the 70 to 283 nucleotide region (gorilla data not shown). Macaque DNA produced a strong amelogenin-X peak and two small out-of-marker-range peaks in PET (data not shown).

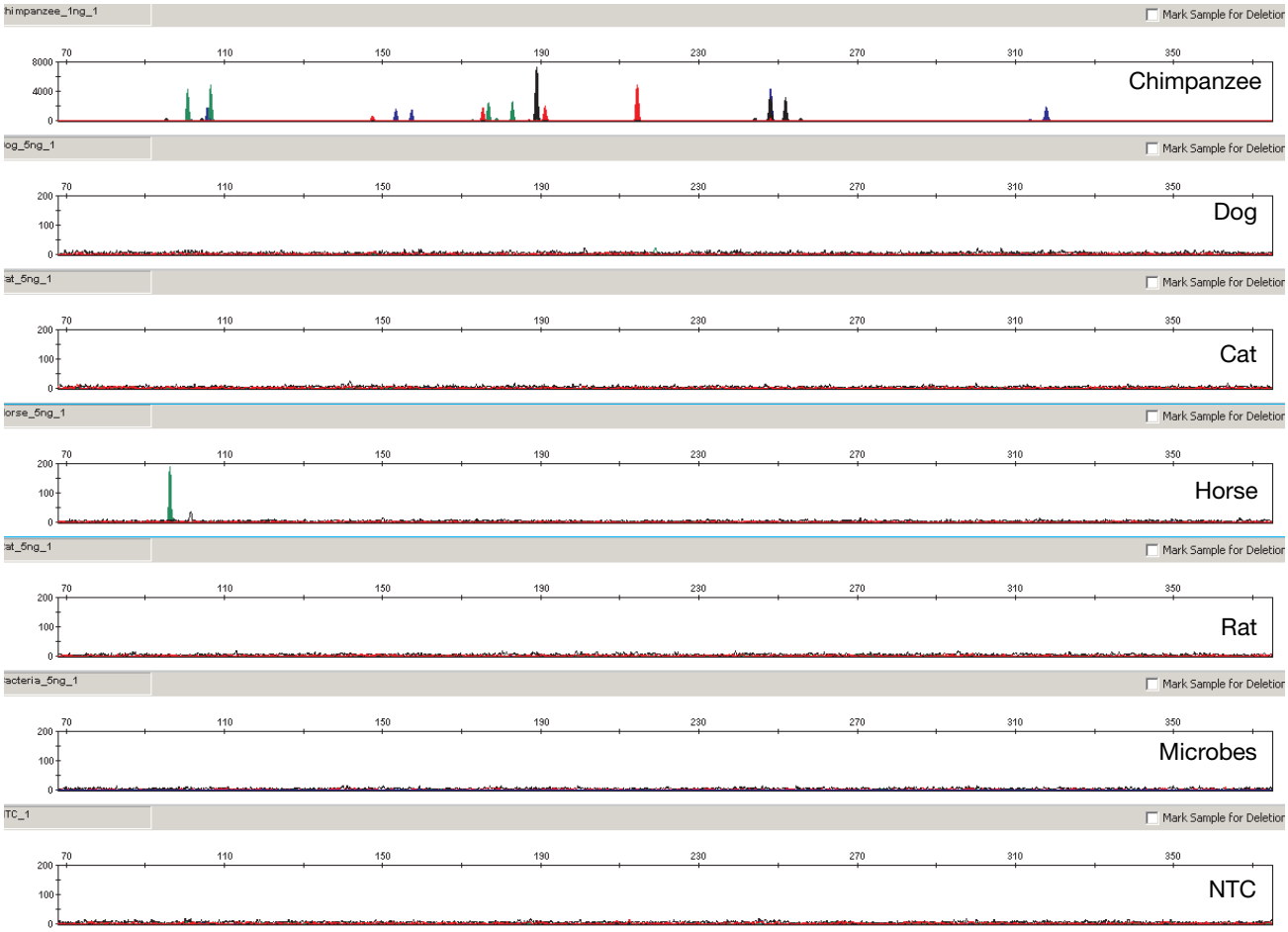


Figure 30 Representative electropherograms from a species specificity study including positive and non-template controls (NTC)

The microorganisms, cow, sheep, pig, dog, cat, chicken, hamster, mouse, rabbit, and rat did not yield detectable products. Of the non-primates, only horse DNA produced a 96-bp fragment near the amelogenin locus in the VIC® dye.

Sensitivity

SWGDAM guideline 2.3

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

Importance of quantification

The recommended amount of input DNA for the AmpFℓSTR® NGM™ Kit is 1.0 ng, based on quantification using either the Quantifiler® Human or Quantifiler® Duo Quantification kit and individual laboratories should determine the optimum input DNA amount according to the quantification method in use in the laboratory. If the sample contains degraded or inhibited DNA, amplification of a higher concentration of DNA may be beneficial. In Figure 31 on page 100, the control DNA 007 was serially diluted from 2.0 ng to 0.062 ng. Full profiles (32 PCR products) were consistently obtained at 0.125 ng, but occasional partial profiles missing 1 to 3 alleles were observed at 0.062 ng.

Effect of DNA quantity on results

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

- Fluorescence intensity that exceeds the linear dynamic range for detection by the instrument ("off-scale" data).
- Off-scale data. Off-scale data is a problem because:
  - quantification (peak height and area) for off-scale peaks is not accurate. For example, an allele peak that is off-scale can cause the corresponding stutter peak to appear higher in relative intensity, thus increasing the calculated percent stutter.
  - Multicomponent analysis of off-scale data is not accurate. This inaccuracy results in poor spectral separation ("pull-up").
- Incomplete +A nucleotide addition.

To address these issues, reamplify the sample using less DNA.

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

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

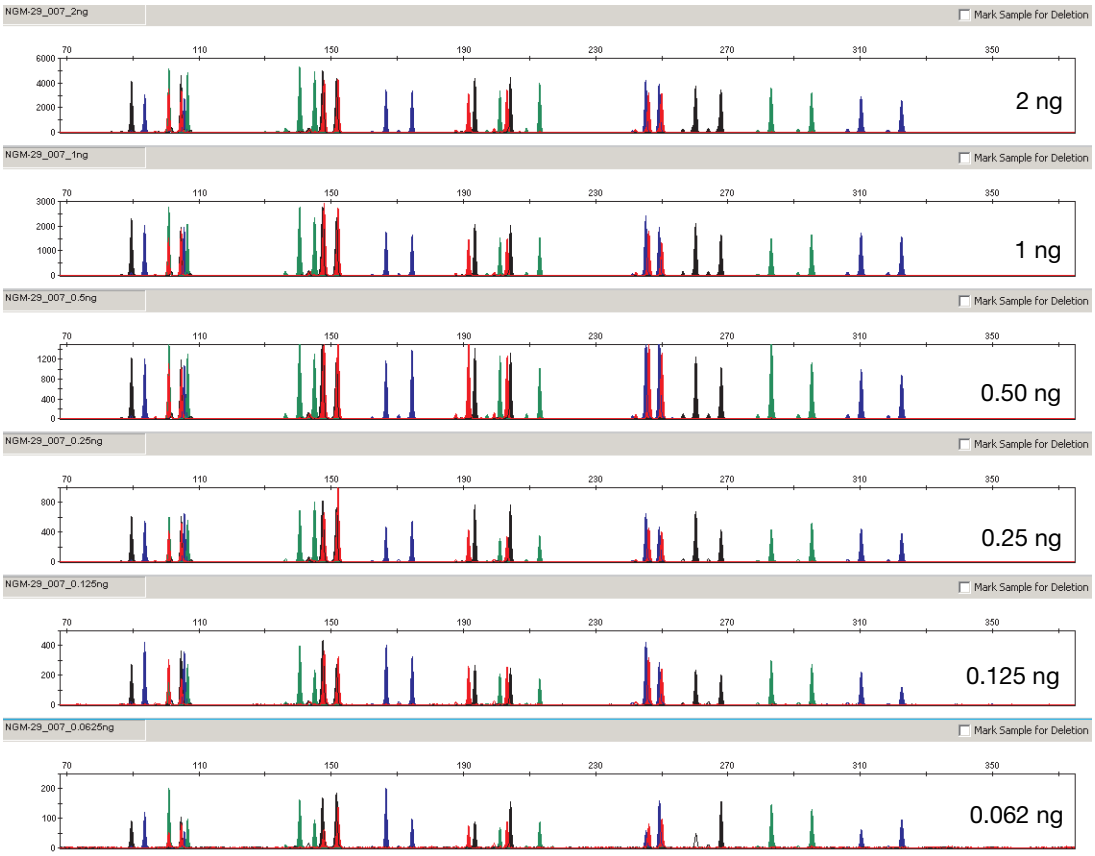


Figure 31 Electropherograms for amplifications using 2 ng, 1 ng, 0.50 ng, 0.25 ng, 0.125 ng, and 0.062 ng of control DNA 007. Electrophoresis was performed on an Applied Biosystems 3130*xl* Genetic Analyzer. Note that the y-axis scale is magnified for the smaller input amounts of DNA.

Stability

SWGDAM guideline 2.4

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

Degraded DNA

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

Degraded DNA was prepared to examine the potential for differential amplification of loci. High-molecular-weight Raji DNA was sonicated and incubated with increasing doses of DNase I (0 to 6 Units) for 20 minutes (Bender *et al.,* 2004). The DNA was examined by agarose gel analysis to determine the average size of the DNA fragments at each time point.

Amplification of 1 ng of degraded DNA using the AmpFℓSTR® NGM™ Kit was performed. As the DNA became progressively degraded, the loci failed to amplify robustly in order of decreasing size. Preferential amplification was not observed.

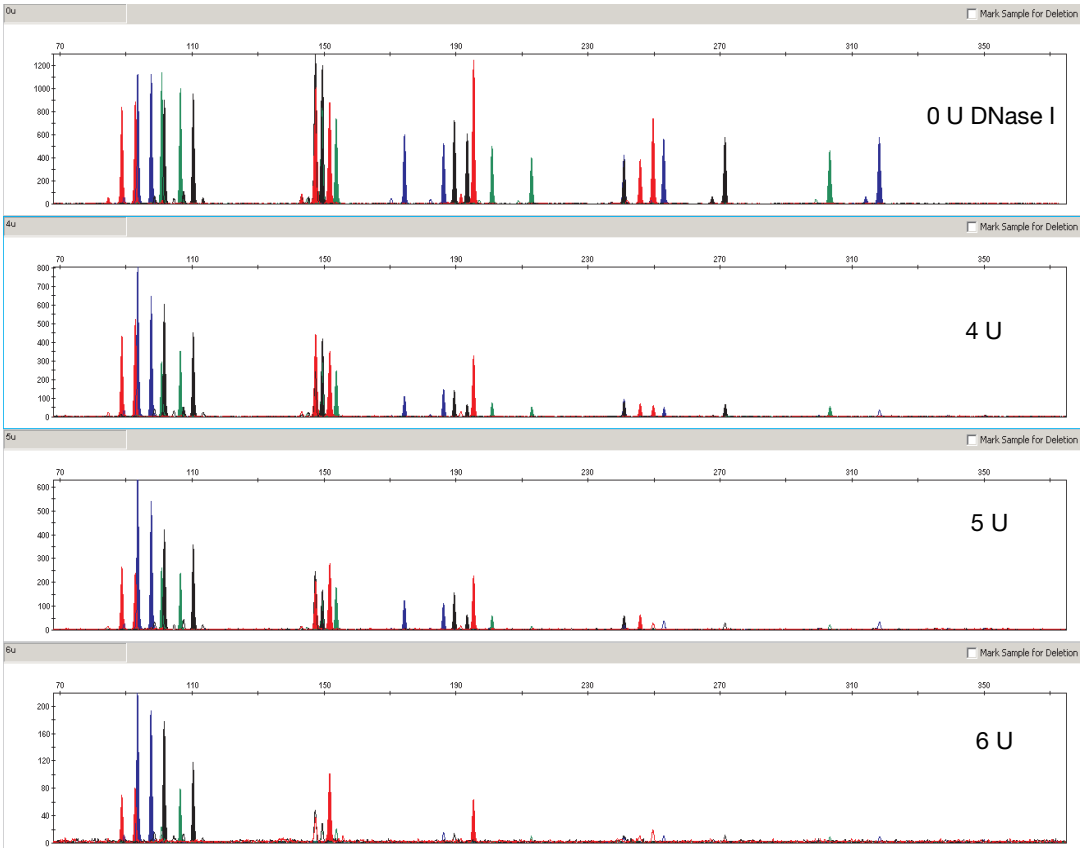


Figure 32 Amplification of Raji DNA samples sonicated and incubated with increasing doses of DNase I. Panels 1, 2, 3, and 4 correspond to 0, 4, 5, and 6 units of DNase I. Note that the y-axis scale is magnified for more degraded samples, which generate lower peak heights.

Effect of inhibitors — hematin

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

To examine the effects of hematin on the performance of the AmpFℓSTR® NGM™ Kit, 1 ng of control DNA 007 was amplified in the presence of increasing concentrations of hematin for 29 cycles of amplification (Figure 33 on page 103).The concentrations of hematin used were 0 µM, 50 µM, 100 µM, 150 µM, and 200 µM (see Table 9 on page 103).

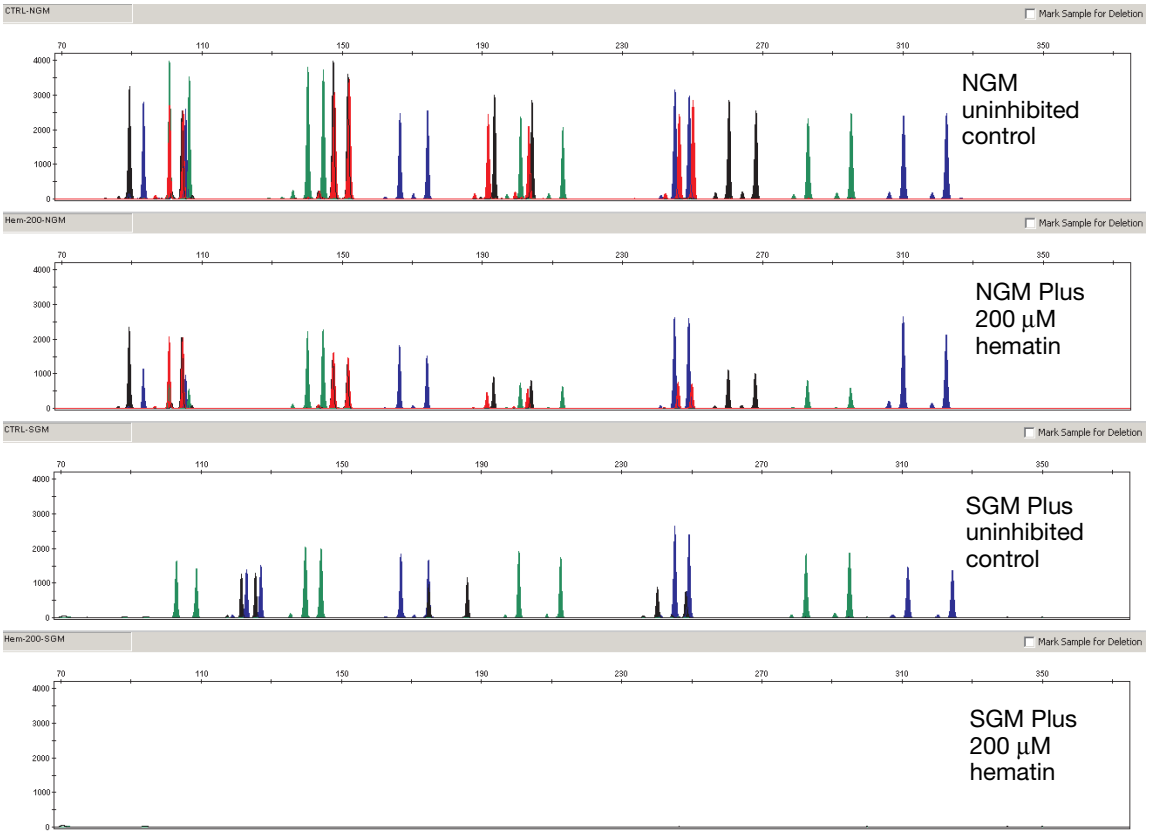


Figure 33 Electropherograms for the AmpFℓSTR NGM and SGM Plus kits show the improved performance of the NGM kit in the presence of hematin compared with previous AmpFℓSTR kits. In order from top to bottom, the panels show profiles for: NGM Kit uninhibited control, NGM Kit with 200 µM hematin, SGM Plus Kit uninhibited control and SGM Plus Kit with 200 µM hematin.

Table 9 NGM kit performance in simulated hematin inhibition (n = 3)

Hematin Concentration (µM)	Number of Alleles Detected‡
0	32, 32, 32
50	32, 32, 32
100	32, 32, 32
150	32, 32, 32
200	32, 32, 32

‡ Only those peaks >50 RFU were counted. A complete profile with control DNA 007 yields 32 peaks using the AmpFℓSTR® NGM™ Kit.

Effect of inhibitors — humic acid

Traces of humic acid may inhibit the PCR amplification of DNA evidence collected from soil. Amplification of 1 ng of control DNA 007 in the presence of increasing amounts of humic acid was performed using the AmpFℓSTR® NGM™ Kit for 29 cycles of amplification (see Figure 34). The concentrations of humic acid tested were 0, 20, 40, 60, and 80 ng/µL (see Table 10 on page 104).

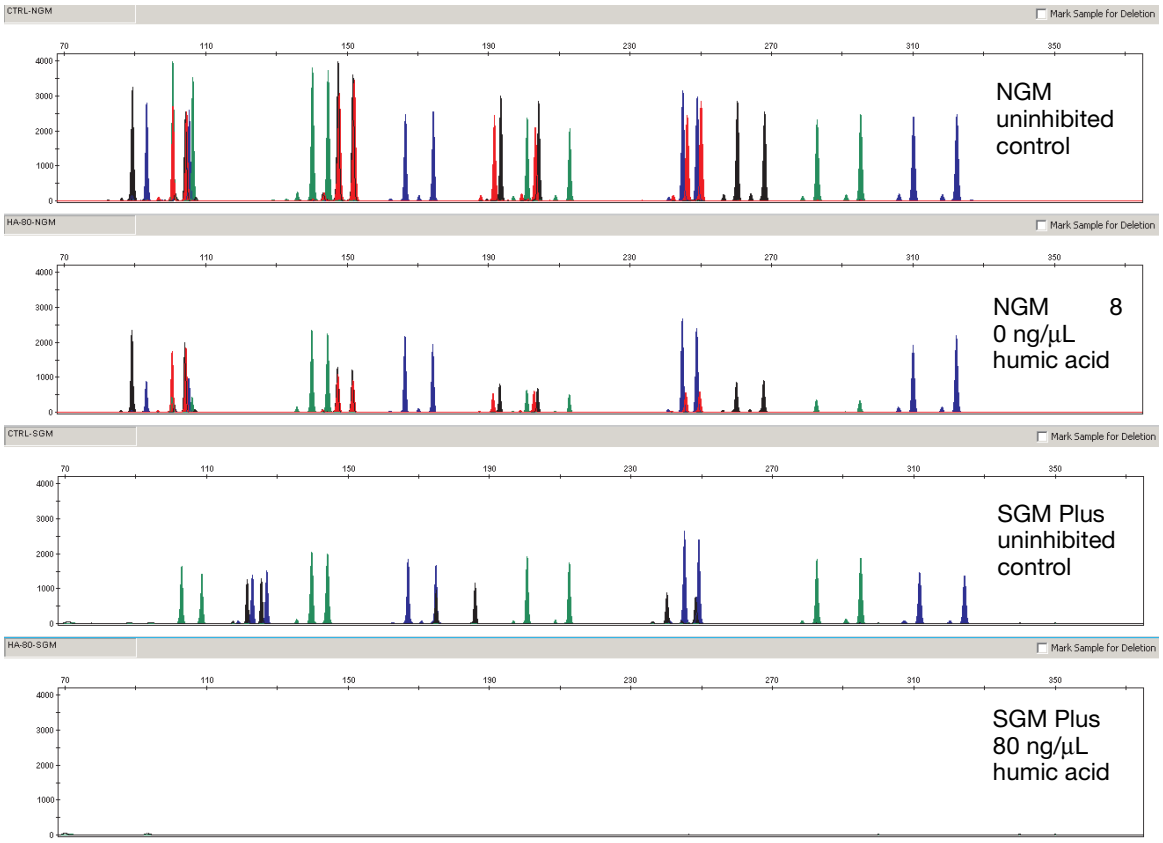


Figure 34 Electropherograms for the AmpFℓSTR NGM and SGM Plus kits show improved performance of the NGM kit in the presence of humic acid compared to previous AmpFℓSTR kits. In order from top to bottom, the panels show profiles for: NGM Kit uninhibited control, NGM Kit with 80 ng/µL humic acid, SGM Plus Kit uninhibited control and SGM Plus Kit with 80 ng/µL humic acid.

Table 10 NGM kit performance in simulated model of humic acid inhibition (n = 3)

Humic Acid Concentration (ng/µL)	Number of Alleles Detected‡
0	32, 32, 32
20	32, 32, 32
40	32, 32, 32
60	32, 32, 32
80	32, 32, 32

‡ Only those peaks >50 RFU were counted. A complete profile with control DNA 007 yields 32 peaks using the AmpFℓSTR® NGM™ Kit.

Mixture studies

SWGDAM guideline 2.8

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

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

Mixture Studies

Evidence samples that contain body fluids and/or tissues originating from more than one individual are an integral component of forensic casework. Therefore, it is essential to ensure that the DNA typing system is able to detect DNA mixtures. Mixed samples can be distinguished from single-source samples by:

- The presence of more than two alleles at a locus
- The presence of a peak at a stutter position that is significantly greater in percentage than typically observed in a single-source sample
- Significantly imbalanced alleles for a heterozygous genotype

The peak height ratio is defined as the height of the lower peak (in RFU) divided by the height of the higher peak (in RFU), expressed as a percentage. Mean, median, minimum, and maximum peak height ratios observed for alleles in the AmpFℓSTR® NGM™ Kit loci in unmixed population database samples are shown in Figure 35 on page 106.

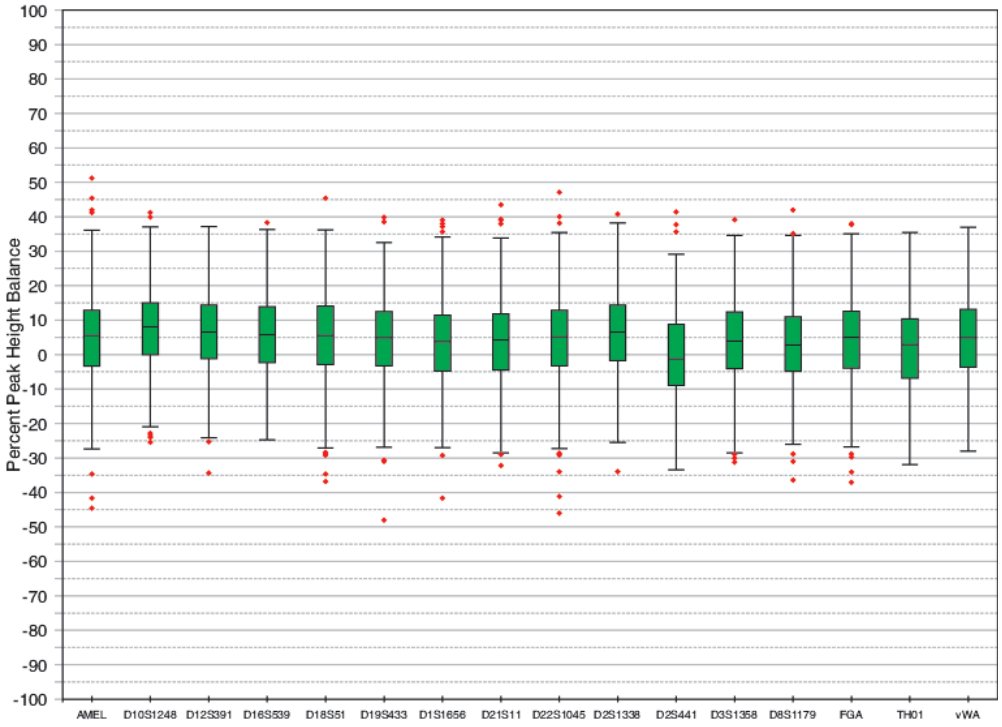


Figure 35 Heterozygote ratios for 1 ng of input DNA. The distribution of intra-locus peak height ratios are expressed as plus and minus percent, by locus. Green boxes show the middle 50% or interquartile range (IQR). Box halves below and above median show the second and third quartile, respectively. "Whiskers" indicate 1.5 IQR from the upper and lower margins of the IQR. Red diamonds are outlier data points more than 1.5 IQR from the median.

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

- Degraded DNA
- Presence of inhibitors
- Extremely low amounts of input DNA
- A mutation in one of the primer binding sites
- Presence of an allele containing a rare sequence that does not amplify as efficiently as the other allele

Resolution of genotypes in mixed samples

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

- Heterozygote + heterozygote, no overlapping alleles (four peaks)

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

Specific genotype combinations and input DNA ratios of the samples contained in a mixture determine whether or not it is possible to resolve the genotypes of the major and minor component(s) at a single locus.

The ability to obtain and compare quantitative values for the different allele peak heights on Applied Biosystems instruments provides additional valuable data to aid in resolving mixed genotypes.

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

The results of the mixed DNA samples are shown in Figure 36 on page 108 where samples A and B were mixed according to the ratios indicated. The minor component allele calls at non-overlapping loci are highlighted. Detection of full profiles for the minor contributor was possible at ratios of 3:1 (0.750:0.250 ng) and 7:1 (0.875:0.125 ng). Generally, 15:1 ratios resulted in partial profiles for the minor component. The profiles of these samples are described in Table 11 on page 109.

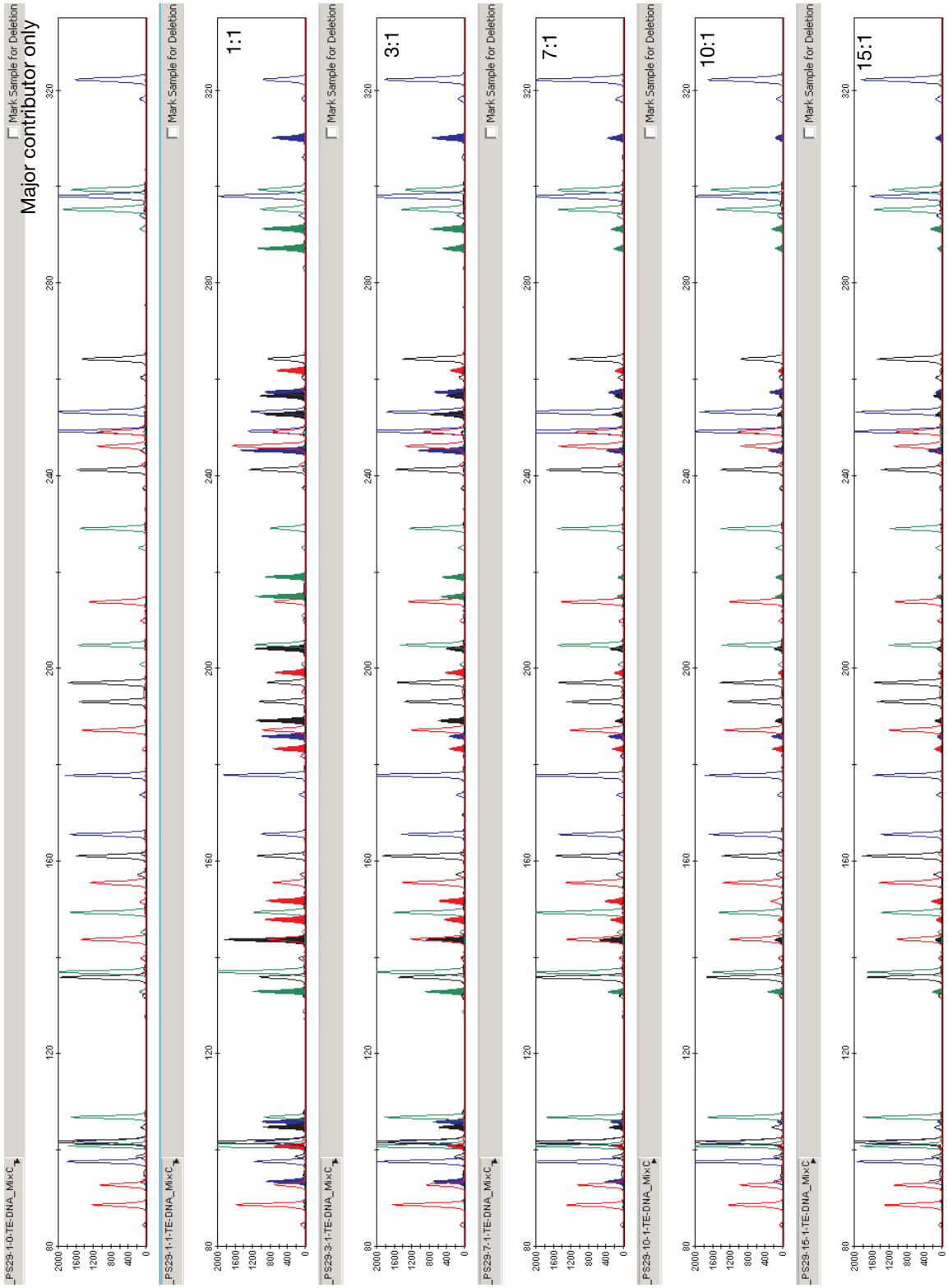


Figure 36 Amplification of DNA mixtures at various ratios. Panels show electropherograms for (top to bottom): Major contributor only, 1:1 mixture (maj:min), 3:1 mixture, 7:1 mixture, 10:1 mixture and 15:1 mixture.

Table 11 Genotypes of mixed DNA samples

Locus	Sample A Genotype	Sample B Genotype
D10S1248	13, 14	12, 15
vWA	14, 17	17, 19
D16S539	10, 11	9, 12
D2S1338	17, 23	17, 20
AMEL	X, Y	X
DS1179	11,14	10,11
D21S11	29, 35	31.2, 32.2
D18S51	15, 16	13, 14
D22S10	45, 15	15, 16
D19S433	11, 17.2	13
TH01	7, 8	6, 9.3
FGA	19, 25	22, 23
D2S441	11, 12	11, 14
D3S1358	14, 17	15, 16
D1S1656	12, 18.3	11, 15
D12S391	18, 18.3	18, 22

Population data

SWGDAM guideline 2.7

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

Overview

The AmpFℓSTR® NGM™ PCR Amplification Kit contains loci for which extensive population data are available. For additional information on 11 loci shared between the kits, see the population data and additional studies section of the *AmpFℓSTR SGM Plus® PCR Amplification Kit User's Manual* (PN 44309589).

Population samples used in these studies

The AmpFℓSTR® NGM™ PCR Amplification Kit was used to generate the population data provided in this section. Whole blood samples, provided by the Insterstate Blood Bank (Memphis, Tennessee), were collected in the United States (with no geographical preference) from randomly selected individuals of known ethnicities. Ethnicities of sample donors were:

- African-American – 350 samples
- Caucasian – 350 samples
- Hispanic – 296 samples

DNA was extracted using an ABI PRISM® 6100 Nucleic Acid PrepStation.

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

Mutation rate

Estimating germ-line mutations

Estimation of spontaneous or induced germ-line mutation at genetic loci can be achieved by comparing the genotypes of offspring to those of their parents. From such comparisons the number of observed mutations are counted directly.

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

Additional mutation studies

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

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

Probability of identity

Table 12 shows the Probability of Identity (PI) values of the AmpFℓSTR® NGM™ Kit loci individually and combined.

Table 12 Probability of identity values for the AmpFℓSTR® NGM™ Kit STR loci

Locus	African-American (N=350)	Caucasian (N=350)	Hispanic (N=293)
D10S1248	0.070	0.095	0.105
vWA	0.062	0.066	0.087
D16S539	0.072	0.104	0.080
D2S1338	0.023	0.032	0.031
D8S1179	0.075	0.064	0.067
D21S11	0.045	0.053	0.051
D18S51	0.031	0.031	0.028
D22S1045	0.054	0.132	0.149
D19S433	0.040	0.085	0.052
TH01	0.095	0.080	0.083
FGA	0.033	0.039	0.030
D2S441	0.101	0.097	0.099
D3S1358	0.100	0.075	0.087
D1S1656	0.034	0.022	0.025
D12S391	0.039	0.023	0.029
D10S1248	0.070	0.095	0.105
Combined	6.52×10^{-20}	2.78×10^{-19}	2.59×10^{-19}

The P_I value is the probability that two individuals selected at random will have an identical AmpFℓSTR® NGM™ Kit genotype (Sensabaugh, 1982). The P_I values for the populations described in this section are then approximately $1/1.53 \times 10^{19}$ (African-American), $1/3.60 \times 10^{18}$ (U.S. Caucasian), and $1/3.86 \times 10^{18}$ (U.S. Hispanic).

Probability of paternity exclusion

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

Table 13 Probability of Paternity Exclusion values for the AmpFℓSTR® NGM™ PCR Amplification Kit® STR loci

Locus	African-American (N=350)	Caucasian (N=350)	Hispanic (N=293)
D10S1248	0.659	0.572	0.488
vWA	0.599	0.652	0.622
D16S539	0.557	0.582	0.523
D2S1338	0.795	0.748	0.749
D8S1179	0.588	0.614	0.553
D21S11	0.726	0.731	0.681
D18S51	0.784	0.754	0.777
D22S1045	0.670	0.454	0.417
D19S433	0.637	0.506	0.641
TH01	0.512	0.506	0.565
FGA	0.726	0.663	0.681
D2S441	0.522	0.492	0.494
D3S1358	0.483	0.531	0.477
D1S1656	0.743	0.807	0.708
D12S391	0.738	0.812	0.668
Combined	0.9999999193	0.9999998447	0.9999994515

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

Troubleshooting

A

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

Observation	Possible causes	Recommended actions
Faint or no signal from both the 007 and the DNA test samples at all loci	Incorrect volume or absence of either AmpFℓSTR® NGM™ Master Mix or AmpFℓSTR® NGM™ Primer Set	Repeat amplification using correct reagent volumes.
	No activation of enzyme	Repeat amplification, making sure to hold reactions initially at 95 °C for 11 min.
	Master Mix not vortexed thoroughly before aliquoting	Vortex Master Mix thoroughly.
	AmpFℓSTR NGM™ Primer Set exposed to too much light	Store Primer Set protected from light.
	GeneAmp® PCR System malfunction	Refer to the thermal cycler user's manual and check instrument calibration.
	Incorrect thermal cycler parameters	Check the protocol for correct thermal cycler parameters.
	Tubes/plate not seated tightly in the thermal cycler during amplification	Push reaction tubes/plate firmly into contact with block after first cycle. Repeat test.
	Wrong PCR reaction tubes or plate	Use Applied Biosystems MicroAmp Reaction. Tubes with Caps or the MicroAmp Optical 96-Well Reaction Plate for the GeneAmp® PCR System 9700.
	MicroAmp™ Base used with tray/retainer set and tubes in GeneAmp® PCR System 9700	Remove MicroAmp Base from tray/retainer set and repeat test.
	Insufficient PCR product electrokinetically injected	**For ABI PRISM® 3100-*Avant* or Applied Biosystems 3100/3130*xl* runs:** Mix 1.0 µL of PCR product and 9 µL of Hi-Di™ Formamide/ GeneScan™ 500 LIZ® solution. **For ABI PRISM® 310 instrument runs:** Mix 0.75 µL of PCR product and 24.24 µL of Hi-Di™ Formamide/GeneScan™ 500 LIZ® solution.
	Degraded formamide	Check the storage of formamide; do not thaw and refreeze multiple times. Try Hi-Di™ Formamide.

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	Quantify DNA and add 1.0 ng of DNA. Repeat test.
	Test sample contains high concentration of PCR inhibitor (for example, heme compounds, certain dyes	Quantify 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 two alleles present at a locus	Presence of exogenous DNA	Use appropriate techniques to avoid introducing foreign DNA during laboratory handling.
	Amplification of stutter product	Interpret according to laboratory procedures. **Note:** Additional information will be provided on completion of validation.
	Mixed sample	
	Incomplete 3´ A base addition (n-1 nt position)	Addition of excess DNA to the reaction will contribute to the occurrence of incomplete 3' base addition. Quantify DNA and add 1.0 ng of DNA to the reaction. Repeat test. Also be sure to include the final extension step of 60 °C for 10 min in the PCR.
	Signal exceeds dynamic range of instrument (off-scale data)	Ensure cycle number is optimized according to instructions on page 20. Repeat PCR amplification using fewer PCR cycles or use your laboratory's SOP to analyze off-scale data.
	Poor spectral separation (bad matrix)	Follow the steps for creating a spectral file.
		Confirm that Filter Set G5 modules are installed and used for analysis.
	Too much DNA in reaction	Use recommended amount of template DNA (1.0 ng).
	Incomplete denaturation of double stranded DNA	Use the recommended amount of Hi-Di™ Formamide and perform heat denaturation according to instructions on page 30.
Poor peak height balance	Incorrect thermal cycler parameters	Check the protocol for correct thermal cycler parameters.
	GeneAmp® PCR System 9700 with Aluminum 96-Well block or third-party thermal cyclers	Use Applied Biosystems GeneAmp® PCR System 9700 with silver or gold-plated silver blocks only.

Safety

B

This appendix covers:

Chemical safety

Chemical hazard warning

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

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

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

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See “About MSDSs” on page 120.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer’s cleanup procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

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

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

Obtaining MSDSs

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

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **MSDS**.
2. In the Keyword Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

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

Chemical waste safety

Chemical waste hazards

WARNING! HAZARDOUS WASTE. Refer to Material Safety Data Sheets and local regulations for handling and disposal.

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

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

Chemical waste safety guidelines

To minimize the hazards of chemical waste:

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

Waste disposal

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

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.

- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

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

Biological hazard safety

General biohazard

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

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

Additional information about biohazard guidelines is available at:

www.cdc.gov

Chemical alerts

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

General alerts for all chemicals

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

Specific chemical alerts

CAUTION! CHEMICAL HAZARD. AmpFℓSTR® NGM™ PCR Amplification Kit may cause eye, skin, and respiratory tract irritation. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

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

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

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

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

WARNING! CHEMICAL HAZARD. POP-7™ Polymer for the 3730 Genetic Analyzer is harmful by inhalation and if swallowed and irritating to eyes, respiratory system, and skin.

Documentation

Related documentation

For additional documentation, see "How to obtain support" on page 128.

Document title	Part number
ABI PRISM® 3100/3100-Avant Data Collection v2.0 User Guide	4347102
ABI PRISM® 3100/3100-Avant Genetic Analyzers Using Data Collection Software v2.0 User Bulletin	4350218
ABI PRISM® 3100 Genetic Analyzer User Manual (Data Collection v1.1)	4315834
ABI PRISM® 3100/3100-Avant Genetic Analyzers Protocols for Processing AmpFℓSTR® PCR Amplification Kit PCR Products User Bulletin	4332345
AmpFℓSTR® PCR Amplification Kit - PCR Setup Quick Reference Card	4442401
AmpFℓSTR® PCR Amplification Kit - CE Quick Reference Card	4442693
Applied Biosystems 3130/3100xl Genetic Analyzers Using Data Collection Software v3.0 User Bulletin	4363787
Applied Biosystems 3130/3130xl Genetic Analyzers Getting Started Guide	4352715
Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide	4352716
Applied Biosystems 3130/3130xl Genetic Analyzers Quick Reference Card	4362825
Applied Biosystems 3130/3130xl Genetic Analyzers AB Navigator Software Administrator Guide	4359472
Applied Biosystems 3130/3100xl DNA Analyzers User Guide	4331468
Applied Biosystems 3730/3730xl Genetic Analyzer Getting Started Guide	4359476
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
GeneMapper® ID-X Software Version 1.1 (Mixture Analysis) Quick Reference Guide	4402094

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

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

How to obtain support

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

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

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

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Index

Symbols

A

B

C

D

E

F

G

H

I

K

L

M

N

O

P

Q

R

S

T

U

V

W

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