AmpF*l*STR® SEfiler™

PCR Amplification Kit

User's Manual

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AmpFℓSTR SEfiler PCR Amplification Kit User's Manual

Preface

About This Manual

The *AmpFℓSTR® SEfiler™ PCR Amplification Kit User's Manual* can be used as a reference to operate the Applied Biosystems instruments, chemistries, associated software, and procedures detailed in this user's manual. Refer to the specific chapters for information on related software, chemistries and their use.

Audience

This manual is intended for users of protocols for processing AmpFℓSTR® PCR Amplification Kit PCR products with ABI PRISM® instruments and software. Some examples of instruments and software are listed below:

- ABI PRISM® 310 Genetic Analyzer for both the Macintosh® and Microsoft® Windows NT® operating systems
- ABI PRISM® 377 DNA Sequencer for Windows NT operating system
- ABI PRISM® 377 DNA Sequencer with XL Upgrade (ABI PRISM 377XL) for Windows NT operating system
- ABI PRISM® 377 DNA Sequencer with 96-Lane Upgrade (377-96 instrument) for Windows NT operating system
- ABI PRISM® 3100 Genetic Analyzer, using ABI PRISM® 3100 Data Collection Software Version 1.1
- ABI PRISM® GeneScan® Analysis Software for the Windows NT operating system

Related Documentation

The following related documents are shipped with the system:

- *AmpFℓSTR® SEfiler PCR Amplification Kit User's Manual*
- AmpFℓSTR® SEfiler Kit Template 8 CD

Typefaces and Symbols

The following table describes the typefaces that are used to indicate selections on windows and the meaning of text symbols in this user's manual.

Typeface Conventions

Typeface or Symbol	Meaning	Example
Menu item, **icon**, or **button**	Menu items, icons, or buttons on the windows that activate other processes are shown in bold when you are instructed to select them.	Click **Execute**.
Italics	Names of manuals or documents are shown in italics.	*AmpFlSTR® SEfiler PCR Amplification Kit User's Manual*
>	This symbol represents the menu and next item to select on a submenu.	Select **File** > **New** to open a new document.
Shortcuts	Keyboard shortcuts for window operations are shown in two formats: 1. Commas between commands. When commands are shown in this format, press each key sequentially without holding them down. 2. Plus sign (+) shown between commands. When commands are shown in this format, press and hold the keys together.	Alt, W, T Press **Alt**, then press **W**, then **T** to tile the open windows. Ctrl+N Press and hold **Ctrl** and **N** together to open the New Document dialog box.

Obtaining Technical Support

Applied Biosystems Web Site

A services and support page is available on the Applied Biosystems Web site. To access this, go to:

http://www.appliedbiosystems.com

and click the link for services and support.

At the services and support page, you can:

• 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

In addition, the services and support page provides worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Send Us Your Comments

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

techpubs@appliedbiosystems.com

Safety and EMC Compliance Information

General Safety

Documentation User Attention Words

Five user attention words appear in the text of all Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below.

Note: Calls attention to useful information.

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 which, 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 which, if not avoided, could result in death or serious injury.

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

Site Preparation and Safety Guide

A site preparation and safety guide is a separate document sent to all customers who have purchased an Applied Biosystems instrument. Refer to the guide written for your instrument for information on site preparation, instrument safety, chemical safety, and waste profiles.

Chemical Safety

Chemical Hazard Warning

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

- 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.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* 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 (*e.g.*, 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 on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical Waste Hazard Warning

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

- 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.
- Handle chemical wastes in a fume hood.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* 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 (*e.g.*, fume hood). For additional safety guidelines, consult the MSDS.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

General Biohazard

⚠️ **WARNING** **BIOHAZARD**. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Read and follow the guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (http://bmbl.od.nih.gov) and in the Occupational Safety and Health Standards, Bloodborne pathogens (http://www.access.gpo.gov/nara/cfr/ waisidx_01/ 29cfr1910a_01.htm). Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves.

Electric Shock

⚠️ **WARNING** **ELECTRICAL SHOCK HAZARD**. To reduce the chance of electrical shock, do not remove covers that require tool access. No user serviceable parts are inside. Refer servicing to Applied Biosystems qualified service personnel.

Laser Exposure

⚠️ **WARNING** **LASER HAZARD**. Exposure to direct or reflected laser light can burn the retina and leave permanent blind spots. Never look directly into the laser beam. Remove jewelry and other items that can reflect the beam into your eyes. Protect others from exposure to the beam.

About MSDSs

Some of the chemicals used with this instrument may be listed as hazardous by their manufacturer. When hazards exist, warnings are prominently displayed on the labels of all chemicals.

Chemical manufacturers supply a current MSDS before or with shipments of hazardous chemicals to new customers and with the first shipment of a hazardous chemical after an MSDS update. MSDSs provide you with the safety information you need to store, handle, transport and dispose of the chemicals safely.

We strongly recommend that you replace the appropriate MSDS in your files each time you receive a new MSDS packaged with a hazardous chemical.

⚠ **WARNING** **CHEMICAL HAZARD**. Be sure to familiarize yourself with the MSDSs before using reagents or solvents.

Ordering MSDSs You can order free additional copies of MSDSs for chemicals manufactured or distributed by Applied Biosystems using the contact information below.

To obtain documents through the Applied Biosystems Web site:

1.	Go to **http://docs.appliedbiosystems.com/msdssearch.html**
2.	In the **SEARCH** field, type in the chemical name, part number, or other information that will appear in the MSDS and click **SEARCH**. **Note:** You may also select the language of your choice from the drop-down list.
3.	When the **Search Results** page opens, find the document you want and click on it to open a PDF of the document.

For chemicals not manufactured or distributed by Applied Biosystems, call the chemical manufacturer.

Introduction

1

In This Chapter   This chapter describes the contents of the AmpFℓSTR® SEfiler™ PCR Amplification Kit, and provides an overview of the kit.

About This User's Manual   This user's manual provides users of the AmpFℓSTR SEfiler PCR Amplification Kit with protocols and summaries of experiments performed by Applied Biosystems. Applied Biosystems recommends that users conduct similar experiments in their laboratory to evaluate the DNA typing system consisting of the AmpFℓSTR SEfiler PCR Amplification Kit, reagents, software, and ABI PRISM instrument(s). Applied Biosystems suggests that users apply the standards established by the community for which this test will be used to further evaluate this DNA typing system.

This user's manual describes:

- Materials and equipment required to use the AmpFℓSTR SEfiler kit
- How to use the kit to amplify DNA samples
- How to perform automated detection
- How to analyze results

Product Overview

Purpose   The AmpF*l*STR SEfiler PCR Amplification Kit is a short tandem repeat (STR) multiplex assay that amplifies 11 tetranucleotide repeat loci, including the SE33 locus. The kit simultaneously coamplifies the seven ENFSI loci, the highly polymorphic SE33 (ACTBP2) locus, the Amelogenin locus, and the D2S1338 and D19S433 loci.

Five-Dye DNA Fragment Analysis   The SEfiler kit uses a five-dye fluorescent system for automated DNA fragment analysis. Applied Biosystems PET™ and LIZ® dyes expand the spectral detection range that can be used on ABI PRISM® instruments. Together with 6-FAM™, VIC®, and NED™ dyes, the spectral emission for this five-dye set extends to 660 nm.

About the Primers   The AmpF*l*STR SEfiler kit employs the same primer sequences as used in all previous AmpF*l*STR kits. Also included are primers for the SE33 locus.

Multicomponent Analysis Overview

About Multicomponent Analysis

Multicomponent analysis is the process that separates the five different fluorescent dye colors into distinct spectral components. The four dyes used in the AmpF*l*STR SEfiler 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 Size Standard.

How Multicomponent Analysis Works

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

Figure 1-1 shows emission spectra of the five dyes used in the AmpF*l*STR SEfiler PCR Amplification Kit.

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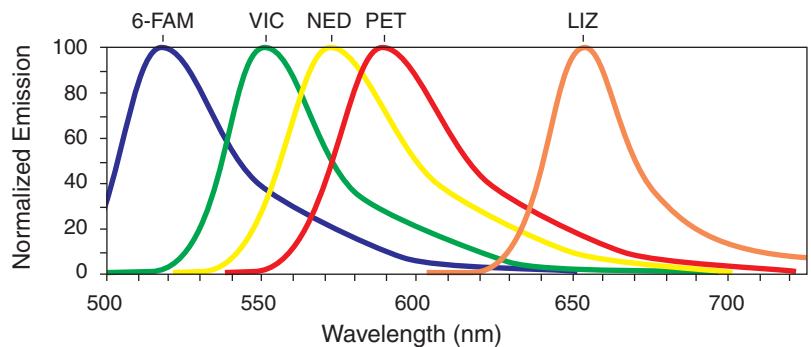
Figure 1-1   Emission spectra of the five dyes

Allelic Ladder and Control DNA Information

Table 1-1 shows the loci amplified and the corresponding dyes used. The AmpFℓSTR SEfiler Allelic Ladder is used to genotype the analyzed samples. The alleles contained in the allelic ladder and the genotype of the AmpFℓSTR Control DNA 007 are listed in the table.

Table 1-1   AmpFℓSTR SEfiler Kit Loci and Alleles

Locus Designation	Chromosome Location	Dye Label	AmpFℓSTR Allelic Ladder Alleles	AmpFℓSTR Control DNA 007 Genotype
D2S1338	2q35–37.1	6-FAM	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28	20, 23
D3S1358	3p	6-FAM	12, 13, 14, 15, 16, 17, 18, 19	15, 16
D8S1179[a]	8	VIC	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	12, 13
D16S539	16q24-qter	6-FAM	5, 8, 9, 10, 11, 12, 13, 14, 15	9, 10
D18S51	18q21.3	PET	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27	12, 15
D19S433	19q12–13.1	NED	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2	14, 15

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

Table 1-1   AmpF𝑙STR SEfiler Kit Loci and Alleles *(continued)*

Locus Designation	Chromosome Location	Dye Label	AmpF𝑙STR Allelic Ladder Alleles	AmpF𝑙STR Control DNA 007 Genotype
D21S11	21q11.2–q21	PET	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38	28, 31
FGA	4q28	NED	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2	24, 26
SE33	6	VIC	4.2, 6.3, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 20.2, 21, 21.1, 21.2, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, 35, 35.2, 36, 37	17, 25.2
TH01	11p15.5	NED	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3	7, 9.3

Table 1-1   AmpF*l*STR SEfiler Kit Loci and Alleles *(continued)*

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

Materials for the Kit

Kit Contents The AmpF*l*STR SEfiler kit (P/N 4335129) contains sufficient quantities of the following reagents to perform 100 50-μL amplifications:

Table 1-2   Reagents

Component	Description	Volume
AmpF*l*STR PCR Reaction Mix	Two tubes containing $MgCl_2$, deoxynucleotide triphosphates, and bovine serum albumin in buffer with 0.05% sodium azide	1.1 mL/tube
AmpF*l*STR SEfiler Primer Set	One tube containing fluorescently labeled primers and non-labeled primers	1.1 mL
AmpliTaq Gold® DNA Polymerase	Two tubes of enzyme with an activity of 5 U/μL	50 μL/tube
AmpF*l*STR Control DNA 007	One tube containing 0.10 ng/μL human male genomic DNA in 0.05% sodium azide and buffer (refer to "Allelic Ladder and Control DNA Information" on page 1-4 for profile)	0.3 mL
AmpF*l*STR SEfiler Allelic Ladder	One tube of AmpF*l*STR SEfiler Allelic Ladder containing amplified alleles. See the table under "Allelic Ladder and Control DNA Information" on pages 1-4 through 1-6 for a list of alleles included in the allelic ladder.	50 μL
Mineral Oil	One dropper bottle	5 mL

Kit Storage and Stability

The table below lists the storage temperature for the kit components.

IMPORTANT! The fluorescent dyes attached to the primers are light-sensitive. Protect the AmpF*l*STR SEfiler Primer Set from light when not in use. Amplified DNA, AmpF*l*STR SEfiler Allelic Ladder and GeneScan-500 LIZ Size Standard should also be protected from light.

Table 1-3   Storage Temperatures

Component	Storage Temperature
AmpF*l*STR PCR Reaction Mix	2 to 8 °C
AmpF*l*STR SEfiler Primer Set	
AmpF*l*STR Control DNA 9947A	
AmpF*l*STR SEfiler Allelic Ladder	
AmpliTaq Gold DNA Polymerase	–15 to –25 °C

PCR Amplification

2

In This Chapter   This chapter describes how to prepare the master mix for amplifying sample DNA using the AmpF*l*STR® SEfiler™ PCR Amplification Kit, how to prepare samples and controls, and how to perform PCR.

PCR Work Areas

Setup Work Area

IMPORTANT! These items should never leave the PCR Setup Work Area. For more information on implementing PCR technology, refer to Appendix B, "Laboratory Setup."

- 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
- Pipet tips, sterile, disposable hydrophobic filter-plugged
- Pipettors
- Tube decapper, autoclavable
- Vortex

Amplified DNA Work Area

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

- GeneAmp PCR System 9700, or
  GeneAmp PCR System 9600
- GeneAmp PCR System 2400
- DNA Thermal Cycler 480

PCR Equipment and Materials

Equipment and Materials Required but not Supplied

The tables below list the equipment and materials required in addition to the reagents supplied with the AmpF*l*STR SEfiler kit for PCR amplification. Refer to GeneAmp PCR Instrument Systems and Accessories on page 2-4.

Required Equipment:

Equipment	Source
GeneAmp PCR System 9700, or GeneAmp PCR System 9600	Applied Biosystems (P/N N805-0001)
Microcentrifuge	Major laboratory supplier (MLS)
Pipettors	MLS
Vortex	MLS

Required Materials:

Materials	Source
MicroAmp® 96-Well Trays for Tubes with Caps	Applied Biosystems (P/N N801-0541)
MicroAmp® Reaction Tubes with Caps, 0.2-mL	Applied Biosystems (P/N N801-0540)
MicroAmp® Reaction Tubes (8 tubes/strip)	Applied Biosystems (P/N N801-0580)
MicroAmp® Caps (8 caps/strip)	Applied Biosystems (P/N N801-0535)
MicroAmp® 96-Well Tray/Retainer Set	Applied Biosystems (P/N 403081)
MicroAmp® 96-Well Base	Applied Biosystems (P/N N801-0531)
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems (P/N N801-0560)
Microcentrifuge tubes, 1.5-mL	MLS
Microcentrifuge tubes, 2.0-mL	MLS

Required Materials: *(continued)*

Materials	Source
Pipet tips, sterile, disposable hydrophobic filter-plugged	MLS
Tape, labeling	MLS
Tube, 50-mL Falcon	MLS
Tube decapper, autoclavable	MLS
Deionized water, PCR grade	MLS
Tris-HCL, pH 8.0	MLS
0.5-M EDTA	MLS

GeneAmp PCR Instrument Systems and Accessories

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

- DNA Thermal Cycler 480 (P/N N801-0100, N801-0101, N801-0102)
- DNA Thermal Cycler 480 Accessories:
  - GeneAmp Autoclaved Thin-Walled Reaction Tubes (P/N N801-0611)
  - GeneAmp Thin-Walled Reaction Tubes (P/N N801-0537)
  - Temperature Verification System (P/N N801-0434)
- GeneAmp PCR System 2400 (P/N N803-0001, N803-0002, N803-0003)
- GeneAmp PCR System 2400 Accessories:
  - MicroAmp® Autoclaved Reaction Tube with Caps (P/N N801-0612)
  - Bulkpack MicroAmp Reaction Tube with Caps (P/N N801-1540)
  - MicroAmp 24-Well Bases (P/N N801-5531)
  - MicroAmp 24-Well Tray (P/N N801-5532)
  - Temperature Verification System   (P/N N801-0435)

Preparing the Reagents

TE Buffer The final concentration of TE buffer is 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0.

To prepare TE buffer:

1.	Mix together: • 10 mL of 1 M Tris-HCl, pH 8.0 • 0.2 mL of 0.5 M EDTA • 990 mL glass-distilled or deionized water ⚠️ **CAUTION** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves. **Note:** Adjust the volumes accordingly for specific needs.
2.	Aliquot and autoclave the solutions.
3.	Store at room temperature.

Master Mix Prepare the master mix by combining AmpF*l*STR® PCR Reaction Mix, AmpliTaq Gold® DNA Polymerase, and AmpF*l*STR SEfiler Primer Set reagents.

IMPORTANT! The fluorescent dyes attached to the primers are light-sensitive. Protect the AmpF*l*STR SEfiler Primer Set from light when not in use. Also protect the AmpF*l*STR SEfiler Allelic Ladder, GeneScan®-500 LIZ® Size Standard and amplified, fluorescently-labeled PCR products from light.

To prepare the master mix:

1.	Determine the total number of samples, including controls.
2.	**IMPORTANT!** Vortex the following reagents at medium speed for 5 seconds: • AmpF*l*STR PCR Reaction Mix • AmpliTaq Gold DNA Polymerase • AmpF*l*STR SEfiler Primer Set ⚠ **CAUTION** **CHEMICAL HAZARD**. **AmpliTaq Gold DNA Polymerase** may cause eye and skin irritation. It may cause discomfort if swallowed or inhaled. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves.
3.	Spin the tubes briefly in a microcentrifuge to remove any liquid from the caps.
4.	Select a clean, unused tube for the master mix. | If you are preparing...	Then use a...
≤ 84 samples and controls	1.5-mL microcentrifuge tube
85–110 samples and controls	2.0-mL microcentrifuge tube
> 110 samples and controls	tube that is appropriate	
5.	Calculate the required amount of components as shown: **Note:** The formulation in the list below provides a slight overfill to allow for volume lost in pipetting. • Number of samples x 21 µL of AmpF*l*STR PCR Reaction Mix • Number of samples x 1.0 µL of AmpliTaq Gold DNA Polymerase • Number of samples x 11 µL of AmpF*l*STR SEfiler Primer Set

To prepare the master mix: *(continued)*

6.	Vortex the master mix at medium speed for 5 sec.
7.	Spin briefly in a microcentrifuge to remove any residue from the caps.
8.	Dispense 30 µL of master mix per PCR tube.

Preparing the DNA Samples

DNA Sample Input

DNA amplification with the AmpF*l*STR® SEfiler™ kit requires 20 µL of DNA at a recommended concentration of 1.0–2.5 ng/µL.

Preparing the Samples

Note: The final volume in each PCR tube is 50 µL.

If you are preparing the...	Then...
DNA test sample tube and the sample DNA concentration is ≤ 0.125 ng/µL	add 20 µL of sample to the PCR tube.
DNA test sample tube and the sample DNA concentration is > 0.125 ng/µL	dilute a portion of the sample with TE buffer (see Appendix , "TE Buffer,"page 2-5 for preparation) so that only 1.0–2.5 ng of total DNA is in a volume of 20 µL (final sample concentration is 0.05–0.125 ng/µL).
Positive Control Tube	1. Vortex the AmpF*l*STR® Control DNA 007 tube (0.10 ng/µL). 2. Spin the tube briefly in a microcentrifuge to remove any liquid from the cap. 3. Add 20 µL (2 ng) of AmpF*l*STR Control DNA 007 to the Positive Control Tube.
Negative Control Tube	add 20 µL of TE buffer to the labeled Negative Control Tube.

Performing PCR

Thermal Cyclers   Use any of the following thermal cyclers to amplify loci using the AmpF*l*STR SEfiler kit:

- GeneAmp® PCR System 9700
- GeneAmp® PCR System 9600
- GeneAmp® PCR System 2400
- DNA Thermal Cycler 480

Amplifying the DNA   **To amplify the DNA:**

1. Program the thermal cycling conditions. The thermal cycling parameters for GeneAmp thermal cyclers and DNA Thermal Cycler 480 are the same.

   IMPORTANT! If using the GeneAmp PCR System 9700, select the 9600 Emulation Mode.

   IMPORTANT! If using DNA Thermal Cycler 480, add one drop of mineral oil to the GeneAmp tubes.

Initial Incubation Step	Denature	Anneal	Extend	Final Extension	Final Step
HOLD	CYCLE (28 cycles)			HOLD	HOLD
95 °C 11 min	94 °C 1 min	59 °C 1 min	72 °C 1 min	60 °C 45 min	4–25 °C (forever)

   Note: If leaving the amplified products in the thermal cycler for more than 18 hr., set the final step to HOLD at 4–25 °C forever. The final step can be held anywhere in this range. Each laboratory should determine the final time and temperature to store PCR products in the thermal cycler.

To amplify the DNA: *(continued)*

2.	Place the tray in the thermal cycler. ⚠ **WARNING** **PHYSICAL INJURY HAZARD.** During instrument operation, the temperature of the heated cover can be as high as 108 °C, and the temperature of the sample block can be as high as 100 °C. Keep hands away from the heated cover and sample block.
3.	Close the heated cover. ⚠ **WARNING** **PHYSICAL INJURY HAZARD.** During instrument operation, the temperature of the heated cover can be as high as 108 °C, and the temperature of the sample block can be as high as 100 °C. Before performing the procedure, keep hands away until the heated cover and sample block reach room temperature.
4.	Start the thermal cycler.
5.	Remove the tubes from the instrument block after the PCR is completed.
6.	Store the amplified DNA. <table><tr><td>**If you are storing the DNA...**</td><td>**Then place at...**</td></tr><tr><td><2 weeks</td><td>2 to 6 °C.</td></tr><tr><td>>2 weeks</td><td>−15 to −25 °C.</td></tr></table> **IMPORTANT!** Protect the amplified products from light.

Amplification Using Bloodstained FTA Cards

FTA™-treated DNA collection cards can be useful for the collection, storage, and processing of biological samples. A small punch of the bloodstained card can be placed directly into an amplification tube, purified, and amplified without transferring the evidence. Our studies have indicated that a 1.2-mm bloodstained punch contains approximately 5–20 ng DNA. Accordingly, an appropriate cycle number for this high quantity of DNA is 25 cycles. It is recommended that each laboratory determine the cycle number based on individual validation studies.

In the example shown in Figure 2-1, a 1.2-mm punch of a bloodstained FTA card was purified using one wash with FTA Purification Reagent and two washes with 1X TE buffer. After drying at room temperature overnight, the punch was amplified directly in the MicroAmp® tube for 25 cycles.
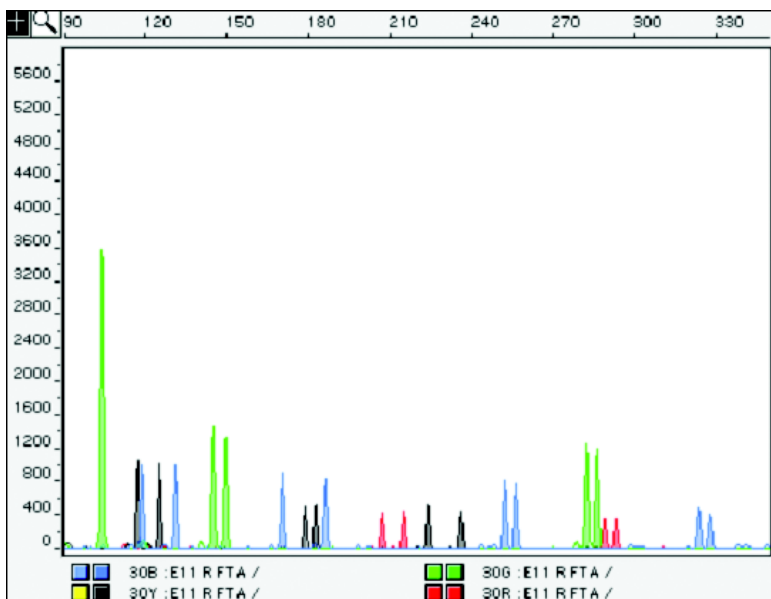
Figure 2-1   AmpF*l*STR SEfiler kit results from a 1.2-mm FTA bloodstain punch (25 cycle amplification), analyzed on the ABI PRISM 310 Genetic Analyzer.

Setting up the ABI PRISM 310 Genetic Analyzer

3

In This Chapter   This chapter is a general overview of the ABI PRISM® 310 Genetic Analyzer. Procedures for setting up the system for both the Macintosh® and Microsoft® Windows NT® operating systems are described in this chapter.

Overview

This chapter provides the steps necessary to install a new electrode on the ABI PRISM 310 Genetic Analyzer including:

- Installing a new electrode
- Cleaning and reloading the syringe
- Removing and priming the pump
- Installing the capillary
- Filling the buffer reservoirs

ABI PRISM 310 Genetic Analyzer

Figure 3-1 shows the ABI PRISM 310 Genetic Analyzer. The parts mentioned in this section are labeled. Refer to the *ABI PRISM® 310 Genetic Analyzer User Guide* (P/N 4317588) and *GeneScan® Analysis Software Version 3.1 User's Manual* (P/N 4306157) for more detailed information on the instrument and software used with this protocol.
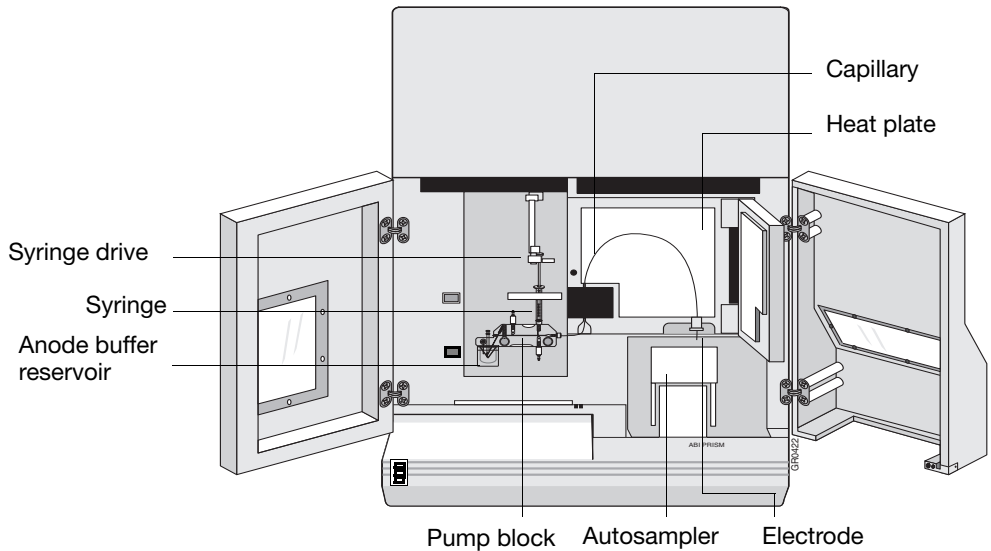
Figure 3-1   ABI PRISM 310 Genetic Analyzer

Setting up the Genetic Analyzer

Installing a New Electrode

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

IMPORTANT! A new electrode must be trimmed to the correct length. Refer to Figure 3-2 for information on trimming the electrode.

Note: Not all electrodes need to be trimmed. Trim only as needed.

To install a new electrode:

1.	Install the new electrode on the instrument as described in the *ABI PRISM® 310 Genetic Analyzer User Guide*.
2.	Select **Manual Control** > **Home Z-Axis**.
3.	Follow these guidelines when using the wire cutter: a. Use the flush-cutting wire cutter (P/N T-6157) provided in the instrument packing kit. b. Hold the cutters with the flat cutting face toward the top of the instrument.

To install a new electrode: *(continued)*

4.	Cut a small amount off the end of the electrode until it is flush with the lower surface of the stripper plate.
	Be careful not to flex the stripper plate upwards while cutting. Do not cut off more than 1mm beyond the lower surface of the stripper plate (Figure 3-2).
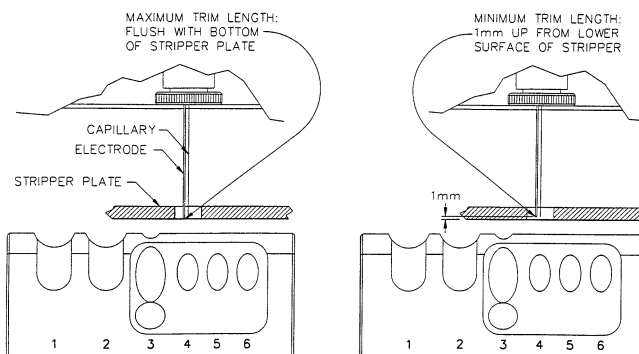
Figure 3-2   Trimming the electrode

Cleaning the Electrode

To clean the electrode:

1.	Wipe the electrode with a lint-free tissue that has been dampened with distilled, deionized water.
2.	Dry the electrode with a fresh lint-free tissue.
	Note: The autosampler should be recalibrated after cleaning the electrode, as described in "Calibrating the Autosampler" of the *ABI PRISM® 310 Genetic Analyzer User Guide*.

Removing the Syringe

To remove the syringe:

1.	Launch the ABI PRISM 310 Data Collection Software.
2.	a. Select **Window** > **Manual Control**. b. Select **Function > Syringe Home**. c. Click **Execute**. **Note:** For all commands in the Manual Control window, the Execute button must be selected to complete the task.
3.	Open the instrument doors and move the syringe drive toggle to the left.
4.	Unscrew the syringe from the pump block.

Checking the Syringe

Verify that the 1.0-mL glass syringe (P/N 4304471) has a small O-ring (P/N 221102) inside the syringe, and that another O-ring is placed around the ferrule-shaped seal. The ferrule should be firmly seated in the end of the 1.0-mL syringe. If the syringe is dirty, clean it before using.

Cleaning the Syringe

To clean the syringe:

1.	Remove the plunger by slowly drawing it from the glass barrel (count to 5, this should take approximately 5 sec.) while keeping the entire syringe submerged in water.
	IMPORTANT! Moving the dry plunger quickly can damage it, resulting in premature failure or leakage around the plunger.
2.	Remove the ferrule from the syringe.
	a. Soak the ferrule in warm (not boiling) water for as long as it takes to remove crystals (if any) in the ferrule.
	b. Rinse the ferrule with deionized water.
3.	Clean the glass barrel with warm water. Dissolve any crystals.
4.	Rinse the glass barrel with distilled, deionized water.
	IMPORTANT! Remove all residual water from the syringe by blowing compressed air through it.
5.	Inspect the O-ring in the stainless steel hub of the syringe for damage, and replace it if necessary.
	IMPORTANT! Make sure the O-ring does not block the hole in the stainless steel hub.
6.	Inspect the O-ring on the ferrule and replace it if necessary.
7.	Place the ferrule back onto the syringe.
	IMPORTANT! The Teflon® tip of the plunger must be damp when you insert it into the barrel (place a drop of distilled deionized water on the Teflon tip), or the Teflon tip could be damaged.
	Note: For syringe storage, the plunger should remain in the syringe barrel.

Loading the Syringe

To load the syringe:

1.	Prime the syringe with approximately 0.1 mL of ABI PRISM® POP-4™ polymer. ⚠️ **WARNING** **CHEMICAL HAZARD. POP-4 polymer** causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	Fill the 1.0-mL syringe manually with a maximum of 0.8 mL of POP-4 polymer. **Note:** The polymer should not stay in the syringe longer than 3 days. Do not return unused polymer to the bottle. **Note:** Before use, the POP-4 polymer should be allowed to equilibrate to room temperature. If precipitate is present in the bottle when removed from cold storage, it should go back into solution at room temperature. Gently mix the polymer thoroughly by inversion before using.
3.	Wipe the outside of the syringe with a lint-free tissue to dry it.
4.	Remove any air bubbles by inverting the syringe and pushing a small amount of polymer out of the tip.

Removing and Cleaning the Pump Block

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

To remove and clean the pump block, see "Cleaning and Maintaining the Instrument" in the *ABI PRISM® 310 Genetic Analyzer User Guide*. Follow the instructions in the sections titled "Removing the Pump Block," "Rinsing the Pump Block," and "Replacing the Pump Block." We do not recommend following the section titled "Rinsing the Pump Block on the Instrument" for this application.

IMPORTANT! Remove all residual water from the pump block and fittings by blowing canned compressed air through the channels. Make sure the can is held upright or the propellant in the can may be shot into the gel block, resulting in poor resolution or high baseline.

Reinstall the pump block on the instrument after cleaning.

Installing the Syringe on the Pump Block

To install the syringe on the pump block:

1.	Move the syringe drive toggle on the instrument to the left to attach the syringe to the pump block.
2.	Place the 1.0-mL syringe through the right-hand port of the plastic syringe guide plate and screw the syringe into the pump block. The syringe should be finger-tight in the block.
3.	Hand-tighten the valves on the pump block to the left of and below the syringe. **Note:** Overtightening can cause microscopic fractures in the pump block. Undertightening may result in a "syringe leak detected" message.

Installing the Capillary

To install the capillary:

1.	Clean the capillary window with 95% ethanol on a lint-free tissue. **Note:** Do not touch the capillary window after cleaning.
2.	Install the 47-cm, 50-µm i.d. capillary (P/N 402839, green mark) as described in the *ABI PRISM® 310 Genetic Analyzer User Guide*. Follow the instructions in the section titled "Installing the Capillary." If a new capillary has been installed, select **Instrument** > **Change Capillary**.
3.	Select **OK** in the Reset window to set the injection counter to zero.
4.	Secure the capillary into place by pressing a piece of thermal tape over it onto the heat plate just above the electrode. ⚠ **WARNING PHYSICAL INJURY HAZARD**. Hot Surface. Use care when working around this area to avoid being burned by hot components. **Note:** The capillary should be approximately flush with, or less than 1 mm below, the end of the electrode.
5.	Calibrate the autosampler. Make sure it is calibrated in the X, Y, and Z directions. The capillary should almost touch the metal calibration points. Refer to "Calibrating the Autosampler" in the *ABI PRISM® 310 Genetic Analyzer User Guide*. **IMPORTANT!** The sample tray must be removed before calibrating the autosampler. If the sample tray is not removed, the electrode may bend.

Filling the Buffer Reservoirs

To fill the buffer reservoirs:

1.	Dilute 5 mL of 10X Genetic Analyzer Buffer with EDTA (P/N 402824) to 1X concentration (50 mL) with distilled, deionized water. Change to fresh buffer every 48 hours or 96 injections, whichever comes first. ⚠ **CAUTION** **CHEMICAL HAZARD. 10X Genetic Analyzer Buffer with EDTA.** May cause eye, skin and respiratory tract irritation. Please read the MSDS, and follow handling instructions. Wear appropriate protective eye wear, clothing, and gloves.
2.	Fill the anode buffer reservoir to the red line with 1X Genetic Analyzer Buffer, then secure the reservoir on the pump block.
3.	a. Fill a 4-mL glass buffer vial (P/N 401955) to the fill line with 1X Genetic Analyzer Buffer. b. Insert the plastic vial lid with attached septum (P/N 402059) into the glass vial. c. Place the buffer vial into position 1 on the autosampler. This will serve as the cathode buffer. **Note:** Overfilling and underfilling one or both buffer reservoir and vial can cause siphoning. Pay close attention to the red fill line.
4.	Fill and position a second buffer: a. Fill a second 4-mL glass buffer vial to the fill line with distilled water. b. Insert the plastic vial lid with attached septum into the glass vial. c. Place the vial into position 2 on the autosampler.
5.	Fill and position a 1.5-mL Eppendorf tube: a. Fill the tube with distilled water until it is full. b. Place it into position 3 on the autosampler. **Note:** Do not use a screw-cap tube. The lids on screw-cap tubes are too high to clear the electrode and capillary. Use a 1.5-mL Eppendorf tube with the lid clipped off.

Priming the Pump Block

To prime the pump block:

1.	Close the buffer valve: a. Select **Window** > **Manual Control**. b. Select **Buffer Valve Close** from the pop-up menu. c. Click **Execute**.
2.	Partly unscrew the capillary filling ferrule.
3.	Manually press down on the 1.0-mL syringe plunger until the ferrule space is filled with polymer. **Note:** This will remove the air bubbles at the ferrule site.
4.	Tighten the ferrule to close.
5.	Partly unscrew the waste valve on the pump block (below the syringe).
6.	Manually press down on the 1.0-mL syringe plunger until the valve space is filled with polymer. **Note:** This procedure removes the air bubbles at this valve site. It should use about 0.1 mL of polymer.
7.	Tighten the waste valve to close.
8.	To open the pin valve at the anode buffer reservoir on the pump block, a. Select **Manual Control** > **Buffer Valve Open**. b. Click **Execute**.
9.	Manually press down on the 1.0-mL syringe plunger to push enough gel through the block so that all of the air bubbles are removed from the polymer channel in the block. (This process should use about 0.2 mL of polymer.) **IMPORTANT!** There should be no air bubbles in the pump block channels.

To prime the pump block: *(continued)*

10.	In the Manual Control window: a. Close the pin valve by selecting **Buffer Valve Close** from the pop-up menu. b. Click **Execute**.
11.	Move the syringe drive toggle to the right so that it is positioned over the syringe plunger.
12.	Lower the syringe plunger: a. In the Manual Control window select **Syringe Down**. b. Select 50-step intervals. Execute until the toggle almost makes contact with the syringe plunger. c. Click **Execute**. d. Select smaller step intervals until the toggle makes contact with the syringe plunger.

Running the 310 Genetic Analyzer on Windows NT OS

4

In This Chapter

AmpF*l*STR® SEfiler™ PCR Amplification Kit products are electrophoretically separated using a capillary filled with ABI PRISM® POP-4™ polymer and detected on the ABI PRISM® 310 Genetic Analyzer. Protocols for analyzing samples on the ABI PRISM 310 Genetic Analyzer using Microsoft® Windows NT® operating system are described in this chapter.

Software Requirements

Data Collection Software for Windows NT Operating System

Before using the instrument, you must install the appropriate software and use the products shown in the table below:

Table 4-1   Products Needed

Product	Needed to
ABI PRISM® 310 Data Collection Software v3.0 (P/N 4326986)	run AmpFℓSTR SEfiler PCR Amplification Kit products and collect five-dye data
6-FAM™, VIC®, NED™, PET™, and LIZ® matrix standard set DS-33 run using the GS STR POP 4 (1 mL) G5 module	create a required matrix file
ABI PRISM® GeneScan® Analysis Software for the Windows NT operating system	—
ABI PRISM® Genotyper® Software v3.7 or higher	analyze SEfiler kit data

Analysis Software

This chapter is written for ABI PRISM® GeneScan® Analysis Software version 3.7.1 or higher.

Refer to the documents listed below for more detailed information on the instrument and software used with these protocols:

- *ABI PRISM® 310 Genetic Analyzer User Guide* (P/N 4317588)
- *ABI PRISM® GeneScan® Analysis Software Version 3.7 User Guide* (P/N 4308923)
- Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS"

Equipment and Supplies

Supplies

The equipment and supplies necessary or recommended for running AmpFℓSTR SEfiler kit data on the ABI PRISM 310 Genetic Analyzer are listed in the tables under .

Setting Up the Run for Windows NT OS

Setting the Run Temperature

Setting the run temperature prior to starting a run is optional; however, this step saves time. This heating step occurs automatically at the beginning of the GS STR POP4 (1 mL) G5 run module.

To set the run temperature:

1.	Close the instrument doors.
2.	Return to the ABI PRISM 310 Data Collection Software.
3.	Set the temperature: a. Select **Window** > **Manual Control**. b. Select **Temperature Set** from the pop-up menu. c. Set the temperature to 60 °C. d. Click **Execute**. **Note:** It takes up to 30 min for the instrument to reach the 60 °C run temperature. You can prepare samples while the instrument is heating.

Setting the Parameters

To select a five-dye sample sheet:

1.	**Note:** This is an optional step.

Set the standard color:

a. Launch the 310 Data Collection Software v3.0.

b. Select **Window** > **Preferences**, then select **GeneScan Sample Sheet Defaults**.

c. Set the size standard color to orange (**O**) as shown in the figure below.
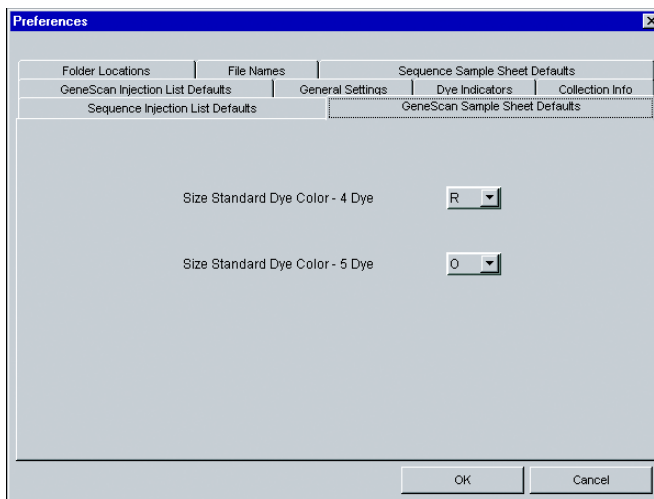
Figure 4-1   Preferences window

To select a five-dye sample sheet: *(continued)*

2.	Select **Windows** > **Preferences**, then select **GeneScan Injection List Defaults** from the drop-down menu. The following window opens.
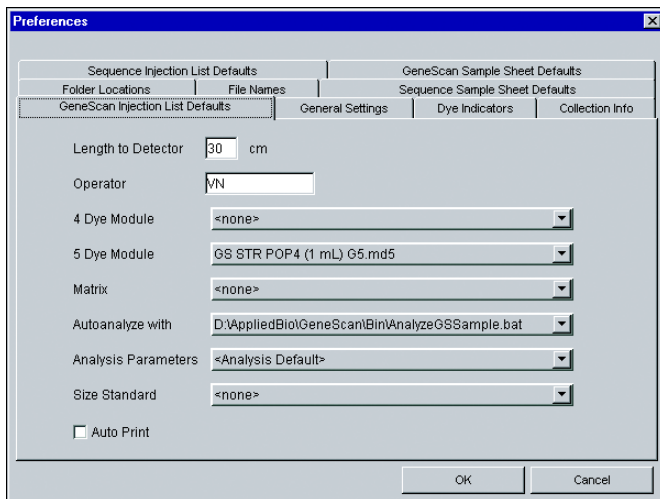
Figure 4-2   Preferences window showing defaults

3.	Make the following selections in the Preferences window: a. Select GS STR POP4 (1 mL) G5 for the five-dye module. b. Select an appropriate matrix file. c. Make sure the **AnalyzeGSSample.bat** file is selected if you wish to autoanalyze. If you do not wish to autoanalyze your data, select **Autoanalyze with > none**. **Note:** When you create a new sample sheet, a portion of the form is automatically filled in for you. You can modify the automatic defaults in the Preferences file.
4.	Once you have finished making changes to the **Preferences** pages, click **OK** to save your changes.
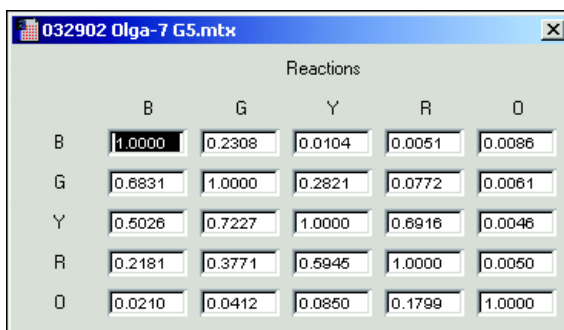
Running Matrix Samples

The precise spectral overlap between the five dyes is measured by analyzing DNA fragments labeled with each of the dyes (6-FAM, VIC, NED, PET or LIZ dye) in separate injections on a capillary. These dye-labeled DNA fragments are called matrix standard samples. See Chapter 1 for a general description of multicomponent analysis.

The ABI PRISM GeneScan Analysis Software v3.7.1 or higher analyzes the data from each of these five samples and creates a matrix file. The matrix file contains a table of numbers with five columns and five rows. These numbers are normalized fluorescence intensities that represent a mathematical description of the spectral overlap that is observed between the five dyes (Figure 4-3 on page 4-6).

The rows in the matrix file table represent the virtual filters and the columns represent the dye-labeled DNA fragments, indicated as "Reactions" in Figure 4-3 below. The matrix file table contains the values obtained on a particular ABI PRISM 310 System. The values obtained are unique for each instrument. The top left-hand value, 1.0000, represents the normalized fluorescence of blue (6-FAM-labeled) DNA fragments in the blue filter. It follows that all matrix tables should have values of 1.0000 on the diagonal from top left to bottom right, as shown in Figure 4-3.

032902 Olga-7 G5.mtx					×
			Reactions		
	B	G	Y	R	O
B	1.0000	0.2308	0.0104	0.0051	0.0086
G	0.6831	1.0000	0.2821	0.0772	0.0061
Y	0.5026	0.7227	1.0000	0.6916	0.0046
R	0.2181	0.3771	0.5945	1.0000	0.0050
O	0.0210	0.0412	0.0850	0.1799	1.0000

Figure 4-3   Matrix file table from an ABI PRISM 310 system

All the other values in Figure 4-3 should be less than 1.0000. These values represent the amount of spectral overlap observed for each dye in each virtual filter. For example, the values in the first column reflect quantitatively the amount of blue dye detected in each virtual filter. These matrix file values will vary between different instruments, virtual filter sets, and run conditions on a single instrument. A matrix file must be made for each instrument and for a particular set of run conditions.

You can apply the appropriate matrix file to data on subsequent runs on the same instrument, as long as the running conditions are constant from run to run. This is because the spectral overlap between the five dyes is reproducible under constant run conditions. However, it is recommended that you make a new matrix once a month to use with the AmpFℓSTR products or when changing polymer, capillaries, and buffer.

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

Filter Set G5 Module Files

Data Collection Software Modules

The ABI PRISM 310 Data Collection Software v3.0 collects light intensities from five specific areas on the CCD camera, each area corresponding to the emission wavelength of a particular fluorescent dye. Each of these areas on the CCD camera is referred to as a "virtual" filter since no physical filtering hardware (*e.g.*, band pass glass filter) is used.

The information that specifies the appropriate virtual filter settings for a particular set of fluorescent dyes is contained in each appropriate ABI PRISM Data Collection Software module file.

The GS STR POP4 (1 mL) G5 module file must be installed and used for dye set DS-33 (6-FAM, VIC, NED, PET, LIZ dyes) on the ABI PRISM 310 Genetic Analyzer. The configuration is POP-4 polymer with 1-mL syringe.

IMPORTANT! Filter Set G5 module files must be installed on the instrument's computer before making a matrix file using the 6-FAM, VIC, NED, PET, and LIZ matrix standards. Filter Set G5 module files must also be used on all subsequent runs. Samples that are run on a capillary using Filter Set G5 must be analyzed using a matrix file that was created using Filter Set G5.

Five-Dye Data Collection Software

The ABI PRISM 310 Data Collection Software v3.0 enables collection of five-dye data for DNA fragment analysis applications. This section provides detailed information on sample sheet and injection lists.

Creating a Five-Dye Sample Sheet and Injection List

To create a five-dye sample sheet:
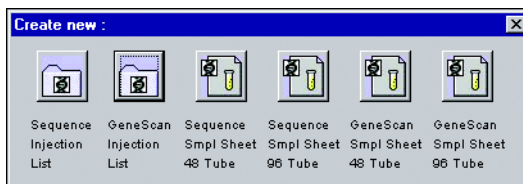
1.	Select **File** > **New** to open the **Create new** window. **Figure 4-4 Create new window**
2.	Click the icon corresponding to an appropriate GeneScan Sample Sheet configuration. A Sample Sheet window opens. **Figure 4-5 Example of a Sample Sheet window**
3.	Select the **5 Dyes** option from the drop-down menu in the upper-right corner of the window.
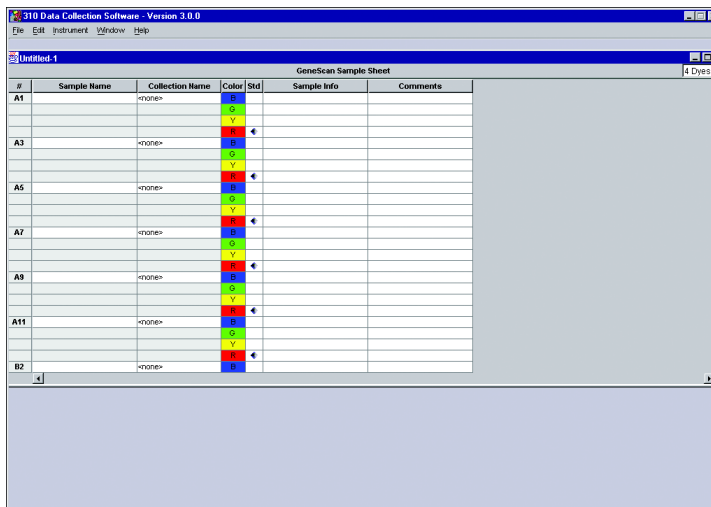
To create a five-dye sample sheet: *(continued)*

4. In the five-dye Sample Sheet:

   a. Enter the sample name, sample information and comments.

   b. Designate color for the appropriate size standard.

   c. Save the sample sheet.

   Be sure to select the orange dye as the designated size standard for all five-dye samples. Under Preferences, this feature can be preset. See Figure 4-2 on page 4-5. Figure 4-6 shows a completed five-dye sample sheet.

Figure 4-6   Five-dye Sample Sheet

Note: Setting up five-dye samples requires the use of a five-dye sample sheet. You may not set up both four-dye and five-dye samples in a five-dye sample sheet. All four-dye samples must be set up separately in a four-dye specific sample sheet.

To create a five-dye sample sheet: *(continued)*

5.	To create a new injection list, select **File** > **New**, then select a new injection list in the Create new window. **Figure 4-7 Create new window**
6.	Click the **GeneScan Injection List** icon.
7.	From the Sample Sheet drop-down menu (in the GeneScan Injection List), import the appropriate sample sheet. **Note:** To access five-dye modules, you must first import a five-dye sample sheet into the injection list. **Figure 4-8 Injection List window**
8.	After setting the appropriate injection parameters, save the injection list.
9.	To start the sequence of injections, click the **Run** option in the **Injection List** window.
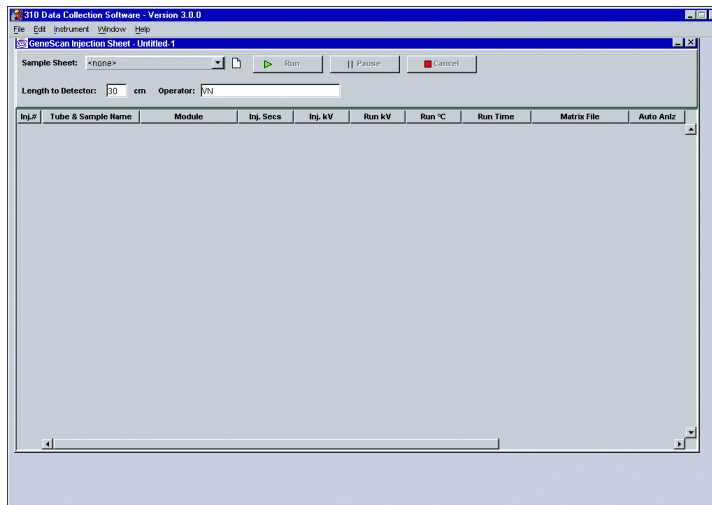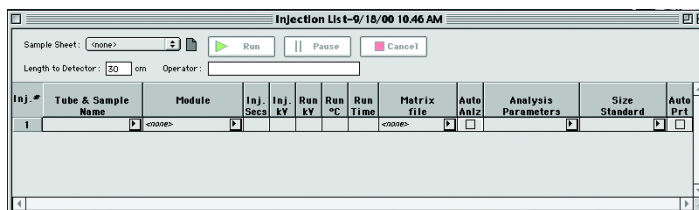
Making a Matrix File

Matrix Standards The matrix standards are supplied in the Matrix Standard Set DS-33 (6-FAM, VIC, NED, PET and LIZ) for use with the 310/377 system (P/N 4318159).

Making a Matrix File on the ABI PRISM 310 If you need information on required supplies for the procedures below, refer to Dedicated Equipment and Supplies on .

To make the matrix file:

1.	• Combine 1 µL of each matrix standard with 25 µL of Hi-Di™ Formamide (P/N 4311320). • Prepare one tube for each matrix standard sample. ⚠ **WARNING** **CHEMICAL HAZARD**. **Formamide** is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves. **IMPORTANT!** Do not include the GeneScan 500 LIZ Size Standard in the preparation of the matrix standards.
2.	a. Denature the samples at 95 °C for 3 min. b. Quick-chill on ice for 3 min. c. Place tubes in the appropriate sample tray. **Note:** Be careful not to carry-over any water on the outside of the tubes. Water on the autosampler tray may promote arcing.
3.	Launch the ABI PRISM 310 Collection application.

To make the matrix file: *(continued)*

4.	Select **File** > **New** and click the **GeneScan Smpl Sheet 48 Tube** or **GeneScan Smpl Sheet 96 Tube** icon, as appropriate.
5.	Complete the sample sheet as described in the *ABI PRISM® 310 Genetic Analyzer User Guide*. a. Enter the sample names/numbers for each row in the **Sample Name** column to identify which sample is in which tube of the sample tray. b. Close and save the sample sheet.
6.	Select **File** > **New** and click the **GeneScan Injection List** icon.
7.	a. In the **Injection List**, select the appropriate sample sheet from the **Sample Sheet** pop-up menu. b. Select **Module** > **GS STR POP4 (1 mL) G5** for every injection. c. Select **None** in the **Matrix File** column for each matrix standard sample. **Note:** Review data of each matrix standard. Re-inject if necessary.
8.	Click **Run**.

To make the matrix file: *(continued)*

9.	When the injections are done, create the matrix using GeneScan Analysis Software:
	a. Select **File** > **New**.
	b. Click the **Matrix** icon and select five dyes from the number of dyes pop-up window.
	c. In the window that opens, indicate the sample files that correspond to each matrix standard dye color.
	d. Select starting scan numbers for each sample to exclude the primer peak.
	e. Select the number of points so that at least these five peaks are contained in the scanned region (approximately 2500 scan data points). Avoid spikes or artifacts, if possible, when selecting the range.
	f. Click **OK** to create the matrix and open the matrix file table.
10.	Save the matrix file in the ABI folder:
	`D:\AppliedBio\Shared\Analysis\SizeCaller\Matrix`

To verify the accuracy of the matrix file:

1.	Apply the new matrix file to the Matrix Standard Sample Files as follows:
	a. In the **Analysis Control** window, highlight the **Sample File** column by clicking in the **Sample File** title row.
	b. Select **Sample** > **Install New Matrix**.
	c. Select the new matrix file (located in the ABI folder in the System folder), and click **Open**.
2.	Analyze the matrix standard samples as follows:
	a. Select **Settings** > **Analysis Parameters**, and verify that the settings are correct.
	b. In the **Analysis Control** window, select all five colors in each sample row for all of the matrix standard samples.
	c. Click the **Analyze** button.

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

3.	In the **Results Control** window, examine the results for all five colors for each of the matrix standard samples.
	For example, the 6-FAM matrix standard results should have peaks for blue. Evaluate the baseline. A pattern of pronounced peaks or dips in any of the other four colors indicates that the color separation may not be optimal.
4.	If this verification test fails, then the capillary may not have been aligned properly in the instrument during the run. To correct this problem:
	a. Repeat the experiment, making sure that the capillary is placed carefully in the laser detection window.
	b. Tape the capillary to the heat plate so that the capillary is immobilized during the run.

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

Running DNA Samples

Preparing Samples and AmpF*l*STR SEfiler Allelic Ladder

To prepare the samples:

1.	Combine the necessary amount of Hi-Di Formamide and GeneScan-500 LIZ Size Standard (P/N 4322682) in a single microcentrifuge tube as shown: • (Number of samples + 2) × 24.5 µL Hi-Di Formamide • (Number of samples + 2) × 0.5 µL GeneScan-500 LIZ Size Standard If you are using a multi-channel pipettor or processing many samples, you may want to prepare additional master mix. ⚠ **WARNING** **CHEMICAL HAZARD**. **Formamide** is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves. Be sure to include at least one injection of AmpF*l*STR SEfiler Allelic Ladder per run in the calculations.
2.	a. Vortex the tube to mix. b. Spin the tube briefly in a microcentrifuge.
3.	a. Label tubes appropriately. b. Aliquot 25 µL of Hi-Di Formamide/GeneScan-500 LIZ solution into 0.2-mL or 0.5-mL Genetic Analyzer sample tubes. **Note:** To pipet the Hi-Di Formamide/size standard solution, we recommend using a repeating pipettor.
4.	Add 1.5 µL of PCR product or AmpF*l*STR SEfiler Allelic Ladder per tube, and mix by pipetting up and down.
5.	Seal each tube with a septum.

To prepare the samples: *(continued)*

6.	Vortex the sample tray and spin briefly in a microcentrifuge. **Note:** Ensure that there are no bubbles.
7.	Denature each sample for 3 minutes at 95 °C.
8.	Chill tubes for at least 3 minutes on ice. **Note:** Be careful not to carry-over any water on the outside of the tubes. Water on the autosampler tray may promote arcing.

Loading Samples

To load samples:

1.	Open the instrument door and press the **Tray** button to present the autosampler.
2.	Place a 48-well or 96-well sample tray on the autosampler. For a 48-well autosampler tray, tube #1 will go into sample tray position A1, tube #2 into sample tray position A3, and so on. For a 96-well autosampler tray, tube #1 will go into sample tray position A1, tube #2 into sample tray position A2, and so on.
3.	Press the **Tray** button on the instrument to retract the autosampler.
4.	Close the instrument door.

Running Sample Electrophoresis	**To run the samples:**

1.	If not already open, launch the ABI PRISM 310 Data Collection Software v3.0.
2.	Select **File** > **New** and click the appropriate **GeneScan Sample Sheet** icon. **Note:** The 310 Data Collection Software v3.0 must be installed for use with the AmpF*l*STR SEfiler PCR Amplification Kit.
3.	Complete the sample sheet. The sample sheet can be prepared at any time before the preparation of samples and saved in the Sample Sheet folder. a. Select 5-dyes from the drop-down menu. b. Enter sample names/numbers for each injection in the **Sample Name** column. This column will indicate which sample is in which tube of the sample tray. c. Enter the sample description for each row in the **Sample Info** column (for Blue, Green, Yellow, and Red for each appropriate sample). This entry is necessary for the AmpF*l*STR® SEfiler™ Template 1 File to build tables containing the genotypes for each sample. d. Type the word **ladder** for the Blue, Green, Yellow, and Red rows for the AmpF*l*STR SEfiler Allelic Ladder injection. **Note:** Software requires the word "ladder." Alternatively: a. Select 5-dyes from the drop-down menu. b. Enter the sample names and numbers for each injection in the **Sample Name** column. c. Select **Edit** > **Copy**, and copy all sample names at one time by highlighting the Sample Name header and paste by highlighting the Sample Info header. The sample name will appear in the blue, green, yellow, red, and orange Sample Info column for each sample.
4.	Select **File** > **New** and click the **GeneScan Injection List** icon.

To run the samples: *(continued)*

5.	Select the appropriate sample sheet from the **Sample Sheet** pop-up menu (at the top left of the Injection List window).
6.	Select **Module** > **Module GS STR POP4 (1 mL) G5** for every injection.
	a. Click the arrow in the Module column for the first sample/injection to view the pop-up menu and select the **GS STR POP4 (1 mL) G5** module file.
	b. Select the entire Module column by clicking the **Module** column heading, then select **Edit** > **Fill Down**.
	Note: You do not need to perform this step if the preferences were set as described in "Setting the Parameters" on page 4-4.
7.	Select matrix files for the injections:
	a. From the **Matrix** file pop-up menu, select the appropriate matrix file for every injection.
	b. Click the arrow in the **Matrix** column for the first sample/injection to view the pop-up menu and select the appropriate matrix file. Select the entire Matrix column by clicking the Matrix column heading, then select **Edit > Fill Down**.
	IMPORTANT! The matrix file must be one that was made using the 6-FAM, VIC, NED, PET, and LIZ matrix standards and Filter Set G5 module. If you wish to autoanalyze, you must place a copy of the matrix file in the ABI folder located in the System Folder.
	Note: You will not need to perform this step if the preferences were set as described in "Setting the Parameters" on page 4-4.
8.	Click the **Run** button.
	Note: If you have not preheated the heat plate, the module has an initial step in which the plate is heated to 60 °C before running the first sample. This step takes up to 30 min. Once the plate reaches 60 °C, the run will begin.

Setting Up Software Parameters

Setting the Analysis Parameters

Perform the following steps in ABI PRISM GeneScan Analysis Software v3.7.1 or higher.

To set analysis parameters:

1.	Launch the GeneScan Software v3.7.1.
2.	Select **Settings** > **Analysis Parameters**. **Note:** A more detailed discussion for each of the six Analysis Parameters is in Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," and the *ABI PRISM® GeneScan® Analysis Software Version 3.7 User Guide*.
3.	Enter parameters in the dialog box:

Parameter	Entry
Analysis Range	1. Click the **This Range (Data Points)** radio button. 2. Enter Start and Stop data point numbers in the entry fields. Select the **Start** data point just before the first peak of interest, the 75 bp size standard peak. At a minimum, select the **Stop** data point just after the last peak of interest, the 450 bp size standard peak. 3. Look at the raw data and enter the values that are appropriate for all sample files in the project. These data points affect data in the results display.
Smooth Options	The default parameter for Smooth Options is light. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* (P/N 4335617) for more information on how to set the appropriate value for smooth options.

To set analysis parameters: *(continued)*

3. *(continued)*

Parameter	Entry
Peak Detection	1. Select a Peak Amplitude Threshold (PAT) for each dye color. 2. Use the active scroll bar to enter the PATs for each of the five colors. 3. After analysis, the GeneScan table contains data for all peaks with a height above that specified by the PAT. **Note:** We suggest that you determine the PATs appropriate for your analysis. Sensitivity experiments should be conducted in your laboratory with each instrument to evaluate the PATs used for analysis.
Polynomial Degree	The default parameter for polynomial degree is 3. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* for more information on how to set the appropriate value for the polynomial degree.
Min. Peak Half Width	The Min Peak Half Width for use with the AmpFℓSTR products is 2 Pts. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* for more information on how to set the appropriate value for Min. Peak Half Width.
Peak Window Size	The default parameter for peak window size is 15. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* for more information on how to set the appropriate value for Peak Window Size.

To set analysis parameters: *(continued)*

3. *(continued)*

Parameter	Entry
Size Call Range	Click the **This Range (Base Pairs)** radio button and enter the values of **75** for Min and **450** for Max.
Size Calling Method	Click the **Local Southern Method** radio button for sizing of the AmpF*l*STR products. This method determines the sizes of fragments by using the reciprocal relationship between fragment length and mobility.
Baselining	The default setting for the baseline window size is 51 pts. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* (P/N 4335617) for more information on how to set the appropriate value for the baseline window size.
Slope Threshold	The default parameter for slope threshold for peak start and peak end should be 0.0. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* (P/N 4335617) for more information on how to set the appropriate value for slope threshold.
Auto Analysis Only	Refer to the user bulletin (P/N 4335617) for more information.

4. Click **OK** when done.

To set analysis parameters: *(continued)*

5. Assign a size standard:

a. Click the arrow in the Size Standard column for a sample file to view the pop-up menu and select **New**.

– For more information on defining a size standard, refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan® Analysis Version 3.7 User Guide*.

– Do not assign a size for the 250-bp peak for data generated on the ABI PRISM® 310 Genetic Analyzer (*i.e.*, skip a row or assign a size of zero). This peak can be used as an indicator of precision within a run. Twelve size standard peaks should be viewed at this step, as shown below.
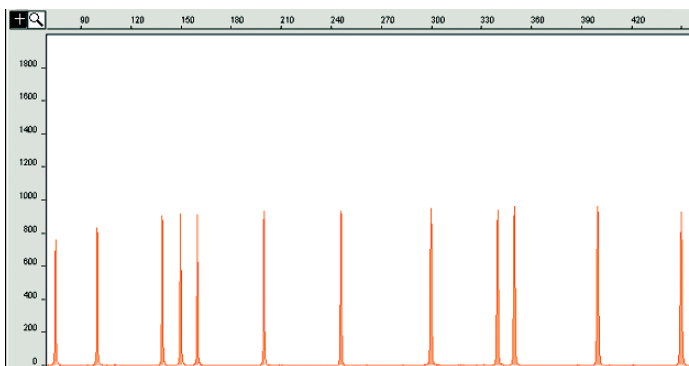
Figure 4-9   Size standard peaks

– Save the size standard for this sample in:

`D:\AppliedBio\Shared\Analysis\SizeCaller\SizeStandards`

in the ABI PRISM GeneScan Version 3.7.1 Software folder.

b. To apply this standard to all injections, select the appropriate standard in the Size Standard column header (above sample 1) in the **Analysis Control** window.

To set analysis parameters: *(continued)*

6.	Analyze sample files:
	a. Highlight the blue, green, yellow, red, and orange columns.
	Note: Confirm that the orange box is the standard (a diamond symbol should appear in all orange boxes where a size standard is included with the sample files). If the diamond symbol is not in the orange boxes, **Ctrl-click** to place a diamond in the box.
	b. Click the **Analyze** button.
7.	After the analysis is complete, confirm that the sizes for the peaks in the GeneScan-500 LIZ Size Standard have been correctly assigned.
	a. Select **Window > Results Control** and examine the orange GeneScan-500 LIZ Size Standard peaks in overlapping groups of 16 samples (Quick Tile Off). Be sure to select **View > Align By Size**.
	b. While the samples are tiled, check the 250-bp peaks (sized as approximately 246 bp) in the enlarged view window. Remember that this peak was not defined in the size standard. The tiled 250-bp peaks should size consistently, *i.e.*, should all overlap. In a typical run, the 250-bp peaks all fall within a size window of approximately 1 bp. Temperature fluctuations in the laboratory may cause variations >1 bp.
	Note: Laboratory temperature variations can cause size shifts. If the temperature of the laboratory varies, try injecting the AmpF*l*STR SEfiler Allelic Ladder approximately every 10 injections, or 5 hours.
	c. Scroll through the tables to verify correct GeneScan-500 LIZ peak assignments.
	d. Check the GeneScan-500 LIZ Size Standard peaks in the remaining samples, taking note of which samples (if any) have incorrect peak assignments.
8.	View AmpF*l*STR SEfiler kit results (using the Results Control window).
	Refer to the *ABI PRISM® GeneScan® Analysis Version 3.7 User Guide* for printing options.

GeneScan Software Results

GeneScan Analysis Software

After the sample files have been analyzed, use the Results Control window to display the results from each lane of a gel or each injection into a capillary. The Results Control window displays the newly analyzed sample files and allows the user to specify the format of the results. Selecting both the Electropherogram and Tabular Data icons is recommended for reviewing the results. For more information on displaying the results, refer to the *ABI PRISM® GeneScan® Analysis Software Version 3.7 User Guide.*

Information Provided in the Electropherogram and Table

Both the electropherogram and the tabular data can be displayed. See .

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

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

The columns in the table list the following:

- Column 1 lists the Dye/Sample and Peak (*e.g.*, "4B, 1" indicates the first blue peak in project sample 4).
- Column 2 lists the time it took the dye-labeled fragment to reach the detector.
- Column 3 lists the base pair size of the peak, as calculated using the GeneScan-500 LIZ Size Standard curve.
- Column 4 lists the height in RFU of the peak.
- Column 5 lists the relative peak area, which is the integral of the RFU times the data point (scan number). This value depends on the velocity of the dye labeled fragment as it passes the detector.

Column 6 lists the data point (scan number) of the dye labeled fragment at its maximum peak height; the data point correlates with the number of laser scans (or data points collected) from the beginning of the run until the time that the peak maximum is detected. Figure 4-10 is a GeneScan electropherogram of AmpF*l*STR SEfiler alleles in AmpF*l*STR Control DNA 007 analyzed on the ABI PRISM 310 Genetic Analyzer.
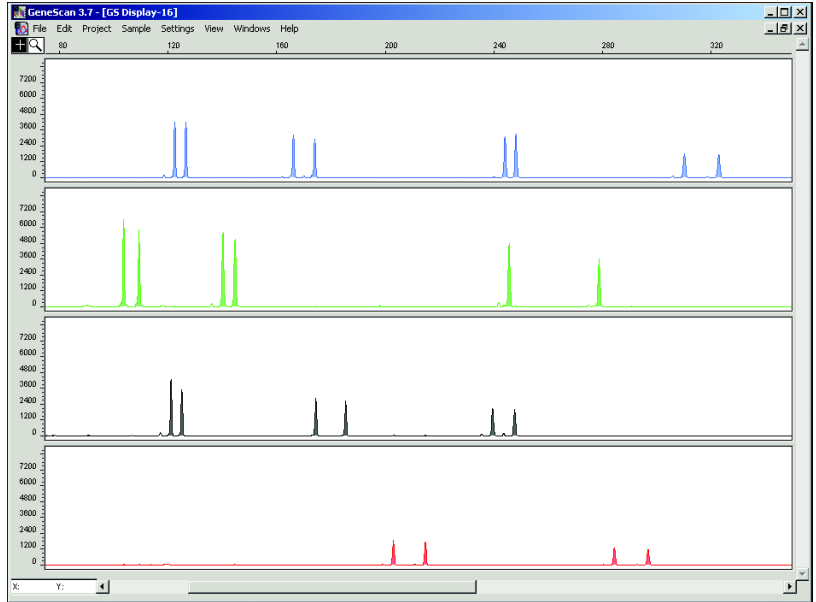
Figure 4-10   GeneScan electropherogram of AmpF*l*STR SEfiler alleles in AmpF*l*STR Control DNA 007 analyzed on the ABI PRISM 310 Genetic Analyzer

Results Display Options

The GeneScan Software v3.7.1 or higher offers two main options in the Results Control window for electropherogram viewing formats: Quick Tile Off and Quick Tile On.

- The Quick Tile Off format provides the option of displaying results either for multiple colors within a single lane or injection, or from multiple lanes or injections in the same panel (*i.e.*, the results are overlaid), as shown in panel 1 of Figure 4-11.
- The Quick Tile On format displays each color of each lane or injection separately, as shown in panels 2–5 of Figure 4-11.

The Quick Tile Off and On feature offers the user versatility in customizing the display of results. Up to eight panels can be tiled at a single time and up to 16 electropherograms may be overlaid in one panel at the same time.
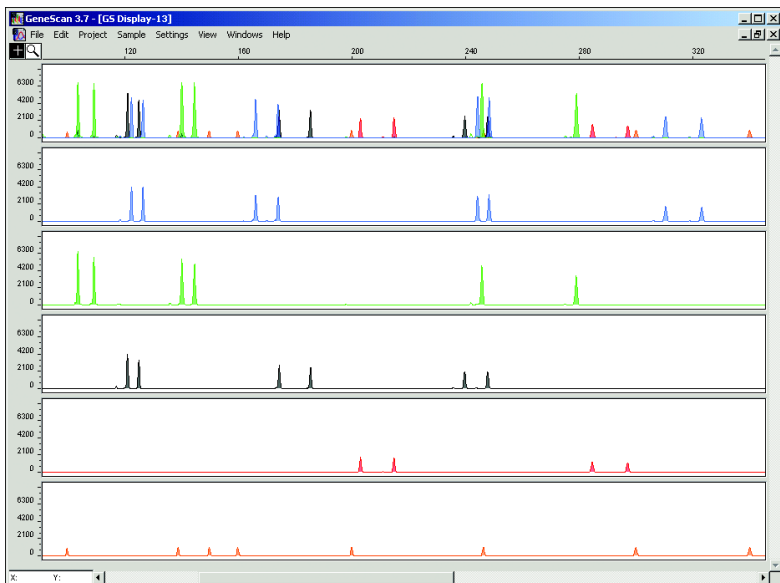
Figure 4-11   Quick Tile Off and Quick Tile On options

Panel A is an example of one sample displayed with Quick Tile Off. Panels B–F are examples of the same sample file with Quick Tile On using the AmpFℓSTR Control DNA 007 analyzed on the ABI PRISM 310 Genetic Analyzer.

Note: For a more detailed description refer to the *ABI PRISM® GeneScan® Analysis Software Version 3.7 User Guide*.

Standards for Samples

For the SEfiler kit, the panel of standards needed for PCR amplification, PCR product base pair sizing, and genotyping are as follows:

- The AmpF*l*STR Control DNA 007 provides a positive control for the efficiency of the amplification step and STR genotyping using the AmpF*l*STR SEfiler Allelic Ladder.

- The AmpF*l*STR SEfiler Allelic Ladder was developed by Applied Biosystems for accurate characterization of the alleles amplified by the AmpF*l*STR SEfiler kit. The AmpF*l*STR SEfiler Allelic Ladder contains the majority of alleles reported for the 15 loci. Refer to Table 1-1, "AmpF*l*STR SEfiler Kit Loci and Alleles," on page 1-4 for a list of the alleles included in the AmpF*l*STR SEfiler kit.

- GeneScan-500 LIZ Size Standard is used for obtaining base pair sizing results. The GeneScan-500 LIZ Size Standard is designed for sizing DNA fragments in the 35–500 bp range, and it contains 16 single-stranded fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250 (not assigned when used on the ABI PRISM 310 Genetic Analyzer), 300, 340, 350, 400, 450, 490, and 500 bases. This standard has been evaluated as an internal lane size standard and it yields extremely precise sizing results of AmpF*l*STR SEfiler kit PCR products. Order the GeneScan-500 LIZ Size Standard (P/N 4322682) separately.

Shutting Down the Instrument

Ending the Run   If the instrument is not going to be in use for 3 or more consecutive days, it is recommended that the instrument be cleaned and shut down.

To shut down the instrument:

1.	Remove and clean the syringe and block as previously described.
2.	Discard unused polymer in the proper waste container. ⚠ **CAUTION** **CHEMICAL HAZARD. POP polymers** may cause eye, skin, and respiratory tract irritation. Read the MSDS for the polymer you are using, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Use for research and development purposes only. **IMPORTANT!** Do not put unused polymer back into the bottle. Polymer in the syringe decomposes over time at room temperature. ⚠ **WARNING** **CHEMICAL WASTE HAZARD**. Dispose of it in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.
3.	In the **Manual Control** window, select **Autosampler Home X, Y Axis** and click **Execute**.
4.	Select **Autosampler Home Z Axis** and click **Execute**.
5.	Turn off the instrument.

Dedicated Equipment and Supplies

Required Equipment

The equipment and supplies necessary or recommended for running AmpF*l*STR SEfiler kit data on the ABI PRISM 310 Genetic Analyzer are listed in the tables below.

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

Table 4-2   Equipment

ABI PRISM 310 Genetic Analyzer
ABI PRISM 310 Genetic Analyzer Accessories: • ABI PRISM 310 Genetic Analyzer Capillary, L_t = 47 cm, L_d = 36 cm, i.d. = 50 µm (P/N 402839), labeled with a green mark • ABI PRISM 310 Genetic Analyzer Vials, 4.0 mL (P/N 401955) • ABI PRISM 310 Genetic Analyzer 0.5-mL Sample Tubes (P/N 401957) • ABI PRISM 310 Genetic Analyzer Septa for 0.5-mL Sample Tubes (P/N 401956) • Syringe, Kloehn 1.0-mL (P/N 4304471)
Benchkote absorbent protector sheets
Flush-cutting wire cutter (P/N T-6157)
Freezer, –15 to –25 °C, non-frost-free
Gloves, disposable, powder-free
Glassware
Ice bucket
Lint-free tissues
Lab coat
Microcentrifuge tubes, 1.5-mL
Microtube racks
Nalgene filter apparatus, 150–mL, 0.2-µm CN filter

Table 4-2   Equipment *(continued)*

Permanent ink pen
Pipet bulb
Pipets, serological
Pipet tips, sterile, disposable hydrophobic filter-plugged
Pipettors, adjustable, 1–10 µL, 2–20 µL, 20–200 µL, and 200–1000 µL
Refrigerator
Repeat pipettor and Combitips that dispense 25–125 µL (optional)
Sink
Syringe, 35 cc (optional)
Tape
Thermal cycler
Tube, 50 mL Falcon
Tube decapper, autoclavable

Required Reagents

Table 4-3   Reagents

ABI PRISM 310 10X Genetic Analyzer Buffer with EDTA (P/N 402824)
Deionized water, PCR grade
Hi-Di™ Formamide (P/N 4311320)
GeneScan-500 LIZ Size Standard (P/N 4322682)
Matrix Standard Set DS-33 [6-FAM, VIC, NED, PET, LIZ dyes] for use with the 310/377 system (P/N 4318159)
POP-4 polymer (P/N 402838)

Software and User Documentation Required

Table 4-4   Software and User Documentation

ABI PRISM 310 Collection Software, Version 3.0 or higher
ABI PRISM® 310 Genetic Analyzer User Guide (P/N 4317588)
ABI PRISM 310 Module GS STR POP4 (1 mL) G5
GeneScan Software v3.7.1 or higher
ABI PRISM® GeneScan® Analysis Software Version 3.7.1 User Bulletin (P/N 4335617)

Running the 310 Genetic Analyzer on Mac OS

5

In This Chapter   Protocols for analyzing samples on the ABI PRISM® 310 Genetic Analyzer on a Macintosh® OS are described in this chapter.

Software Requirements

Data Collection Software for Macintosh®

Before using the instrument, you must install the appropriate software and use the products shown in the table below:

Table 5-1   Products Needed

Product	Needed to
ABI PRISM® Data Collection Software v2.1 (P/N 4324229)	run AmpFℓSTR® SEfiler™ PCR Amplification Kit products and collect five-dye data
6-FAM™, VIC®, NED™, PET™, and LIZ® matrix standard set DS-33 run using the GS STR POP 4 (1 mL) G5 module	create a required matrix file
ABI PRISM® Genotyper® Software v2.5.2 or higher	analyze SEfiler kit data

Analysis Software

This chapter is written for ABI PRISM® GeneScan® Analysis Software version 3.1.2 or higher.

Refer to the documents listed below for more detailed information on the instrument and software used with these protocols:

- *ABI PRISM® 310 Genetic Analyzer User Guide* (P/N 4317588)
- *ABI PRISM® GeneScan® Analysis Software Version 3.7 User Guide* (P/N 4308923)

Equipment and Supplies

Supplies

The equipment and supplies necessary or recommended for running AmpFℓSTR SEfiler kit data on the ABI PRISM 310 Genetic Analyzer are listed in the tables under .

Setting Up the Run for a Macintosh Computer

Setting the Run Temperature

Setting the run temperature prior to starting a run is optional; however, this step saves time. This heating step occurs automatically at the beginning of the GS STR POP4 (1 mL) G5 run module.

To set the run temperature:

1.	Close the instrument doors.
2.	Return to the ABI PRISM 310 Data Collection Software.
3.	Set the temperature: a. Select **Window** > **Manual Control**. b. Select **Temperature Set** from the pop-up menu. c. Set the temperature to 60 °C. d. Click **Execute**. **Note:** It takes up to 30 min for the instrument to reach the 60 °C run temperature. You can prepare samples while the instrument is heating.

Setting the Parameters

To select a five-dye sample sheet:

1.	**Note:** This is an optional step.

Set the standard color:

a. Launch the 310 Data Collection Software v2.1.
b. Select **Window** > **Preferences**, then select the **GeneScan Sample Sheet Defaults**.
c. Set the size standard color to orange (**O**) as shown in the figure below.
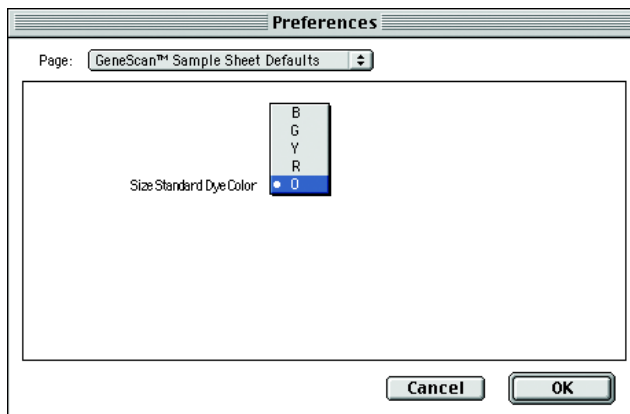
Figure 5-1   Preferences window

To select a five-dye sample sheet: *(continued)*

2.	Select **Page** > **GeneScan Injection List Defaults** to open the Preferences window. Figure 5-2 Preferences window with defaults **Note:** When you create a new sample sheet, a portion of the form is automatically filled in for you. You can modify the automatic defaults in the Preferences file.
3.	Make the following selections in the Preferences window: a. Select **GS STR POP4 (1 mL) G5** for the five-dye module. b. Select a default matrix file. c. Make sure the Genescan Analysis application is selected if you wish to autoanalyze. If you do not wish to autoanalyze your data, deselect the box next to the **Autoanalyze with** option.
4.	Once you have finished making changes to the Preferences pages, click **OK** to save your changes.
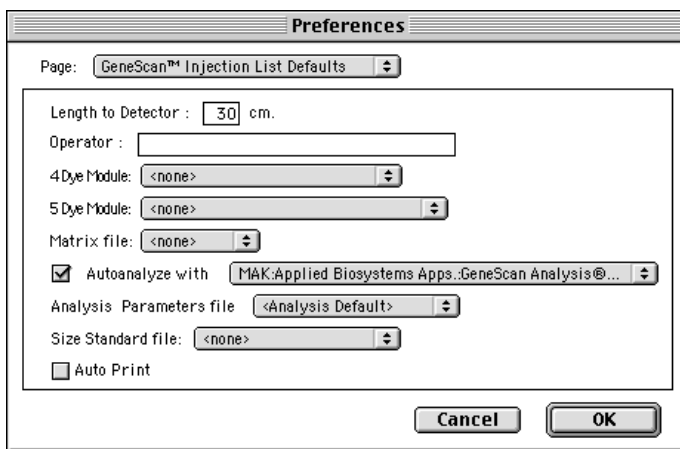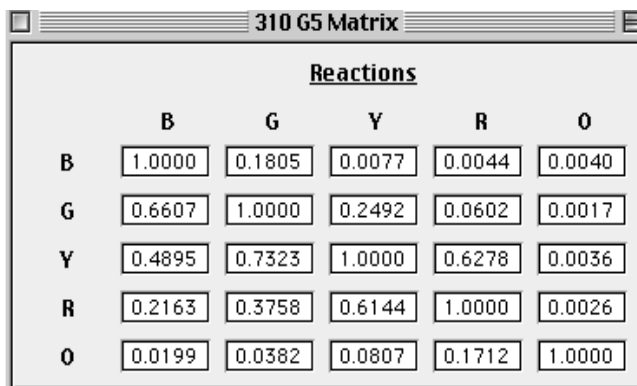
Running Matrix Samples

The precise spectral overlap between the five dyes is measured by analyzing DNA fragments labeled with each of the dyes (6-FAM, VIC, NED, PET or LIZ dye) in separate injections on a capillary. These dye-labeled DNA fragments are called matrix standard samples. See "Multicomponent Analysis Overview" on page 1-3 for a general description of multicomponent analysis.

The ABI PRISM GeneScan Analysis Software v3.1 or higher analyzes the data from each of these five samples and creates a matrix file. The matrix file contains a table of numbers with five columns and five rows. These numbers are normalized fluorescence intensities representing a mathematical description of the spectral overlap that is observed between the five dyes (Figure 5-3 on page 5-6).

The rows in the matrix file table represent the virtual filters and the columns represent the dye-labeled DNA fragments, indicated as "Reactions" in Figure 5-3 below. The top left-hand value, 1.0000, represents the normalized fluorescence of blue (6-FAM-labeled) DNA fragments in the blue filter. It follows that all matrix tables should have values of 1.0000 on the diagonal from top left to bottom right.

The matrix file table shown in Figure 5-3 contains the values obtained on a particular ABI PRISM 310 System. These values are unique for each instrument.

310 G5 Matrix

Reactions

	B	G	Y	R	O
B	1.0000	0.1805	0.0077	0.0044	0.0040
G	0.6607	1.0000	0.2492	0.0602	0.0017
Y	0.4895	0.7323	1.0000	0.6278	0.0036
R	0.2163	0.3758	0.6144	1.0000	0.0026
O	0.0199	0.0382	0.0807	0.1712	1.0000

Figure 5-3   Matrix file table

All the other values in Figure 5-3 should be less than 1.0000. These values represent the amount of spectral overlap observed for each dye in each virtual filter. For example, the values in the first column reflect quantitatively the amount of blue dye detected in each virtual filter. These matrix file values vary between different instruments, virtual filter sets, and run conditions on a single instrument. A matrix file must be made for each instrument and for a particular set of run conditions.

You can apply the appropriate matrix file to data on subsequent runs on the same instrument, as long as the running conditions are constant from run to run. This is because the spectral overlap between the five dyes is reproducible under constant run conditions. However, it is recommended that a new matrix be made once a month for use with the AmpF*l*STR products or when changing polymer, capillaries, and buffer.

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

Filter Set G5 Module Files

The ABI PRISM 310 Data Collection Software v2.1 collects light intensities from five specific areas on the CCD camera, each area corresponding to the emission wavelength of a particular fluorescent dye. Each of these areas on the CCD camera is referred to as a "virtual" filter since no physical filtering hardware (*e.g.*, band pass glass filter) is used.

The information that specifies the appropriate virtual filter settings for a particular set of fluorescent dyes is contained in each appropriate ABI PRISM Data Collection Software module file.

The GS STR POP4 (1 mL) G5 module file must be installed and used for dye set DS-33 (6-FAM, VIC, NED, PET, LIZ dyes) on the ABI PRISM 310 Genetic Analyzer. The configuration is POP-4™ polymer with 1-mL syringe.

IMPORTANT! Filter Set G5 module files must be installed on the instrument's computer before making a matrix file using the 6-FAM, VIC, NED, PET, and LIZ matrix standards. Filter Set G5 module files must also be used on all subsequent runs. Samples that are run on a capillary using Filter Set G5 must be analyzed using a matrix file that was created using Filter Set G5.

Five-Dye Data Collection Software

The ABI PRISM 310 Data Collection Software v2.1 enables collection of five-dye data for DNA fragment analysis applications. Before you can access the five-dye module, you must import a five-dye sample sheet into the injection list. This section provides detailed information on creating a sample sheet and importing it into injection lists. You may not set up both four-dye and five-dye samples in a five-dye sample sheet. All four-dye samples must be set up separately in a four-dye specific sample sheet.

Creating a Five-Dye Sample Sheet and Injection List

Setting up five-dye samples requires using a five-dye sample sheet.

To create a five-dye sample sheet:

1. Select **File** > **New** to open the **Create new** window.
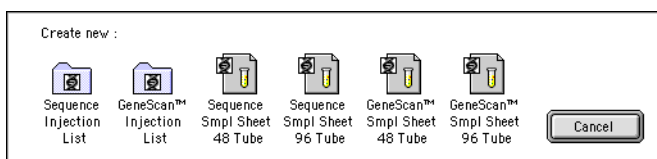
Figure 5-4   Create new window

2. Click the icon corresponding to an appropriate GeneScan Sample Sheet configuration to open the Sample Sheet window as shown in Figure 5-5.
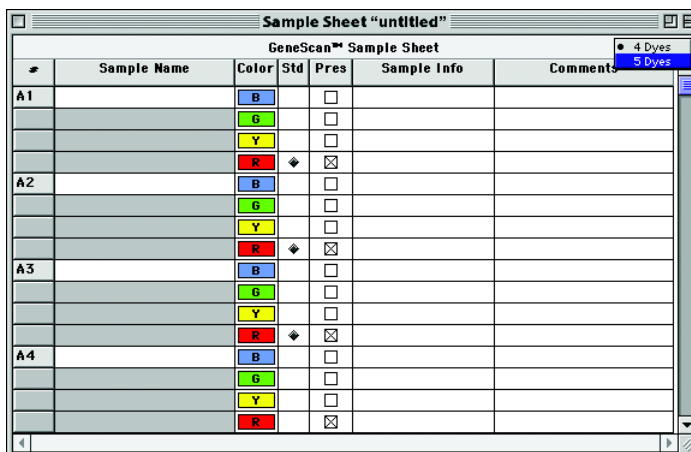
Figure 5-5   Sample Sheet window

To create a five-dye sample sheet: *(continued)*

3.	Select the **5 Dyes** option from the drop-down menu in the upper-right corner of the window.
4.	Complete the five-dye Sample Sheet: a. Enter the sample name, sample information and comments. b. Designate color for the appropriate size standard. c. Save the sample sheet. Be sure to select the orange dye as the designated size standard for all five-dye samples. You can preset this feature in the Preferences window as instructed on page 5-4. Figure 5-6 shows a sample sheet with the orange dye selected. **Figure 5-6 Five-dye Sample Sheet with orange dye selected**
5.	To create a new injection list, select **File** > **New**. Figure 5-7 is a Create new window to create a new injection list. **Figure 5-7 Create new window**
6.	Click the **GeneScan Injection List** icon.
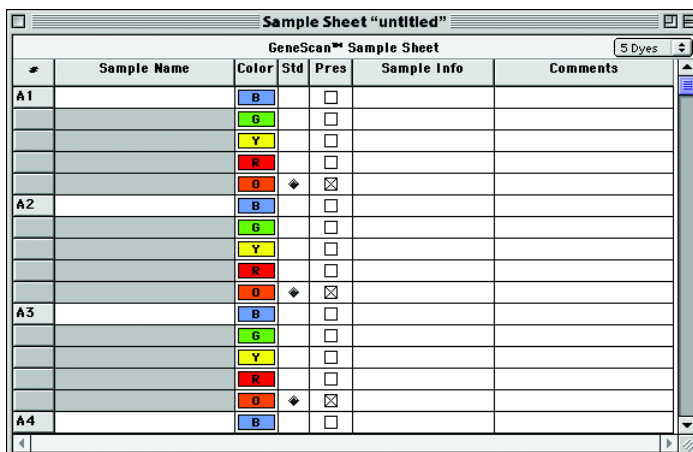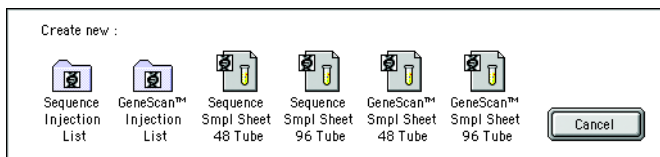
To create a five-dye sample sheet: *(continued)*

7.	From the **Sample Sheet** menu in the GeneScan Injection List, import the appropriate sample sheet. **Note:** To access five-dye modules, you must first import a five-dye sample sheet into the injection list. **Figure 5-8 Sample Injection List**
8.	After setting the appropriate injection parameters, save the injection list.
9.	To start the sequence of injections, click the **Run** option in the **Injection List** window.
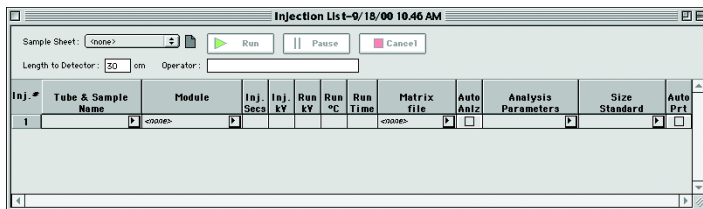
Making a Matrix File

Matrix Standards The matrix standards are supplied in the Matrix Standard Set DS-33 (6-FAM, VIC, NED, PET and LIZ dyes) for use with the 310/377 system (P/N 4318159).

Making a Matrix File on the ABI PRISM 310

To make the matrix file:

1.	Prepare the matrix standards: • Combine 1 µL of each matrix standard with 25 µL of Hi-Di™ Formamide (P/N 4311320). • Prepare one tube for each matrix standard sample. ⚠ **WARNING** **CHEMICAL HAZARD**. **Formamide** is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves. **IMPORTANT!** Do not include the GeneScan® 500 LIZ® Size Standard in the preparation of the matrix standards.
2.	Prepare the samples: a. Denature the samples at 95 °C for 3 min. b. Quick-chill on ice for 3 min. c. Place tubes in the appropriate sample tray. **Note:** Be careful not to carry over any water on the outside of the tubes. Water on the autosampler tray may promote arcing.
3.	Launch the ABI PRISM 310 Data Collection Software.
4.	Select **File** > **New** and click the **GeneScan Smpl Sheet 48 Tube** or **GeneScan Smpl Sheet 96 Tube** icon, as appropriate.

To make the matrix file: *(continued)*

5.	Complete the sample sheet as described in the *ABI PRISM® 310 Genetic Analyzer User Guide*.
	a. Enter the sample names/numbers for each row in the **Sample Name** column to identify which sample is in which tube of the sample tray.
	b. Close and save the sample sheet.
6.	Select **File** > **New** and click the **GeneScan Injection List** icon.
7.	Select the sample sheet:
	a. In the **Injection List**, select the appropriate sample sheet from the **Sample Sheet** pop-up menu.
	b. Select **Module** > **GS STR POP4 (1 mL) G5** for every injection.
	c. Select **None** in the **Matrix File** column for each matrix standard sample.
	Note: Review data of each matrix standard. Re-inject if necessary.
8.	Click **Run**. Figure 5-9 exhibits the raw data of each matrix standard, analyzed on the ABI PRISM 310 Genetic Analyzer.
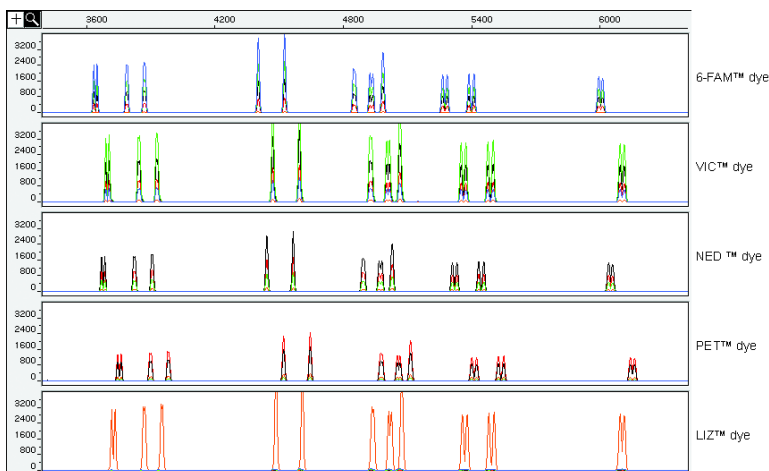
Figure 5-9   Raw data of each matrix standard

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To make the matrix file: *(continued)*

9.	When the injections are done, create a matrix using GeneScan Analysis Software:
	a. Select **File** > **New**.
	b. Click the **Matrix** icon. Select five dyes from the number of dyes pop-up window. In the window that opens, indicate the sample files that correspond to each matrix standard dye color.
	c. Select starting scan numbers for each sample to exclude the primer peak, as shown in Figure 5-9.
	d. Select the number of points so that at least these five peaks are contained in the scanned region (this is approximately 2500 scan data points). Avoid spikes or artifacts, if possible, when selecting the range.
	e. Click **OK**. The software makes the matrix and the matrix file table opens.
10.	Save the matrix file in the ABI folder in the System folder.

Verifying the Matrix File

To verify the accuracy of the matrix file:

1.	Apply the new matrix file to the Matrix Standard Sample Files as follows:
	a. In the **Analysis Control** window, highlight the **Sample File** column by clicking the **Sample File** title row.
	b. Select **Sample** > **Install New Matrix**.
	c. Select the new matrix file (located in the ABI folder in the System folder), and click **Open**.
2.	Analyze the matrix standard samples as follows:
	a. Select **Settings** > **Analysis Parameters**, and verify that the settings are correct.
	b. In the **Analysis Control** window, select all five colors in each sample row for all of the matrix standard samples.
	c. Click the **Analyze** button.

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

Figure 5-10 contains the data of each matrix standard, analyzed on the ABI PRISM 310 Genetic Analyzer.
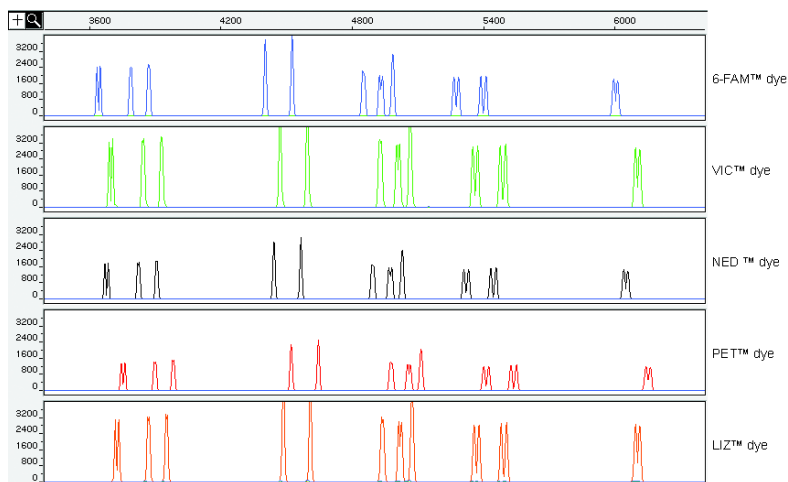
Figure 5-10   Analyzed data of each matrix standard

3.	In the **Results Control** window, examine the results for all five colors for each of the matrix standard samples.
	For example, the 6-FAM matrix standard results should have peaks for blue. Evaluate the baseline. A pattern of pronounced peaks or dips in any of the other four colors indicates that the color separation may not be optimal.
4.	If this verification test fails, then the capillary may not have been aligned properly in the instrument during the run. To correct this problem:
	a. Repeat the experiment, making sure that the capillary is placed carefully in the laser detection window.
	b. Tape the capillary to the heat plate so that the capillary is immobilized during the run.

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

Running DNA Samples

Preparing Samples and AmpF*l*STR SEfiler Allelic Ladder

To prepare the samples:

1.	Combine the necessary amount of Hi-Di Formamide and GeneScan-500 LIZ Size Standard (P/N 4322682) in a single microcentrifuge tube as shown: • (Number of samples + 2) × 24.5 µL Hi-Di Formamide • (Number of samples + 2) × 0.5 µL GeneScan-500 LIZ Size Standard If you are using a multi-channel pipettor or processing many samples, you may want to prepare additional master mix. ⚠ **WARNING** **CHEMICAL HAZARD**. **Formamide** is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves. Be sure to include at least one injection of AmpF*l*STR® SEfiler™ Allelic Ladder per run in the calculations.
2.	a. Vortex the tube to mix. b. Spin the tube briefly in a microcentrifuge.
3.	a. Label tubes appropriately. b. Aliquot 25 µL of Hi-Di Formamide/GeneScan-500 LIZ solution into 0.2-mL or 0.5-mL Genetic Analyzer sample tubes. **Note:** To pipet the Hi-Di Formamide/size standard solution, we recommend using a repeating pipettor.
4.	Add 1.5 µL of PCR product or AmpF*l*STR SEfiler Allelic Ladder per tube, and mix by pipetting up and down.
5.	Seal each tube with a septum.

To prepare the samples: *(continued)*

6.	Vortex the sample tray and spin briefly in a microcentrifuge. **Note:** Ensure that there are no bubbles.
7.	Denature each sample for 3 min at 95 °C.
8.	Chill tubes for at least 3 min on ice. **Note:** Be careful not to carry-over any water on the outside of the tubes. Water on the autosampler tray may promote arcing.

Loading Samples

To load samples:

1.	Open the instrument door and press the **Tray** button to present the autosampler.
2.	Place a 48-well or 96-well sample tray on the autosampler. For a 48-well autosampler tray, tube #1 goes into sample tray position A1, tube #2 into sample tray position A3, and so on. For a 96-well autosampler tray, tube #1 goes into sample tray position A1, tube #2 into sample tray position A2, and so on.
3.	Press the **Tray** button on the instrument to retract the autosampler.
4.	Close the instrument door.

Sample Electrophoresis

To run the samples:

1.	If not already open, launch the ABI PRISM 310 Data Collection Software v2.1.
2.	Select **File** > **New** and click the appropriate **GeneScan Smpl Sheet** icon. **Note:** The 310 Data Collection Software v2.1 must be installed for use with the AmpFℓSTR SEfiler PCR Amplification Kit.

To run the samples: *(continued)*

3.	Complete the sample sheet. The sample sheet can be prepared at any time before the preparation of samples and saved in the Sample Sheet folder.
	a. Select 5-dyes from the drop-down menu.
	b. Enter sample names/numbers for each injection in the **Sample Name** column. This column will indicate which sample is in which tube of the sample tray.
	c. Enter the sample description for each row in the **Sample Info** column (for blue, green, yellow, and red for each sample). This entry is necessary for the AmpF*l*STR SEfiler Template File to build tables containing the genotypes for each sample.
	Type the word **ladder** for the Blue, Green, Yellow, and Red rows for the AmpF*l*STR SEfiler Allelic Ladder injection.
	Note: Software requires the word "ladder." See .
	Alternatively:
	a. Select 5-dyes from the drop-down menu.
	b. Enter the sample names and numbers for each injection in the **Sample Name** column.
	c. Using the copy feature under the **Edit** menu, copy all sample names at one time by highlighting the Sample Name header and paste by highlighting the Sample Info header. The sample name will appear in the blue, green, yellow, red, and orange Sample Info column for each sample.
4.	Select **File** > **New** and click the **GeneScan Injection List** icon.
5.	Select the appropriate sample sheet from the **Sample Sheet** pop-up menu (at the top left of the Injection List window).

To run the samples: *(continued)*

6.	Select **Module** > **Module GS STR POP4 (1 mL) G5** for every injection.
	a. Click the arrow in the Module column for the first sample/injection to view the pop-up menu and select the **GS STR POP4 (1 mL) G5** module file.
	b. Select the entire Module column by clicking the **Module** column heading and select **Edit** > **Fill Down**.
	Note: You do not need to perform this step if you set the preferences as described in "Setting the Parameters" on page 5-4.
7.	Select the appropriate matrix file:
	a. From the **Matrix** file pop-up menu, select the appropriate matrix file for every injection.
	b. Click the arrow in the **Matrix** column for the first sample/injection to view the pop-up menu and select the appropriate matrix file. Select the entire Matrix column by clicking the **Matrix** column heading, then **Edit** > **Fill Down**.
	IMPORTANT! The matrix file must be one that was made using the 6-FAM, VIC, NED, PET, and LIZ matrix standards and Filter Set G5 module. If you wish to autoanalyze, you must place a copy of the matrix file in the ABI folder located in the System Folder.
8.	Click the **Run** button.
	Note: If you have not preheated the heat plate, the module has an initial step in which the plate is heated to 60 °C before running the first sample. This step takes up to 30 min. Once the plate reaches 60 °C, the run will begin.

Setting Up Software Parameters

Setting the Analysis Parameters

Perform the following steps in ABI PRISM GeneScan Analysis Software v3.1 or higher.

To set the analysis parameters:

1.	Launch the GeneScan Analysis Software.
2.	Select **Settings** > **Analysis Parameters**. **Note:** A more detailed discussion for each of the six Analysis Parameters is in the *ABI PRISM® GeneScan® Analysis Software Version 3.1 User's Manual.*
3.	Fill in the dialog box for the analysis parameters: • **Analysis Range**: – Click **This Range (Data Points)** radio button. – Enter start and stop data point numbers in the entry fields. Select the **Start** data point just before the first peak of interest, the 75 bp size standard peak. At a minimum, select the **Stop** data point just after the last peak of interest, the 450 bp size standard peak. – Look at the raw data and enter the values that are appropriate for all sample files in the project. These data points affect data displayed in the results display. • **Data Processing**: – Select the **Baseline** and the **MultiComponent** check boxes. – Select a **Smooth Option**. Smooth Options can affect peak height and peak definition. The "Light smoothing option" is recommended for use with the AmpF*l*STR products on the Macintosh computer. • **Peak Detection**: – Select a Peak Amplitude Threshold (PAT) for each dye color. – Use the active scroll bar to enter the PATs for each of the five colors. – After analysis, the GeneScan table contains data for all peaks with a height above that specified by the PAT.

To set the analysis parameters: *(continued)*

	3. *(continued)*
	Note: We suggest that you determine the PATs appropriate for your analysis. Sensitivity experiments should be conducted in your laboratory with each instrument to evaluate the PATs used for analysis.
	– The Min Peak Half Width for use with the AmpF*l*STR products is 3 Pts.
	• **Size Call Range**:
	Click the **This Range (Base Pairs)** radio button and enter the values of **75** for Min and **450** for Max.
	• **Size Calling Method**:
	Click the **Local Southern Method** radio button for sizing of the AmpF*l*STR products. This method determines the sizes of fragments by using the reciprocal relationship between fragment length and mobility.
	• **Split Peak Correction**:
	Click the **None** radio button; no correction is needed for use with the AmpF*l*STR products.
4.	Click **OK** when done.

To set the analysis parameters: *(continued)*

5.	Assign a size standard:
	
	a. Click the arrow in the Size Standard column for a sample file to view the pop-up menu and select **Define New**.
	
	   – For more information on defining a size standard, refer to the *ABI PRISM® GeneScan® Analysis Software Version 3.1 User's Manual*.
	
	   – Do not assign a size for the 250-bp peak for data generated on the ABI PRISM® 310 Genetic Analyzer (*i.e.*, assign a size of zero). This peak can be used as an indicator of precision within a run. Twelve size standard peaks should be viewed at this step, as shown below.
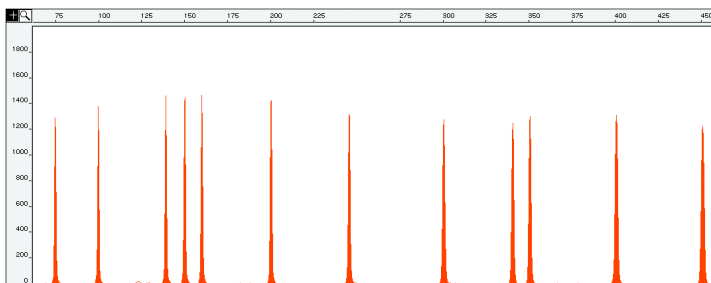	
	Figure 5-11 Standard size peaks
	   – Save the size standard for this sample in the GS Standards Folder in the ABI PRISM GeneScan Version 3.1 Software folder.
	
	b. To apply this standard to all injections, select the appropriate standard in the Size Standard column header (above sample 1) in the **Analysis Control** window.

6.	Analyze sample files: a. Highlight the blue, green, yellow, red, and orange columns. **Note:** Verity that the orange box is indicated as the standard (a diamond symbol should appear in all orange boxes where a size standard is included with the sample files). If the diamond symbol is not in the orange boxes, ⌘-click places a diamond in the box. b. Click the **Analyze** button.

To set the analysis parameters: *(continued)*

7.	After the analysis is complete, confirm that the sizes for the peaks in the GeneScan-500 LIZ Size Standard have been correctly assigned.
	a. Select **Window** > **Results Control** and examine the orange GeneScan-500 LIZ Size Standard peaks in overlapping groups of 16 samples (Quick Tile Off). Be sure to use **View** > **Align By Size** option.
	b. While the samples are tiled, check the 250-bp peaks (sized as approximately 246 bp) in the enlarged view window. Remember that this peak was not defined in the size standard. The tiled 250-bp peaks should size consistently, *i.e.*, should all overlap. In a typical run, the 250-bp peaks all fall within a size window of approximately 1 bp. Temperature fluctuations in the laboratory may cause variations > 1 bp.
	Note: Laboratory temperature variations can cause size shifts. If the temperature of the laboratory varies, try injecting the AmpF*l*STR SEfiler Allelic Ladder approximately every 10 injections, or 5 hours.
	c. Scroll through the tables to verify correct GeneScan-500 LIZ peak assignments.
	d. Check the GeneScan-500 LIZ Size Standard peaks in the remaining samples, taking note of which samples (if any) have incorrect peak assignments.
8.	If the size standard peak assignments are incorrect for one injection, define a new size standard for that sample using the peaks in that injection. To do so, select **Size Standard** > **Define New** for that sample.
9.	Re-analyze any incorrectly sized samples (select the blue, green, yellow, red, and orange boxes) using the newly defined GeneScan-500 LIZ Size Standard file. Re-analyzing creates a new standard file for each of these samples, replacing the previous analysis results for those samples only.

To set the analysis parameters: *(continued)*

10.	Confirm that the GeneScan-500 LIZ Size Standard peaks are now correctly assigned in the re-analyzed samples.
11.	View AmpF*l*STR SEfiler kit results (using the Results Control window).
	Refer to the *ABI PRISM® GeneScan® Analysis Software Version 3.1 User's Manual* for printing options.

GeneScan Software Results

GeneScan Analysis Software

After the sample files have been analyzed, use the Results Control window to display the results from each lane of a gel or each injection into a capillary. The Results Control window displays the newly analyzed sample files and allows the user to specify the format of the results. Selecting both the Electropherogram and Tabular Data icons is recommended for reviewing the results. For more information on displaying the results, refer to the *ABI PRISM® GeneScan® Analysis Software Version 3.1 User's Manual* (P/N 4306157).

Information Provided in the Electropherogram and Table

Both the electropherogram and the tabular data can be displayed. See .

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

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

The columns in the table list the following:

• Column 1 lists the Dye/Sample and Peak (*e.g.*, "4B, 1" indicates the first blue peak in project sample 4).

• Column 2 lists the time it took the dye-labeled fragment to reach the detector.

• Column 3 lists the base pair size of the peak, as calculated using the GeneScan-500 LIZ Size Standard curve.

• Column 4 lists the height in RFU of the peak.

• Column 5 lists the relative peak area, which is the integral of the RFU times the data point (scan number). This value depends on the velocity of the dye labeled fragment as it passes the detector.

• Column 6 lists the data point (scan number) of the dye labeled fragment at its maximum peak height; the data point correlates with the number of laser scans (or data points collected) from the beginning of the run until the time that the peak maximum is detected.

Figure 5-12 is a GeneScan electropherogram of AmpF*l*STR SEfiler alleles in AmpF*l*STR Control DNA 007 analyzed on the ABI PRISM 310 Genetic Analyzer.

Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
7B, 2	13.57	122.74	4056	23339	3700
7B, 4	13.70	126.79	4014	23199	3735
7B, 7	14.87	166.45	3329	21367	4054
7B, 9	15.11	174.33	3110	20212	4119
7B, 11	17.21	244.41	3845	26831	4692
7B, 12	17.33	248.47	3186	23122	4724
7B, 14	19.04	310.51	2033	16232	5193
7B, 16	19.38	323.34	1751	14184	5285
7G, 4	12.95	103.99	6632	38445	3532
7G, 7	13.14	109.57	6172	35266	3583
7G, 11	14.12	140.56	5704	35600	3850
7G, 12	14.25	145.01	5232	32432	3884
7G, 15	17.24	245.68	4195	29233	4702
7G, 18	18.19	278.80	3720	27205	4959
7Y, 5	13.52	121.25	3296	18267	3687
7Y, 7	13.65	125.16	3224	17467	3721
7Y, 12	15.12	174.69	2413	14248	4122
7Y, 14	15.46	185.77	2487	15652	4214
7Y, 18	17.08	240.12	1888	13232	4658
7Y, 21	17.32	248.22	1599	11359	4722
7R, 5	15.99	203.27	1653	10962	4360
7R, 7	16.35	215.11	1625	10965	4457
7R, 9	18.36	284.85	1329	9782	5005
7R, 11	18.70	297.33	1280	9972	5099

Figure 5-12   Electropherogram and tabular data of AmpF*l*STR SEfiler alleles

Results Display Options

The GeneScan Software v3.1 or higher offers two main options in the Results Control window for electropherogram viewing formats: Quick Tile Off and Quick Tile On.

- The Quick Tile Off format provides the option of displaying results either for multiple colors within a single lane or injection or from multiple lanes or injections in the same panel, (*i.e.*, the results are overlaid), as shown in panel 1 of Figure 5-13.

- The Quick Tile On format displays each color of each lane or injection separately, as shown in panels 2–5 of Figure 5-13.

  The Quick Tile Off and On feature offers the user versatility in customizing the display of results. Up to eight panels can be tiled at a single time and up to 16 electropherograms may be overlaid in one panel at the same time.
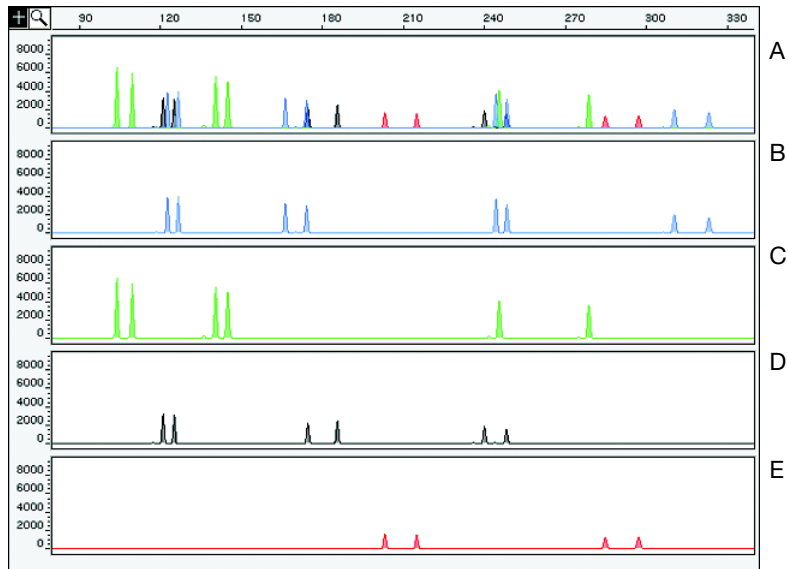
Figure 5-13   Quick Tile Off and Quick Tile On options

Panel A in Figure 5-13 is an example of one sample displayed with Quick Tile Off. Panels B–F are examples of the same sample file with Quick Tile On using the AmpF*l*STR Control DNA 007 analyzed on the ABI PRISM 310 Genetic Analyzer.

Note: For a more detailed description see the *ABI PRISM® GeneScan® Software Version 3.1 User's Manual*.

Standards for Samples

For the SEfiler kit, the panel of standards needed for PCR amplification, PCR product base pair sizing, and genotyping are as follows:

- The AmpF*l*STR Control DNA 007 provides a positive control for the efficiency of the amplification step and STR genotyping using the AmpF*l*STR SEfiler Allelic Ladder.

- GeneScan-500 LIZ Size Standard is used for obtaining base pair sizing results. The GeneScan-500 LIZ Size Standard is designed for sizing DNA fragments in the 35–500 bp range, and it contains 16 single-stranded fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250 (not assigned when used on the ABI PRISM 310 Genetic Analyzer), 300, 340, 350, 400, 450, 490, and 500 bases. This standard has been evaluated as an internal lane size standard and it yields extremely precise sizing results of AmpF*l*STR SEfiler kit PCR products.

- The AmpF*l*STR SEfiler Allelic Ladder was developed by Applied Biosystems for accurate characterization of the alleles amplified by the AmpF*l*STR SEfiler kit. The AmpF*l*STR SEfiler Allelic Ladder contains the majority of alleles reported for the 12 loci.

Off-Scale Data

Overview   If too much sample DNA is added to the PCR reaction mixtures, the fluorescence intensity from the PCR products may exceed the linear dynamic range for detection by the instrument, resulting in "off-scale" data. Multicomponent analysis cannot be performed accurately on data that is off-scale. Samples with off-scale peaks will exhibit raised baselines and/or excessive "pull-up" of one or more colors under the off-scale peaks.

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

Off-Scale Data on the ABI PRISM 310

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

1.	In the GeneScan Analysis Software, highlight the sample file row for the questionable sample in the **Analysis Control** window. Alternatively, select **View** > **Show Offscale Regions** to highlight off-scale data with a red bar. The width of the red bar corresponds to the amount of data that is off-scale.
2.	Select **Sample** > **Raw Data**.
3.	Examine the fluorescence intensity for the raw data peaks. Any peaks that are greater than 8191 relative fluorescence units (RFU) are off-scale.
4.	Re-amplify the sample, if necessary. **Note:** DNA samples with off-scale data should be diluted and re-amplified.

Shutting Down the Instrument

Ending the Run   If the instrument is not going to be in use for three or more consecutive days, it is recommended that the instrument be cleaned and shut down.

To shut down the instrument:

1.	Remove and clean the syringe and block as previously described.
2.	Discard unused polymer in the proper waste container. ⚠️ **CAUTION** **CHEMICAL HAZARD**. **POP polymers** may cause eye, skin, and respiratory tract irritation. Read the MSDS for the polymer you are using, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Use for research and development purposes only. **IMPORTANT!** Do not put unused polymer back into the bottle. Polymer in the syringe decomposes over time at room temperature.
3.	In the Manual Control window, select **Autosampler Home X, Y Axis** and click **Execute**.
4.	Select **Autosampler Home Z Axis** and click **Execute**.
5.	Turn off the instrument.

Dedicated Equipment and Supplies

Required Equipment

The equipment and supplies necessary or recommended for running AmpFℓSTR SEfiler kit data on the ABI PRISM 310 Genetic Analyzer are listed in the tables below.

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

Table 5-2   Equipment Required

ABI PRISM 310 Genetic Analyzer
ABI PRISM 310 Genetic Analyzer Accessories: • ABI PRISM 310 Genetic Analyzer Capillary, L_t = 47 cm, L_d = 36 cm, i.d. = 50 µm (P/N 402839), labeled with a green mark • ABI PRISM 310 Genetic Analyzer Vials, 4.0 mL (P/N 401955) • ABI PRISM 310 Genetic Analyzer 0.5-mL Sample Tubes (P/N 401957) • ABI PRISM 310 Genetic Analyzer Septa for 0.5-mL Sample Tubes (P/N 401956) • Syringe, Kloehn 1.0-mL (P/N 4304471)
Benchkote absorbent protector sheets
Flush-cutting wire cutter (P/N T-6157)
Freezer, –15 to –25 °C, non-frost-free
Gloves, disposable, powder-free
Glassware
Ice bucket
Lint-free tissues
Lab coat
Microcentrifuge tubes, 1.5-mL
Microtube racks
Nalgene filter apparatus, 150-mL, 0.2-µm CN filter

Table 5-2 Equipment Required *(continued)*

Permanent ink pen
Pipet bulb
Pipets, serological
Pipet tips, sterile, disposable hydrophobic filter-plugged
Pipettors, adjustable, 1–10 µL, 2–20 µL, 20–200 µL, and 200–1000 µL
Refrigerator
Repeat pipettor and Combitips that dispense 25–125 µL (optional)
Sink
Syringe, 35 cc (optional)
Tape
Thermal cycler
Tube, 50 mL Falcon
Tube decapper, autoclavable

Required Reagents

Table 5-3 Reagents Required

ABI PRISM 310 10X Genetic Analyzer Buffer with EDTA (P/N 402824)
AG501 X8 ion exchange resin (Bio-Rad)
Deionized water, PCR grade
Hi-Di™ Formamide (P/N 4311320)
GeneScan-500 LIZ Size Standard (P/N 4322682)
Matrix Standard Set DS-33 [6-FAM, VIC, NED, PET, LIZ] for use with the 310/377 system (P/N 4318159)
POP-4 polymer (P/N 402838)

Software and User Documentation Required

Table 5-4   Software and User Documentation

ABI PRISM 310 Data Collection Software, Version 2.1 or higher
ABI PRISM® 310 Genetic Analyzer User's Manual (P/N 903565)
ABI PRISM 310 Module GS STR POP4 (1 mL) G5
GeneScan Software v3.1 or higher
Genotyper Software Version 2.5.2 or higher

Protocols for ABI PRISM 377 DNA Sequencer with Windows NT OS

6

In This Chapter   AmpF*l*STR® SEfiler™ PCR Amplification Kit products are run on 36-cm well-to-read plates on the ABI PRISM® 377 DNA Sequencer, the ABI PRISM 377 DNA Sequencer with XL Upgrade (ABI PRISM 377XL), or the ABI PRISM 377 DNA Sequencer with 96-Lane Upgrade (377-96 instrument). Protocols for analyzing samples on these configurations with the Microsoft® Windows NT® operating system are included in this chapter.

Analysis Software

Software Version   This chapter was written for use with ABI PRISM® GeneScan® Analysis Software Version 3.7.1 or higher. Refer to the *ABI PRISM® 377 DNA Sequencer User Guide* (P/N 4325703) and *ABI PRISM® GeneScan® Analysis Software Version 3.7 for the Windows NT® User Guide* (P/N 4308923), or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* (P/N 4335617) for more detailed information on the instrument and software used with these protocols.

You must use Filter Set G5 for data collection of AmpFℓSTR SEfiler kit PCR products. Install Filter Set G5 module files in the Modules folder within the ABI PRISM 377 or ABI PRISM 377XL folder on the instrument's Microsoft Windows NT computer before following the protocols in this chapter.

IMPORTANT! Before running AmpFℓSTR SEfiler kit PCR products on the instrument, you must make a matrix file using the 6-FAM™, VIC®, NED™, PET™, and LIZ® matrix standards and Filter Set G5.

Equipment and Supplies

Supplies   For a list of required equipment, supplies, chemicals, reagents, software, and documentation to use with the ABI PRISM 377 DNA Sequencer, refer to "Dedicated Equipment and Supplies" on page 6-29.

36-cm Well-to-Read Gel Assembly

Preparing Plates for the ABI PRISM 377 DNA Sequencer

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

1.	Clean 36-cm well-to-read gel plates with Alconox® detergent using a dedicated wash cloth or lint-free tissues. Rinse with deionized water and air dry.
	To obtain the MSDS for Alconox, go to:
	http://www.jtbaker.com/msds/a2052.htm
2.	Place the larger, unnotched plate with the center etch facing down on a covered benchtop. The narrow width is the bottom portion of the plate.
3.	Place 0.2-mm spacers on either side of the plate, with the notched end of the spacer at the top of the plate and the notch facing the center of the plate.
4.	Place the notched ("rabbit eared") plate over spacers, making sure that the side that had been previously in contact with the silicone rubber gasket (of the upper buffer chamber) is external. This is the side with the etched writing on it.
5.	Clamp the plates together using four medium binder clips per side. Place the clamps directly over the spacers.
6.	Elevate the top of the plates approximately 1.0 cm. A pipet tip box top is ideal for this purpose.

Preparing Plates for the ABI PRISM 377 with XL Upgrade

When using the 50-lane comb for the ABI PRISM 377 DNA Sequencer with XL Upgrade, treat the well-forming region of the notched (rabbit-eared) glass plate with Bind-Silane to immobilize the wells and facilitate subsequent loading of the gel. To obtain the MSDS for Bind-Silane, go to:

http://www.promega.com/msds/us/q421.htm

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

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

1.	Clean 36-cm well-to-read gel plates with Alconox detergent using a dedicated wash cloth or lint-free tissues. Rinse with deionized water and air dry.
2.	Place the cleaned, notched (rabbit-eared) glass plate face up on a covered benchtop.
3.	Dip the end of a cotton swab into a bottle of Bind-Silane to wet the tip.
4.	Allow the Bind-Silane to dry on the plate for 1 minute.
5.	Dry the treated region with a lint-free tissue by wiping three times using moderate pressure.
6.	Assemble the plates as described in "Preparing Plates for the ABI PRISM 377 DNA Sequencer" on page 6-3, beginning with step 2.
7.	After running the gel, clean the plates using Alconox detergent. It may be necessary to scrape the gel off the notched plate where it has stuck to the Bind-Silane-treated region.

Preparing Plates for the ABI PRISM 377 DNA Sequencer with 96-Lane Upgrade

To prepare 36-cm well-to-read plates for the ABI PRISM 377 DNA Sequencer with 96-Lane Upgrade:

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

Preparing 36-cm Well-to-Read Gels

We recommend using Long Ranger® gel solutions instead of 4% acrylamide for the 36-cm well-to-read plates. Long Ranger gels form better wells around the comb, facilitating loading and potentially enhancing results. This procedure to prepare 50 mL of 5% Long Ranger/6.0 M urea gel mixture makes enough gel mixture for two 36-cm well-to-read gels.

To prepare the gel mixture:

1.	Combine the following: • Urea 18.0 g • Deionized water 21.5 mL • 5X TBE (Maniatis formulation)[a] 10.0 mL • 50% Long Ranger stock solution 5.0 mL ⚠ **WARNING** **CHEMICAL HAZARD**. **Urea** is a possible mutagen. Do not breathe the dust. Urea can be harmful by inhalation, skin contact, and ingestion. Refer to the MSDS for proper protective equipment. ⚠ **WARNING** **CHEMICAL HAZARD**. **TBE 5X** buffer causes eye, skin, and respiratory tract irritation. Exposure may cause central nervous system depression and kidney damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. ⚠ **WARNING** **CHEMICAL HAZARD**. **Long Ranger gel solution** contains acrylamide. Acrylamide is a neurotoxin. Avoid skin contact with Long Ranger gel solution because acrylamide can be absorbed through the skin. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.

To prepare the gel mixture: *(continued)*

2.	Warm the mixture in a 37 °C water bath, stirring occasionally, to dissolve the urea. Once the urea is dissolved, allow the gel mixture to equilibrate to room temperature. This gel mixture should be used within 12 hours. Keep the gel mixture covered during this step to prevent evaporative loss.
3.	Filter the gel mixture using a 150-mL Nalgene filter apparatus with a 0.2-micron CN filter. Attach to a vacuum source (approximately 20 inches Hg) to pull liquid through filter. This step removes any particulates that may fluoresce or scatter light.
4.	Let stand for 5 minutes, swirling occasionally, with the vacuum on to degas the mixture.
5.	Turn off the vacuum, remove the top of the filter apparatus, and discard. **Note:** The filter apparatus can be rinsed thoroughly with deionized water and air-dried to be reused up to five times.
6.	Add 250 µL of freshly prepared 10% APS and 35 µL TEMED to the gel mixture. Swirl gently to mix. ⚠ **DANGER** **CHEMICAL HAZARD**. **Ammonium persulfate** is an oxidizer, and contact with other materials may cause a fire. Exposure causes burns to the eyes, skin, and respiratory tract. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. ⚠ **WARNING** **CHEMICAL HAZARD**. **TEMED** is extremely flammable, and can be very destructive to the skin, eyes, nose, and respiratory system. Keep TEMED in a tightly closed container. Avoid inhalation and contact with the skin, eyes, and clothing. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves. **IMPORTANT!** Proceed to the next procedure immediately.

a. See "Preparation of Required Reagents" on page 6-33.

To pour gels:

1.	Draw the gel mixture up into a 35-cc syringe. Slowly inject gel mixture into the top center area between the plates. **Note:** Tap the gel plates firmly with your palm while pouring to prevent bubbles and move gel mixture down to the bottom of the plates.
2.	Stop injecting when the gel mixture is near the bottom of the plates. Immediately lay plates flat on a level surface. Each gel requires approximately 20–25 mL of Long Ranger gel mixture.
3.	Follow the procedure in the table below for the instrument you are using:

If using the...	Then...
ABI PRISM 377 DNA Sequencer	Insert a 34-well 0.2-mm square-tooth comb and clamp into place with three large binder clamps over the comb. **Note:** A 24-well square-tooth comb can also be used.
ABI PRISM 377 with XL Upgrade DNA Sequencer	Insert a 50-well 0.2-mm square-tooth comb and clamp into place with three large binder clamps over the comb.
ABI PRISM 377 with 96-Lane Upgrade DNA Sequencer	Insert a 96-lane Mylar 0.4 mm shark's tooth comb into gel, with the flat side of the comb contacting the gel.

To pour gels: *(continued)*

4.	Allow gel to polymerize for at least 2.0 hours.
	Note: The polymerized gel can be stored for up to 24 hours. After polymerization, place a lint-free tissue wetted with deionized water over the top and bottom edges of the gel plates. Do not remove the gel comb. Wrap the top and bottom edges tightly with clear plastic wrap and store at room temperature.
5.	Prepare 1.5 L of 1X TBE (Maniatis formulation) running buffer (89 mM Tris, 89 mM borate, 2 mM EDTA).
	⚠ **WARNING** **CHEMICAL HAZARD**. **TBE 1X** buffer causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Setting Up the Instrument

Setting up the Gel Preferences and Analysis Parameters

To set up the gel preferences:

1.	Launch the Gel Processor 1.0 Software by selecting **Start** > **Applied Biosystems**.
2.	Select **Edit** > **Gel Preferences**.
3.	Fill in the dialog box with the settings shown in Figure 6-1. **Note:** Choose the appropriate comb type. **Figure 6-1 Gel Preferences dialog box** **Note:** You should extract lanes from the full scan range of gel. Primer peaks can be excluded using GeneScan software not the gel processor.
4.	Click **OK** when done.
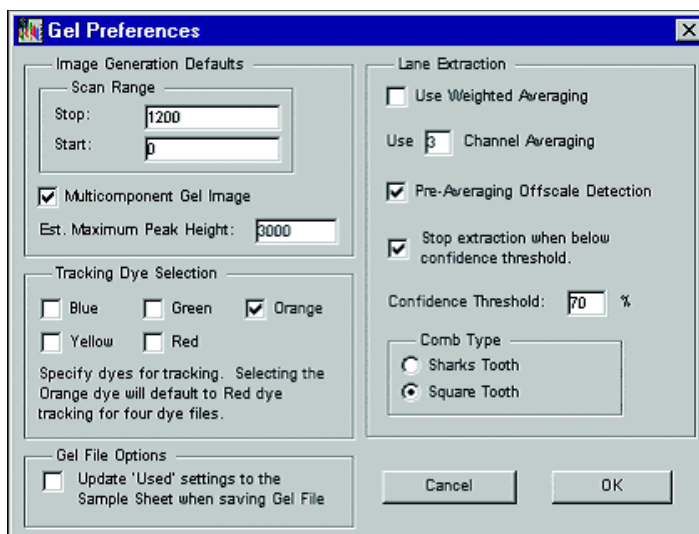
Setting up Analysis Parameters

To set up the analysis parameters:

1.	Launch the GeneScan Analysis Software v3.7.1 by selecting **Start**.
2.	Select **Settings** > **Analysis Parameters**. **Note:** A more detailed discussion for each of the six Analysis Parameters is in Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," and the *ABI PRISM® GeneScan® Analysis Software Version 3.7 User Guide*.
3.	Enter parameters in the dialog box: *(see table below)*

Parameter	Entry
Analysis Range	1. Click the **This Range (Data Points)** radio button. 2. Enter Start and Stop data point numbers in the entry fields. Select the **Start** data point just before the first peak of interest, the 75 bp size standard peak. At a minimum, select the **Stop** data point just after the last peak of interest, the 450 bp size standard peak. 3. Look at the raw data and enter the values that are appropriate for all sample files in the project. These data points affect data in the results display.
Smooth Options	The default parameter for Smooth Options is light. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI Prism® GeneScan Analysis Software Version 3.7.1 User Bulletin* (P/N 4335617) for more information on how to set the appropriate value for smooth options.

To set up the analysis parameters: *(continued)*

3. (continued)

Parameter	Entry
Peak Detection	1. Select a Peak Amplitude Threshold (PAT) for each dye color. 2. Use the active scroll bar to enter the PATs for each of the five colors. 3. After analysis, the GeneScan table contains data for all peaks with a height above that specified by the PAT. **Note:** We suggest that you determine the PATs appropriate for your analysis. Sensitivity experiments should be conducted in your laboratory with each instrument to evaluate the PATs used for analysis.
Polynomial Degree	The default parameter for polynomial degree is 3. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* for more information on how to set the appropriate value for the polynomial degree.
Min. Peak Half Width	The Min Peak Half Width for use with the AmpFℓSTR products is 2 Pts. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* for more information on how to set the appropriate value for Min. Peak Half Width.

To set up the analysis parameters: *(continued)*

3. *(continued)*

Parameter	Entry
Peak Window Size	The default parameter for peak window size is 15. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* for more information on how to set the appropriate value for Peak Window Size.
Size Call Range	Click the **This Range (Base Pairs)** radio button and enter the values of **75** for Min and **450** for Max.
Size Calling Method	Click the **Local Southern Method** radio button for sizing of the AmpFℓSTR products. This method determines the sizes of fragments by using the reciprocal relationship between fragment length and mobility.
Baselining	The default setting for the baseline window size is 51 pts. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* (P/N 4335617) for more information on how to set the appropriate value for the baseline window size.

To set up the analysis parameters: *(continued)*

3. *(continued)*	

Parameter	Entry
Slope Threshold	The default parameter for slope threshold for peak start and peak end should be 0.0. Refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan Analysis Software Version 3.7.1 User Bulletin* (P/N 4335617) for more information on how to set the appropriate value for slope threshold.
Auto Analysis Only	Refer to the user bulletin (P/N 4335617) for more information.

4.	Click **OK** when done.

Setting up the Sample Sheet/ Electrophoresis Parameters

To prepare a sample sheet:

1.	Launch the Data Collection Software by selecting **Start**, then select the data collection appropriate for your 377 instrument: • 377XL • 377 96-well
2.	If a sample sheet needs to be created, select **File** > **New** > **GeneScan Sample**. Otherwise skip to step 1 of the next procedure, on page 6-16.
3.	Select 5 dyes from the sample sheet template window in the upper right corner.
4.	Enter sample names/numbers for each lane in the Sample Name column.
5.	a. Enter the sample description for each row in the Sample Info column (for Blue, Green, Yellow, and Red for each sample). This is necessary for the AmpF*l*STR SEfiler Kit Template File to build tables containing the genotypes for each sample. b. Type the word **ladder** for the Blue, Green, and Yellow rows for the AmpF*l*STR SEfiler Allelic Ladder lane.
6.	Be sure that the diamond symbol in the "std" column indicates the orange sample as the standard in each lane.
7.	Save the sample sheet in the Sample Sheets folder.
8.	Close the sample sheet. **Note:** For more information on how to create and edit a sample sheet, refer to the *ABI PRISM® 377 DNA Sequencer User Guide*.

To create a run file:

1.	Select **File** > **New** and click **GeneScan Run**.
2.	Select a sample sheet that has been prepared previously. The run module cannot be started without a sample sheet.
3.	a. Select **Plate Check Module** > **Plate Check G5.md5** as shown in the figure below. b. Select **PreRun Module** > **GS PR 36G5-2400.md5**. c. Select **Run Module** > **GS Run G5-2400.md5**. d. Click the document icon to the right of the Run Module to verify the electrophoresis settings for the particular run. Make sure the settings are the same as shown in Figure 6-2. **Figure 6-2 Settings for GS Run 36G5-2400.md5 Run Module**
4.	Click **Save**. **Note:** To save these settings as the default, click **Save As Default**.
5.	Make selections from the Lanes pop-up menu: | If using the...	Select...
ABI PRISM 377 DNA Sequencer	34-Well Comb
ABI PRISM 377 DNA Sequencer with XL Upgrade	50-Well Comb
ABI PRISM 377 DNA Sequencer with 96-Lane Upgrade	96-Well Comb	
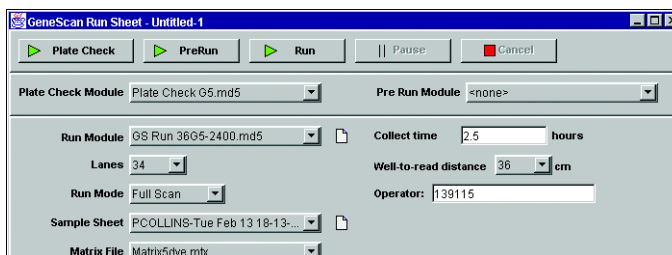
To create a run file: *(continued)*

6.	Select the appropriate matrix file from the gel's **Matrix File** pop-up menu. The matrix file must be previously prepared and saved in the matrix folder located in the D drive so the matrix file can be applied automatically to the gel image: `D:\AppliedBio\Shared\Analysis\SizeCaller\Matrix` **IMPORTANT!** The matrix file must be one that was created using the 6-FAM, VIC, NED, PET, and LIZ® matrix standards and Filter Set G5 module files.
7.	In addition, select the appropriate matrix file for each sample in the Sample Sheet area of the run window. This is necessary to have the matrix applied automatically to the sample files. To do so: a. Click the arrow in the **Matrix File** column for the first sample/lane to view the pop-up menu and select the appropriate matrix file. b. After selecting a matrix file for the first sample/lane, select the entire matrix column by clicking the column heading and selecting **Edit** > **Fill Down**.
8.	a. Verify that the collection time is 2.5 hours. b. Set the well-to-read distance to 36 cm for the GS Run G5-2400 module.
9.	Make selections from the Run Mode pop-up menu:

If using the...	Select...
ABI PRISM 377 DNA Sequencer with comb size of 34 lanes	34-Lane Scan
ABI PRISM 377 with XL Upgrade DNA Sequencer and comb size of 50 lanes	XL Scan
ABI PRISM 377 with 96-Lane Upgrade DNA Sequencer and comb size of 96 lanes	96-Lane Scan

|
| 10. | Select **File** > **Save** to save all collection settings. |

Running the Plate Check and Prerun Modules

To place the gel plates into the instrument:

1.	Remove the comb. **IMPORTANT!** Remove it slowly while lubricating with 1X TBE for the best results. ⚠ **WARNING CHEMICAL HAZARD**. **TBE 1X** buffer causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	Remove any gel that remains around the top of the wells.
3.	Clean the outside of the gel plates with deionized water and wipe dry. **IMPORTANT!** Do not touch the plates in the laser scanning region from this point forward.
4.	Place the gel plates into the ABI PRISM 377 DNA Sequencer cassette. Place the cassette into the instrument, making sure that the lower buffer chamber is already in place.
5.	Make sure the gel plate spacers are pressing against the two positioning pins on the instrument.
6.	Close the instrument door.

To run the plate check:

1.	Click the **Plate Check** button. Five colored, horizontal lines should appear in the scan window after approximately 30 seconds.

If the lines in the scan window...	Then...
are relatively flat and level across the screen	The plates are clean. Click the **Cancel** button to cancel plate check.
show peaks	The plates are not clean. 1. Pause the plate check, remove the cassette, and clean the plates again. 2. Resume plate check.
are flat but not level across the screen	The cassette is not positioned properly in the instrument (*i.e.,* the plates are not flush against the back cooling plate and/or the two positioning pins that set the plates the correct distance from the optics). 1. Pause the plate check and reposition the cassette. 2. Resume the plate check.

2.	After determining that the plates are clean and positioned correctly, clamp the upper buffer chamber onto the gel plates.
3.	Add 1X TBE buffer to the upper buffer chamber and check for leaks. ⚠️ **WARNING** **CHEMICAL HAZARD. TBE 1X** buffer causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Clamp on and connect the cooling plate.

To run the plate check: *(continued)*

5.	Add 1X TBE buffer to the lower buffer chamber.
	Optional: To facilitate the subsequent loading of the gel, drop 15 µL of blue dextran loading buffer (contained in the GeneScan®-500 [LIZ] Size Standard Kit) over the wells in a sweeping motion using a P10 or P20 pipet tip. The dye outlines the walls and bottom of the wells.
6.	Plug in the electrodes and close the instrument door.

Note: Prepare samples while you prerun the gel.

To perform the prerun:

1.	Click the **PreRun** button in the Run window. Prerun the gel for approximately 15 minutes.
2.	Click **Pause** in the Run window to pause the prerun. Pausing stops the electrophoresis, but continues to heat the gel to 51 °C and then maintains that temperature.
	Note: Samples can be loaded onto the gel during this pause. See page 6-23 for sample loading information.
3.	Prior to loading the gel, unplug the cathode and check the Status window to verify that the electrophoresis power has been turned off.

Electrophoresis

Preparing Samples and AmpF*l*STR SEfiler Allelic Ladder

To prepare samples and allelic ladder:

1.	Prepare formamide loading solution (FLS) by combining: • Blue dextran "Loading Buffer" (contained in GeneScan-500 LIZ Size Standard Kit), 100 µL • Deionized formamide, 500 µL ⚠️ **WARNING**　**CHEMICAL HAZARD**. **Formamide** is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling.
2.	Vortex to mix. FLS can be stored at 2–6 °C for up to 2 weeks.
3.	Combine the necessary amount of FLS and GeneScan-500 LIZ Size Standard in a single microcentrifuge tube as shown: • (Number of samples) × 5.0 µL FLS denaturant • (Number of samples) × 0.55 µL GeneScan-500 LIZ Size Standard **Note:** The above formulation provides a slight overfill to allow for volume lost in pipetting. Be sure to include two lanes of AmpF*l*STR SEfiler Allelic Ladder per gel in the calculations.
4.	Vortex the tube to mix, and spin briefly in a microcentrifuge.
5.	Aliquot 5.0 µL of FLS/GeneScan-500 LIZ mixture into 0.5-mL GeneAmp® Thin-walled Reaction Tubes with caps (for use in the DNA Thermal Cycler 480) or 0.2-mL MicroAmp® tubes with caps (for use in the GeneAmp PCR Instrument System 2400, 9600, or 9700). Label one tube per sample.

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

6.	Add 4.0 µL PCR product or AmpF*l*STR SEfiler Allelic Ladder per tube and close the caps. **Note:** You may need to increase the amount of allelic ladder to achieve greater signal intensity.
7.	Heat samples in a thermal cycler for 2 minutes at 95 °C to denature them.
8.	Chill for at least 3 minutes in an ice-water bath. Keep on ice until ready to load.

Loading Samples

To load samples:

1.	Rinse the urea out of each sample well with buffer, using a 0.17-mm flat pipet tip attached to a 35-cc syringe.

⚠ **WARNING** **CHEMICAL HAZARD**. **Urea** may cause eye, skin and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ **WARNING** **CHEMICAL WASTE HAZARD**. Dispose of it in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Note: It may be necessary to cut off the top end of the pipet tip with a razor blade for it to fit on the end of the Luer lock syringe without leaking. |
| 2. | Load the sample for the appropriate instrument as shown in the table below:

If using the...	Then...
ABI PRISM 377 DNA Sequencer	Load 1.5 µL of denatured sample or allelic ladder per well. The AmpF*l*STR SEfiler Allelic Ladder can be loaded into at least two wells on the gel.
ABI PRISM 377 with XL Upgrade DNA Sequencer	Load 1.0 µL of denatured sample or allelic ladder per well. The AmpF*l*STR SEfiler Allelic Ladder can be loaded into at least two wells on the gel.
ABI PRISM 377 with 96-Lane Upgrade DNA Sequencer	Load 1.0 µL of denatured sample or allelic ladder per well. The AmpF*l*STR SEfiler Allelic Ladder can be loaded into at least two wells on the gel.	
3.	Attach the upper buffer chamber lid, plug in the cathode, and close the instrument door.

Sample Electrophoresis

To run samples:

1.	Select **Cancel** to stop the prerun.
	IMPORTANT! Wait 30 seconds before proceeding.
2.	Select **Run** to start gel electrophoresis. Name the Gel file and click **Save**.
3.	Select **Window** > **Status** to check that the run settings are correct and to monitor the run.
	Note: The data collection can be viewed at any time during the run by selecting either Scan or Gel Image from the Window menu.
	IMPORTANT! The Gel Image window should not be left open for long periods of time during the run, as it occupies a large block of memory.

After electrophoresis is completed, the computer automatically launches the Gel Processor 1.0 to generate a gel image on the screen, if it is selected as a preference in the 377 Data Collection Software. The gel preferences last saved in this application are used to generate the gel image.

Analyzing the Data

Using the Gel Processor Software

Note: For more information on data analysis, refer to the *ABI PRISM® 377 Gel Processor Software User Guide* (P/N 4317592).

To analyze data:

1.	Examine the orange GeneScan-500 LIZ Size Standard bands on the gel picture and check that the 75-bp and 450-bp bands are present.
2.	Verify the automatic lane tracking. It may be necessary to adjust the gel contrast for blue, green, and yellow to see all of the bands clearly. Zoom in on the gel image by selecting **View** > **Zoom In**. a. Move the lane tracking line over each sample lane, using the arrow keys on the computer keyboard or the mouse. Change the channel and introduce nodes as necessary. When modifications are made to a lane, the associated diamond lane marker turns from blue to white. b. Mark all used lanes for extraction from the Gel menu (white diamond lane marker) before proceeding to step c. c. Save the changes that have been made by selecting **File** > **Save**.

To analyze data: *(continued)*

3.	Select **Control** > **Extract Lanes**, to create the sample files. Make selections in the dialog box as shown below: **Extract Lanes** Extract from: ○ All 'Used' Lanes ◉ Lanes marked for Extraction (white markers) ○ Use Sample Sheet Settings ☑ Over-Write Original Sample Files ☑ Save Gel after Extraction ☐ Update 'Used' to Sample Sheet Cancel OK **Figure 6-3 Extract Lanes dialog box**
4.	Click **OK** when done.
5.	Open the GeneScan 3.7.1 software.
6.	Create a new project and import the sample files.
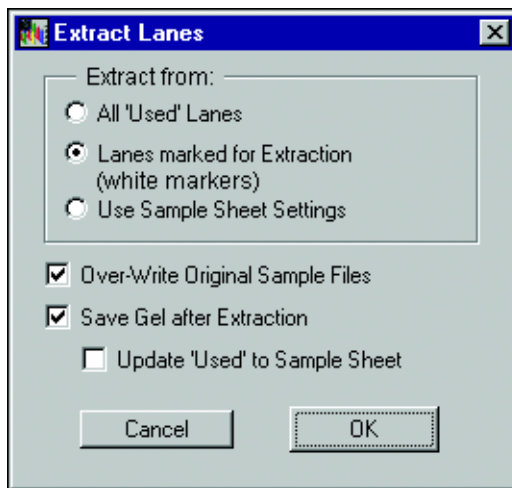
To analyze data: *(continued)*

7. Assign a size standard:

a. Ctrl-click the orange box column in the Analysis Control window to indicate **Orange** as the standard (a diamond symbol should appear in the orange boxes).

b. Click the arrow in the **Size Standard** box for the first sample to view the pop-up menu and select **Define New**, or select a correct standard already stored.

For more information on defining a size standard, refer to Appendix E, "ABI PRISM GeneScan Analysis Software for the Windows NT OS," or the *ABI PRISM® GeneScan® Analysis Software Version 3.7 User Guide*. Refer to the figure below for the sizes of the peaks in the GeneScan-500 LIZ Size Standard. Figure 6-4 shows the markers from 75–450 bp. Tabular data is shown below the electropherogram.
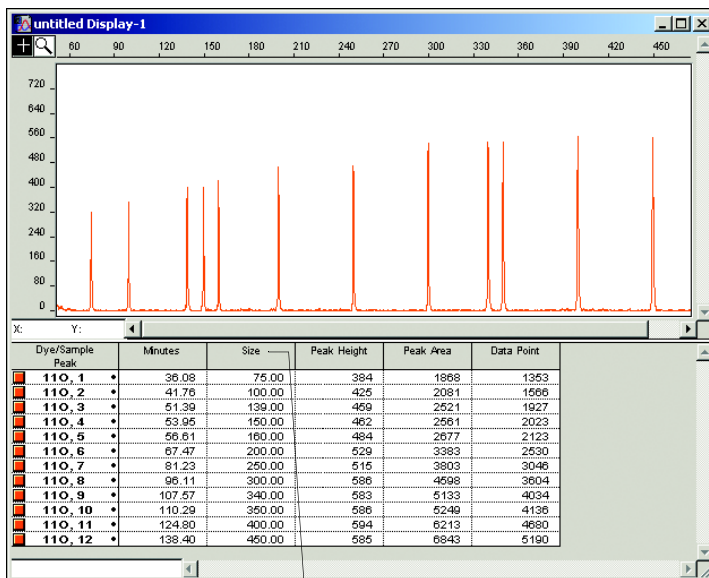
Click here to select the Size column

Figure 6-4   GeneScan-500 LIZ Size Standard

c. To apply one size standard to all lanes, select the **Size Standard** column. Refer to Figure 6-4.

d. Copy the size standard to other rows by using **Ctrl+C** then **Ctrl+V**.

To analyze data: *(continued)*

8.	Analyze the sample files:
	a. Highlight the blue, green, yellow, and red columns.
	b. Click the **Analyze** button.
	This action calculates the base pair size of each fragment and fills the sample files with the analyzed data from each lane.
9.	After the analysis is complete, confirm that the sizes for the peaks in the GeneScan-500 LIZ Size Standard have been correctly assigned.
	a. Select **Window** > **Results Control** and examine the orange GeneScan-500 peaks in overlapping groups (Quick Tile Off). Use Ctrl+ to enlarge the view window for more careful examination; use Ctrl– to reduce it to full range. Be sure to select **View** > **Align By Size**.
	b. Scroll through the tables to verify correct peak assignments (see Figure 6-4 on page 6-27). Check the remaining lanes, taking note of which lanes (if any) have incorrect peak assignments.
	c. If the size standard peak assignments are incorrect in one lane, define a new size standard for that sample using the peaks in that lane. To do so, select the **Define New** option in the Size Standard row for that sample (see step 7b).
	d. Re-analyze any incorrectly sized lanes (select the blue, green, yellow, and red boxes) using the newly defined GeneScan-500 LIZ Size Standard file for that lane. Re-analyzing creates a new standard file for each of these lanes, replacing the previous analysis results for those lanes only.
	e. Confirm that the GeneScan-500 LIZ Size Standard peaks are now correctly assigned in the re-analyzed lanes.
10.	View the AmpF*l*STR SEfiler kit results (using the Results Control window) and print. Refer to the *ABI PRISM® GeneScan® Analysis Software Version 3.7 User Guide* for printing options.

Dedicated Equipment and Supplies

Equipment Required

The equipment and supplies necessary or recommended for running AmpFℓSTR SEfiler kit data on the ABI PRISM 377 DNA Sequencer are listed in the tables below. You can order the part numbers listed below from Applied Biosystems. If no part number is listed, you can order from any major laboratory supplier.

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

Table 6-1   Equipment

ABI PRISM 377 DNA Sequencer, ABI PRISM 377 DNA Sequencer with XL Upgrade, or ABI PRISM 377 DNA Sequencer with 96-Lane Upgrade

ABI PRISM 377 DNA Sequencer Accessories:
• 36-cm well-to-read plates:
  – 36-cm rear glass plate (P/N 401839)
  – 36-cm front glass plate (P/N 401840)
  – 36-cm step plate for 96 wells (P/N 4305384)
  – 36-cm gel spacers, 0.2 mm thick (P/N 401836)
• 34-well square tooth comb, 0.2 mm thick (P/N 401907)
• 50-well square tooth comb, 0.2 mm thick (P/N 402053)
• 96-lane Mylar shark's tooth comb, 0.4 mm (P/N 4305385)
• optional: 24-well square tooth comb, 0.2 mm thick (P/N 401904)
• XL Upgrade for the ABI PRISM 377 DNA Sequencer

Benchkote absorbent protector sheets
Binder clips, medium and large
Freezer, –15 to –25 °C, non-frost-free
Gloves, disposable, powder-free
Glassware
Ice bucket
Lint-free tissues

Table 6-1 Equipment *(continued)*

Lab coat
Lamp, 27-inch gooseneck with magnetic base (Sunnex, P/N 701-27, or office supply store)
Microtube racks
Nalgene filter apparatus, 150-mL, 0.2-µm CN filter
Permanent ink pen
Pipet bulb
Pipets, serological
Pipet tips, sterile, disposable hydrophobic filter-plugged
Pipet tips for gel loading, 0.2 mm flat tips (Rainin, P/N GT1514)
Pipettors, adjustable, 0.5–10 µL, 2–20 µL, 20–200 µL, and 200–1000 µL
Refrigerator
Repeat pipettor and Combitips that dispense 1–5 µL (optional)
Sink
Syringe, 20- or 35-cc (optional)
Thermal cycler
Tube decapper, autoclavable

Required Chemicals and Reagents

Table 6-2   Chemicals and Reagents

Alconox detergent
AG501 X8 ion exchange resin (Bio-Rad)
Ammonium persulfate (APS)
Blue dextran loading buffer (LIZ) (P/N 402055)
Hi-Di™ Formamide, 24 mL (P/N 4311320)
Long Ranger, 50% stock solution (BioWhittaker Molecular Applications (P/N 50611)
Matrix Standards Set DS-33 (P/N 4318159)
GeneScan-500 LIZ Size Standard (P/N 4322682)
TBE, 5X (see "Preparation of Required Reagents" on page 6-33)
N,N,N´,N´-Tetramethylethylenediamine (TEMED)
Urea
Tris base (MLS)
Sodium hydroxide pellets (NaOH)
Water, glass distilled, deionized
Disodium ethylenediaminetetraacetate, dihydrate (Na$_2$EDTA•2H$_2$0) (MLS)

Software and User Documentation Required

Table 6-3   Software and User Documentation

ABI PRISM® 377 DNA Sequencer User Guide For Data Collection Software on the Windows NT® Platform (P/N 4325703)
ABI PRISM® 377 DNA Sequencer with 96-Lane Upgrade User Bulletin (P/N 4313688)
ABI PRISM® 377 DNA Sequencer 96-Lane Upgrade User's Manual (P/N 4305423)
ABI PRISM GeneScan Analysis Software Version 3.7
ABI PRISM® GeneScan® Analysis Software Version 3.7 for the Windows NT® Platform User Guide (P/N 4308923)
Filter Set G5 module files (located in the Modules folder within the ABI PRISM 377, ABI PRISM 377XL, or 377-96 folder): • Plate Check G5 • GS PR 36G5-2400 • GS Run 36G5-2400

Preparation of Required Reagents

To prepare 0.5 M EDTA, pH 8.0:

1.	Slowly add 186.1 g disodium ethylenediaminetetraacetate dihydrate ($Na_2EDTA \cdot 2H_2O$) to 800 mL glass-distilled or deionized water. ⚠ **CAUTION** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	Stir vigorously on a magnetic stirrer.
3.	Adjust to pH 8.0 ± 0.2 by adding NaOH pellets (approximately 20 g). ⚠ **DANGER** **CHEMICAL HAZARD**. **Sodium hydroxide (NaOH)** causes severe eye, skin, and respiratory tract burns. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **Note:** EDTA will not go into solution without pH adjustment.
4.	Adjust the final volume to 1 L with glass-distilled or deionized water.
5.	Autoclave the solution or filter it through a 0.2 µm Nalgene filter. Store at room temperature.

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

⚠️ **WARNING** **CHEMICAL HAZARD**. **TBE 5X** buffer causes eye, skin, and respiratory tract irritation. Exposure may cause central nervous system depression and kidney damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare 5X TBE:

1.	Add approximately 900 mL of deionized water to 20 mL of 0.5 M EDTA, pH 8.0.
2.	Add 54 g Tris base and 27.5 g boric acid to the diluted EDTA solution. Stir vigorously on a magnetic stir plate. ⚠️ **WARNING** **CHEMICAL HAZARD**. **Boric acid** is a hazardous chemical that is harmful if ingested, inhaled, or absorbed through the skin and can be irritating to the eyes, respiratory system, and skin. Handling boric acid while pregnant brings possible risk to the unborn child. Prolonged or repeated exposure can potentially impair fertility. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
3.	Adjust the volume to 1 L with deionized water and mix thoroughly.
4.	Filter the mixture using a 0.2 µm or 0.45 µm Nalgene filter unit to remove particulate matter and prevent formation of a precipitate.
5.	Store in a glass container to facilitate visual inspection for precipitates. If a precipitate forms, discard the 5X TBE buffer and remake it. ⚠️ **WARNING** **CHEMICAL HAZARD**. **TBE 5X** buffer causes eye, skin, and respiratory tract irritation. Exposure may cause central nervous system depression and kidney damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare Deionized Formamide:

1.	Mix 50 mL of formamide and 5 g of AG501 X8 ion-exchange resin. ⚠ **WARNING** **CHEMICAL HAZARD**. **Formamide** is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling. Refer to the supplier's MSDS for more details on handling and storage.
2.	Using a magnetic stirrer and stirbar, stir for 1–3 hours at room temperature.
3.	Filter the Formamide through a 150 mL Nalgene filter apparatus with a 0.2 µM nylon filter. Alternatively, if a filter apparatus is not available, allow the beads to settle to the bottom of the beaker. Remove the supernatant (formamide), taking care not to disturb the beads. **Note:** The conductivity of the solution should be approximately 30 µ siemens or less.
4.	Dispense the deionized formamide into aliquots of 500 µL and store for up to 3 months at –15 to –25 °C.
5.	Use one aliquot per set of samples. Discard any unused deionized formamide.

ABI PRISM 3100 Genetic Analyzer

7

In This Chapter

This chapter describes protocols for processing AmpFℓSTR® PCR Amplification Kit PCR products on the ABI PRISM® 3100 Genetic Analyzer, using ABI PRISM® 3100 Data Collection Software Version 1.1 and GeneScan® Analysis Software Version 3.7.1.

Protocols for Processing AmpF*l*STR PCR Amplification Kit PCR Products

Applicable AmpF*l*STR Kits

PCR products generated from any of the AmpF*l*STR PCR Amplification Kits may be used with the 3100 protocols described in this chapter.

Examples of results obtained using three of the AmpF*l*STR PCR Amplification Kits are in this chapter. See the table below for a list of these kits.

Examples in This Chapter

Examples of results obtained using the AmpF*l*STR PCR Amplification Kits listed below are provided in this chapter. (The examples are shown on .)

Kit	Dyes	Matrix Standard Set
AmpF*l*STR® Identifiler® PCR Amplification Kit	• 6-FAM™ • VIC® • NED™ • PET™ • LIZ®	DS-33
AmpF*l*STR® Profiler Plus™ PCR Amplification Kit AmpF*l*STR® SGM Plus® PCR Amplification Kit	• 5-FAM™ • JOE™ • NED™ • ROX™	DS-32

Process Overview

Flowchart   This flowchart illustrates the procedures required to run the AmpF*l*STR PCR Amplification Kit PCR products on the 3100 Genetic Analyzer.

IMPORTANT! Refer to the reagent warnings on page 7-4 before performing these procedures.

```
• Turn on computer. OrbixWeb™ daemon and software
  automatically launches.
• Turn on 3100 Genetic Analyzer. Wait until the green
  light appears.
• Launch the ABI PRISM 3100 Data Collection Software.
```
↓
```
• Preset Autosampler and place fresh deionized water
  and 1X running buffer in positions 1 to 4.
• Place lower polymer block and anode buffer jar on the
  instrument.
```
↓
```
• Clean the capillary array detection window with
  methanol, if necessary.
• Use the Capillary Array Wizard to either install,
  remove and store, or discard an array. The wizard
  takes you through the following steps:
   – Installing or removing the capillary array
   – Filling a 5-mL reserve syringe and a 250-µL array
     syringe with 3100 POP-4™ polymer
   – Removing bubbles in the polymer block's tubing
```
↓
```
• Perform spatial calibration.
```
— *Fail* →
```
• Repeat spatial calibration
  without filling the capillary.
```
Pass ↓ (from Perform spatial calibration)

Pass → (from Repeat spatial calibration to Prepare spectral standards)

Fail ↓ (from Repeat spatial calibration)
```
• Refill capillary with fresh
  polymer and rerun spatial
  calibration.
```
Pass → (to Prepare spectral standards)

Fail ↓
```
• Remove capillary array and
  clean window.
• Inspect window for damage
  (e.g., scratches or cracks).
```
Pass → (to Prepare spectral standards)

Fail ↓
```
• If the spatial calibration
  continues to fail, refer to the
  troubleshooting chapter in
  the instrument user's
  manual.
```

```
• Prepare spectral standards and perform spectral
  calibration.
```
— *Fail* →
```
• Rerun spectral calibration,
  verify settings and, if
  necessary, prepare new
  standards.
• If spectral calibration
  continues to fail, refer to
  the Troubleshooting
  chapter in the ABI PRISM®
  3100 Genetic Analyzer
  and ABI PRISM®
  3100-Avant Genetic
  Analyzer User Reference
  Guide (P/N 4335393).
```

Pass ↓
```
• Combine PCR product with Hi-Di™ Formamide +
  internal size standard.
• Transfer to 96-well reaction plates.
• Heat denature for 3 min at 95 °C and immediately chill
  on ice for 3 min.
• Assemble plate septa, tray cover, and plate base;
  place on the Autosampler.
```
↓
```
• Complete the plate record and link to the plate
  assignment.
```
↓
```
• Press the green arrow to start the run.
```
↓
```
• Review the extracted sample files in the appropriate
  software.
```

Before You Begin   Before you begin, note the following:

- This protocol has been written for the 96-well reaction plate format only.
- When running the AmpFℓSTR PCR Amplification Kits on the 3100 Genetic Analyzer, we recommend and support the use of:
  - 3100 POP-4 polymer
  - 36-cm capillary array
  - Hi-Di Formamide
- To successfully run the AmpFℓSTR PCR Amplification Kit PCR products on the 3100 Genetic Analyzer, you should perform all of the procedures listed in the flowchart on . Read the reagent warnings below before performing these procedures.

⚠ **CAUTION** **CHEMICAL HAZARD**. **10X Genetic Analyzer Buffer with EDTA** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ **WARNING** **CHEMICAL HAZARD**. **Methanol** is a flammable liquid and vapor. Exposure causes eye and skin irritation, and may cause central nervous system depression and nerve damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ **WARNING** **CHEMICAL HAZARD**. **Formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ **WARNING** **CHEMICAL HAZARD**. **POP-4 polymer** causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

For More Information

The procedures in this chapter provide a broad overview of the steps required to perform a fragment analysis run and to perform data analysis.

If you need more detailed procedures, refer to the following documents:

- *ABI PRISM® 3100 Genetic Analyzer User Guide* (P/N 4334785)
- *ABI PRISM® 3100 Genetic Analyzer and ABI PRISM® 3100-Avant Genetic Analyzer User Reference Guide* (P/N 4335393)
- *ABI PRISM® 3100 Genetic Analyzer Data Collection Software Version 1.1 Upgrade User Bulletin* (P/N 4333533), which describes the new features in the ABI PRISM 3100 Data Collection Software Version 1.1
- *Overview of the Analysis Parameters and Size Caller User Bulletin* (P/N 4335617)
- *ABI PRISM® GeneScan® Analysis Software Version 3.7 for the Windows NT® Platform User Guide* (P/N 4308923)
- *ABI PRISM® Genotyper® 3.7 NT Software User's Manual* (P/N 4309947)

References

- Shadravan, F. 2001. Sizing Precision and Reproducibility Studies of AmpFℓSTR® Kits with ABI PRISM® 3100 Genetic Analyzer. *Proc. Am. Acad. Forensic Sci.* 7:26.
- Shadravan, F., Roby, R.K., Reeder, D.J. 2002. Characterization of AmpFℓSTR® Identifiler™ PCR Amplification Kit for use with ABI PRISM® 3100 Genetic Analyzer. *Proc. Am. Acad. Forensic Sci.* 8:27.

3100 Data Collection Software Version 1.1

The 3100 Data Collection Software Version 1.1 provides several new features/enhancements. Those that pertain to the protocols in this chapter are described in "Selected Features" below.

Note: For a detailed description of all of the new features provided in the 3100 Data Collection Software Version 1.1, refer to the *ABI PRISM® 3100 Genetic Analyzer Data Collection Software Version 1.1 Upgrade User Bulletin* (P/N 4333533).

Selected Features

The 3100 Data Collection Software Version 1.1:

- Allows you to select the active spectral calibration for a dye set from any previous spectral calibration run.
- Incorporates a new spectral calibration algorithm. This algorithm improves the quality of the matrix generated, thereby improving the overall quality of the sample data.
- Provides new options in setting preferences for sample file folders and data extraction folders. You can:
    - Specify a run folder naming format.
    - Specify the data extraction folder name and location.
    - Group extracted files by run or by plate. Grouping sample files by plate puts all the sample files from one plate into a folder. Grouping sample files by run puts all the sample files from one run into a folder.
    - Distinguish between the naming preferences for Sequence Collector and sample files.
- Provides modules that support specific fragment analysis applications using the G5 chemistry:
    - GeneScan36vb_POP4DefaultModule
    - Spect36vb_POP4DefaultModule

Preparing for a Run

Setting Up the Instrument

Refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide* (P/N 4334785) for general instrument setup procedures, including:

- Starting the computer, instrument, and software
- Checking the reagents, and replenishing them, if necessary

Changing a Capillary Array

When to Change a Capillary Array

A capillary array should last approximately 100 runs. The following indications may suggest that a new capillary array is required:

- Poor sizing precision or allele calling
- Poor resolution and/or decreased signal intensity

Changing a Capillary Array

For information on changing a capillary array, refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide*.

Replacing the Syringes

To maintain optimal performance, we recommend that syringes be replaced approximately every three months.

Performing a Spatial Calibration

When to Perform a Spatial Calibration

You should perform a spatial calibration after each time you:

- Install or replace a capillary array
- Temporarily remove the capillary array from the detection block

Performing a Spatial Calibration

For information on performing a spatial calibration, refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide*.

Performing a Spectral Calibration

A spectral calibration creates a matrix to correct for the overlapping of fluorescence emission spectra of the dyes. Application of this matrix to the raw data is called multicomponenting. Multicomponenting occurs as the data are collected; therefore, it is important to generate and use good quality matrices for the individual capillaries.

Performing a spectral calibration can be divided into the following tasks:

When to Perform a Spectral Calibration

You should perform a spectral calibration:

- When you use a new dye set on the instrument
- After the laser or CCD camera has been realigned by a service engineer
- If you begin to see a decrease in spectral separation ("pull-up" and/or "pull-down" peaks)

Choosing the Dye Set and Matrix Standard

Choose the appropriate dye set and matrix standard for the AmpF*l*STR PCR Amplification Kit you are using as shown in the table below.

For kits that use a...	Use...	And use...	Kit Examples
four-dye system, including the following dyes: • 5-FAM™ • JOE™ • NED™ • ROX™	Dye Set F	Matrix Standard Set DS-32	• AmpF*l*STR® COfiler® PCR Amplification Kit • AmpF*l*STR® Profiler™ PCR Amplification Kit • AmpF*l*STR® Profiler Plus™ PCR Amplification Kit • AmpF*l*STR® Profiler Plus™ *ID* PCR Amplification Kit • AmpF*l*STR® SGM Plus® PCR Amplification Kit
five-dye system, including the following dyes: • 6-FAM™ • VIC® • NED™ • PET™ • LIZ®	Dye Set G5	Matrix Standard Set DS-33	• AmpF*l*STR® Identifiler® PCR Amplification Kit • AmpF*l*STR® SEfiler® PCR Amplification Kit

Setting Up the Spectral (Matrix) Calibration Standards

Note: If you need more information on setting up the spectral (matrix) calibration standards, refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide* (P/N 4334785) or the *ABI PRISM® 3100 Genetic Analyzer Data Collection Software Version 1.1 Upgrade User Bulletin* (P/N 4333533).

Matrix Standards for Dye Set F Spectral Calibration

Follow the procedure below if you are setting up spectral (matrix) calibration standards for kits using a four-dye system, including the 5-FAM, JOE, NED, and ROX dyes (*e.g.,* COfiler Kit, Profiler Kit, Profiler Plus Kit, Profiler Plus *ID* Kit, and SGM Plus Kit).

To set up the matrix standards for Dye Set F:

1.	Thoroughly vortex the four Matrix Standard Set DS-32 tubes for Dye Set F.
2.	Spin the tubes briefly in a microcentrifuge.

To set up the matrix standards for Dye Set F: *(continued)*

3.	Prepare Matrix Standard Set DS-32 for Dye Set F by combining the following in a labeled 1.5-mL microcentrifuge tube:

Reagent	Volume (µL)
5-FAM	2.5
JOE	2.5
NED	2.5
ROX	2.5
Hi-Di Formamide	190
Final Volume	**200**

⚠️ **WARNING** **CHEMICAL HAZARD**. **Formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Note: For optimal performance of the new spectral calibration algorithm, Applied Biosystems recommends that you formulate the Matrix Standard Set DS-32 standards as indicated in this chapter. If necessary, add additional matrix standards at increments of 2.5 µL to pass spectral calibration. This chapter recommends a higher concentration of the spectral standards than is suggested in the Matrix Standard Set DS-32 product insert.

4.	Vortex thoroughly.
5.	Spin the mixture briefly in a microcentrifuge.
6.	Heat the tube at 95 °C for 3 min to denature the DNA.
7.	Immediately place the tube on ice for 3 min.

Matrix Standards for Dye Set G5 Spectral Calibration

Follow the procedure below if you are setting up spectral (matrix) calibration standards for kits using a five-dye system, including the 6-FAM, VIC, NED, PET, and LIZ dyes (*e.g.,* Identifiler Kit, SEfiler Kit).

To set up the matrix standards for Dye Set G5:

1.	Thoroughly vortex the Matrix Standard Set DS-33 tube for Dye Set G5.
2.	Spin the tube briefly in a microcentrifuge.
3.	Prepare Matrix Standard Set DS-33 for Dye Set G5 by combining the following in a labeled 1.5-mL microcentrifuge tube: |Reagent|Volume (µL)
Matrix Standard Set DS-33	5
Hi-Di Formamide	195
Final Volume|**200**| ⚠️ **WARNING** **CHEMICAL HAZARD**. **Formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
4.	Vortex thoroughly.
5.	Spin the mixture briefly in a microcentrifuge.
6.	Heat the tube at 95 °C for 3 min to denature the DNA.
7.	Immediately place the tube on ice for 3 min.

Loading the Standards

To load the standards:

1.	Dispense 10 μL of the denatured matrix standard into a 96-well reaction plate, wells A1–H2.
2.	Centrifuge the plate so that each standard is collected at the bottom of its well.

Preparing the Plate Assembly

To prepare the plate assembly:

1.	Insert the 96-well reaction plate into the plate base provided with the instrument.
2.	Prepare the plate assembly. **Note:** For information on preparing the plate assembly, refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide* (P/N 4334785).
3.	Place the plate assembly on the Autosampler.

Performing a Spectral Calibration Run and Reviewing Data	**To perform a spectral calibration run and review data:**

1.	In the Plate View page of the 3100 Data Collection Software, click **New** to access the Plate Editor dialog box as shown below. **Figure 7-1 Plate Editor dialog box**
2.	In the Plate Editor dialog box: a. Type a name for the plate. b. Select **Spectral Calibration**. c. Select **96-Well** as the plate type. d. Click **Finish**. The Plate Editor spreadsheet opens.

To perform a spectral calibration run and review data: *(continued)*

3.	Complete the Plate Editor spreadsheet for the wells you have loaded:
	a. In the Sample Name column, type a name for the matrix sample.
	b. In the Dye Set column, select **F** or **G5**, depending on the AmpF*l*STR PCR Amplification Kit used (see).
	c. In the Spectral Run Module column, select:
	Spect36_POP4DefaultModule for Dye Set F, or
	Spect36vb_POP4DefaultModule for Dye Set G5
	d. In the Spectral Parameters column, select:
	MtxStd{GeneScan-SetF}.par for Dye Set F, or
	MtxStd{GeneScan-SetG5}.par for Dye Set G5
	e. For each of the columns in steps a through d above, click the column header to select the entire column, then select **Edit** > **Fill Down** to apply the information to all of the selected samples.
	f. Click **OK**.
	Completing the Plate Editor spreadsheet creates a plate record for the calibration run in the database. After a few seconds, the entry for the plate record appears in the Pending Plate Records table of the Plate Setup page.
4.	Link your reaction plate and start the run.
	Note: For more information on linking a reaction plate and starting a run, refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide* (P/N 4334785).
5.	At the end of the run, while the data are being analyzed, the Spectral Calibration Result dialog box opens to indicate how many capillaries have passed.
	Click **OK** to acknowledge completion of the run.
6.	If necessary, repeat the spectral calibration run until 14 or more capillaries have passed.

To perform a spectral calibration run and review data: *(continued)*

7.	Select **Tools** > **Display Spectral Calibration**. Review and evaluate the spectral calibration profile for each capillary, even if the Spectral Calibration Results box indicated that all capillaries passed.
	The figure below is a representative spectral display for G5 chemistry in the 3100 Data Collection Software Version 1.1.
	Figure 7-2 Representative spectral display for G5 chemistry
	Note: For more information on reviewing and evaluating a spectral calibration profile, refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide* (P/N 4334785).
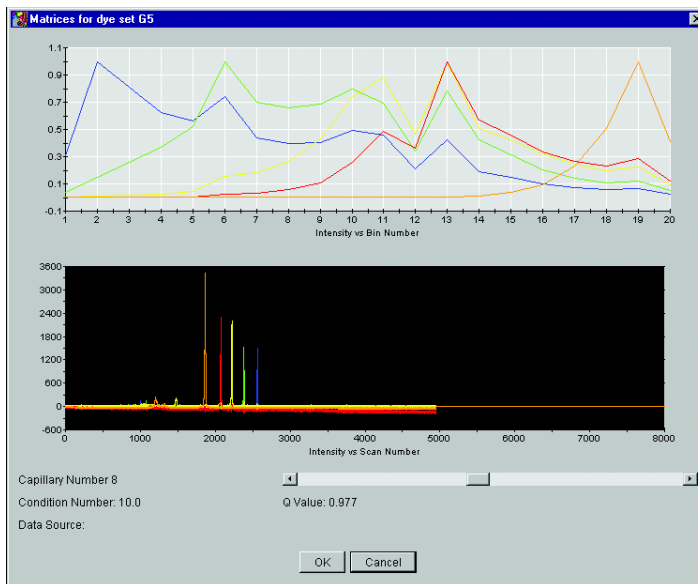
Preparing and Running Your Samples

Preparing samples for a run can be divided into the following tasks:

Note: A run corresponds to a defined set of 16 wells on a 96-well reaction plate.

Performing PCR   To prepare your DNA samples and perform PCR, follow the instructions in the appropriate AmpF*l*STR PCR Amplification Kit user's manual.

Preparing the Formamide:Size Standard Mixture

To prepare the formamide:size standard mixture:

1. You can prepare the formamide:size standard mixture for either each sample or each run.

   For each *sample*, combine the following in a single microcentrifuge tube:

Reagent	Volume (µL)	
	Dye Set F	Dye Set G5
GeneScan™-500 ROX™ Size Standard	0.5	—
GeneScan®-500 LIZ® Size Standard	—	0.3
Hi-Di Formamide	8.5	8.7

   Alternatively, for each *run*, combine the following in a single microcentrifuge tube:

Reagent	Volume (µL)	
	Dye Set F	Dye Set G5
GeneScan-500 ROX Size Standard	8.3	—
GeneScan-500 LIZ Size Standard	—	5
Hi-Di Formamide	141.7	145

   Note: Prepare the appropriate size standard formulation for your dye set.

   IMPORTANT! The amount of size standard listed here is a suggested value only. You should determine the appropriate amount of size standard based on your own results/instruments.

   ⚠️ **WARNING** **CHEMICAL HAZARD**. **Formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

2. Vortex the tube to mix, then spin briefly in a microcentrifuge.

Loading the Samples and Allelic Ladder

To load the samples and allelic ladder:

1.	Dispense 9 µL of the formamide:size standard mixture into each well. **Note:** Add 10 µL of the formamide to each blank well per run. ⚠️ **WARNING** **CHEMICAL HAZARD**. **Formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	Load 1 µL of the sample or allelic ladder into the wells.
3.	Cover the reaction plate with an appropriate septa. Use: • Reservoir septa, or • 96-well plate septa if you prepare samples for more than one run
4.	Briefly spin the reaction plate in a centrifuge to ensure that the contents of each well are mixed and collected at the bottom.
5.	To denature, heat the reaction plate in a thermal cycler at 95 °C for 3 min.
6.	Place the reaction plate immediately on ice for 3 min.

Preparing the Plate Assembly

To prepare the plate assembly:

1.	Insert the 96-well reaction plate into the plate base provided with the instrument.
2.	Prepare the plate assembly. **Note:** For information on preparing the plate assembly, refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide* (P/N 4334785).
3.	Place the plate assembly on the Autosampler.

Performing a Fragment Analysis Run and Analyzing Data

To perform a fragment analysis run and analyze data:

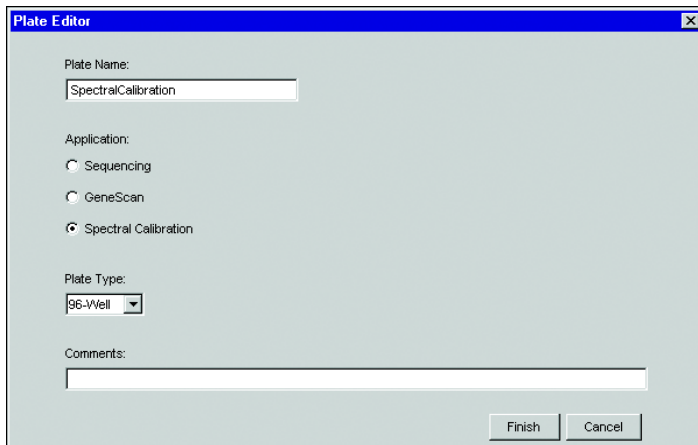|	
1.	In the Plate View page of the 3100 Data Collection Software, click **New** to access the Plate Editor dialog box.
2.	Complete the Plate Editor spreadsheet: a. Type a name for the plate. b. Select **GeneScan**. c. Select **96-Well** as the plate type. d. Click **Finish**. **Figure 7-3 Plate Editor spreadsheet** **Note:** You can reuse plate records by importing data from an existing plate into the current plate. For details, refer to the *ABI PRISM® 3100 Genetic Analyzer Data Collection Software Version 1.1 Upgrade User Bulletin* (P/N 4333533).
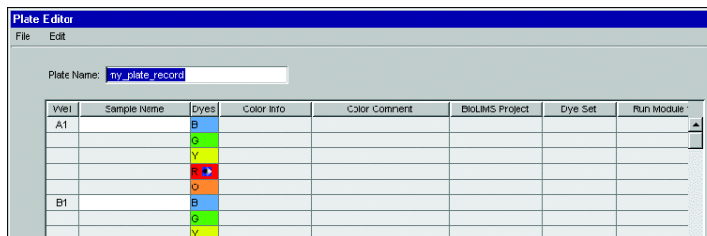
To perform a fragment analysis run and analyze data: *(continued)*

3.	Complete the Plate Editor spreadsheet for the wells you have loaded, then click **OK**.
	For each of the columns, enter information, click the column header to select the entire column, then select **Edit > Fill Down** to apply the information to all of the selected samples.
	a. In the Sample Name column, type a name for the samples.
	b. In the Dyes column, select:
	R for Dye Set F, or
	O for Dye Set G5
	c. In the Color Info column, type the word **ladder** again for each ladder in the Sample Name column.
	Note: Alternatively, you can use the Copy and Paste function.
	d. In the Project Name column, select **3100_Project1** or a project of your choice.
	Note: For information on creating a project name, refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide* (P/N 4334785).
	e. In the Dye Set column, select **F** or **G5**, depending on the AmpFℓSTR PCR Amplification Kit used (see page 9).
	f. In the Run Module 1 column, select:
	GeneScan36_POP4DyeSetFModule for Dye Set F, or
	GeneScan36vb_POP4DyeSetG5Module for Dye Set G5
	Note: If this is your first run, edit the Default Module parameters for Dye Set F (**GeneScan36_POP4DefaultModule**), and Dye Set G5 (**GeneScan36vb_POP4DefaultModule**), changing injection voltage (kV) from 1 to 3, and changing injection time (seconds) from 22 to 10. See "Editing Default Module Parameters in Dye Set F and Dye Set G5" on page 7-22.
	g. In the Analysis Module 1 column, select a module for your size standard (*e.g.,* **GS500Analysis.gsp**).
	After a few seconds, the entry for the plate record appears in the Pending Plate Records table of the Plate Setup page.

To perform a fragment analysis run and analyze data: *(continued)*

4.	Link your reaction plate and start the run. **Note:** For more information on linking a reaction plate and starting a run, refer to the *ABI PRISM® 3100 Genetic Analyzer User Guide* (P/N 4334785).
5.	When the run has completed, view the data. You can view the data: • As color data in the Array View page of the 3100 Data Collection Software **Note:** The electropherogram displayed in the Array View page is the raw, multicomponented data for a selected capillary. • As analyzed sample files in the following default location: **D:\AppliedBio\3100\DataExtractor\ExtractedRuns**
6.	If necessary, re-analyze the data with the GeneScan Analysis Software Version 3.7.1 or later. **Note:** For details, refer to the *Overview of the Analysis Parameters and Size Caller User Bulletin* (P/N 4335617).

Editing Default Module Parameters in Dye Set F and Dye Set G5

If this is your first run, edit the Default Module parameters in Dye Set F and Dye Set G5.

Note: This is a one-time procedure.s

To edit the Default Module parameters:

1.	Click the **Module Editor** button on the toolbar to open the Module Editor dialog box.
2.	From the GeneScan tab, select the appropriate run module to use as a template. Select either **GeneScan36_POP4DyeSetFModule** for Dye Set F, or **GeneScan36vb_POP4DyeSetG5Module** for Dye Set G5.

To edit the Default Module parameters: *(continued)*

3.	Edit the injection parameter values as follows: • Change the injection voltage (kV) from 1 to **3**. • Change the injection time (seconds) from 22 to **10**. **IMPORTANT!** Only whole numbers are accepted. **IMPORTANT!** Be sure that all values are red. Values in black are not saved. Figure 7-4 shows the Default Module parameters (same in both Dye Set F and Dye Set G5).

#	Parameter Name	Value
1	Run Temperature	60
2	Cap Fill Volume	184
3	Maximum Current	300
4	Current Tolerance	100
5	Run Current	100
6	Voltage Tolerance	0.25
7	Pre Run Voltage	15
8	Pre Run Time	180
9	Injection Voltage	3
10	Injection Time	10
11	Run Voltage	15
12	Number of Steps	10
13	Voltage Step Interval	60
14	Data Delay Time	1
15	Run Time	1500

Figure 7-4   Default Module parameters

4.	Click **Save As** and enter the name of new module as follows: **GeneScan36_POP4DyeSetFModule** for Dye Set F, or **GeneScan36vb_POP4DyeSetG5Module** for Dye Set G5
5.	Click **Save** to create a new run module. Enter a unique descriptive name and click **OK**. **Note:** You cannot save default run modules.
6.	When you are finished, click the **Close** button to exit the Module Editor.

Examples of DNA Profiles

Figures 7-5 through 7-12 are examples of DNA profiles obtained on the 3100 Genetic Analyzer. Figures 7-5 and 7-6 below show AmpFℓSTR® Control DNA 9947A (1 ng) amplified with the AmpFℓSTR Identifiler PCR Amplification Kit.
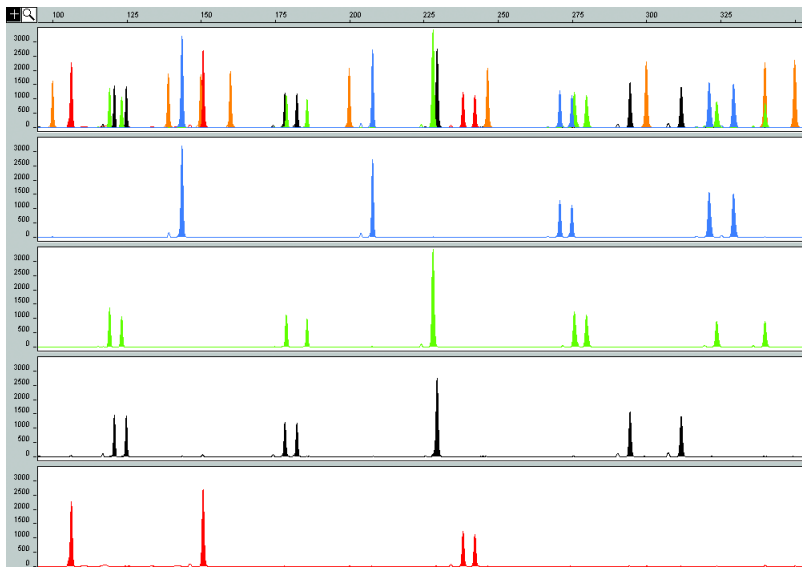
Figure 7-5   Data analyzed using GeneScan Analysis Software Version 3.7.1
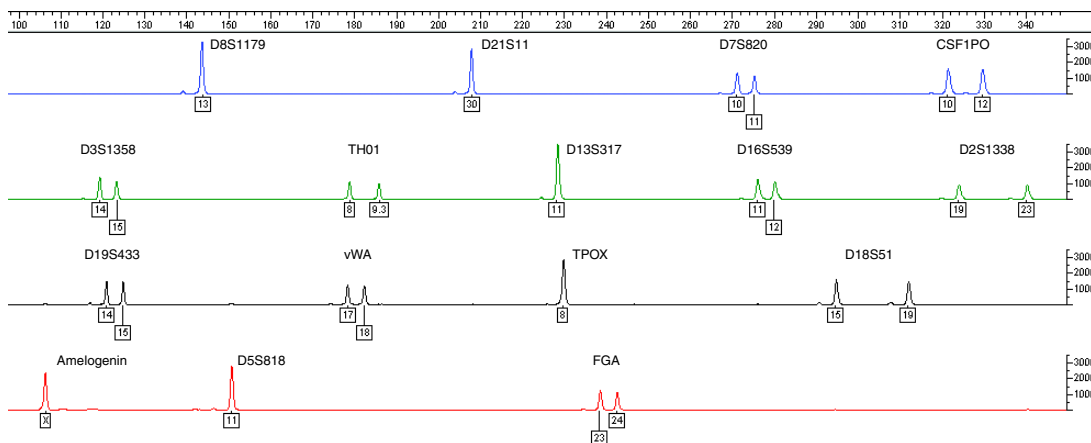
Figure 7-6   The same sample from Figure 7-5, above, analyzed using Genotyper® Software v 3.7
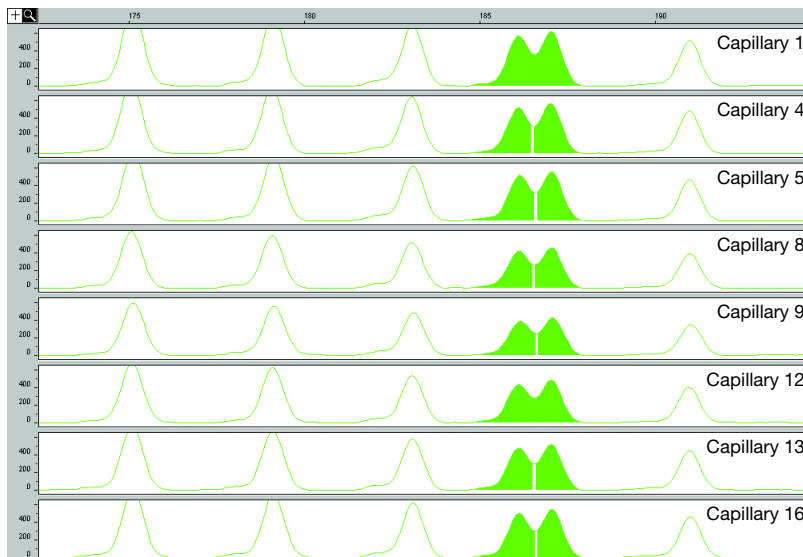
Figure 7-7   Eight (8) capillaries of a 16-capillary array displaying single nucleotide resolution at the 9.3 and 10 alleles (highlighted) of the TH01 locus from the AmpF*l*STR® Identifiler® Allelic Ladder. The 9.3 and 10 alleles were resolved and individually detected in all 16 capillaries.
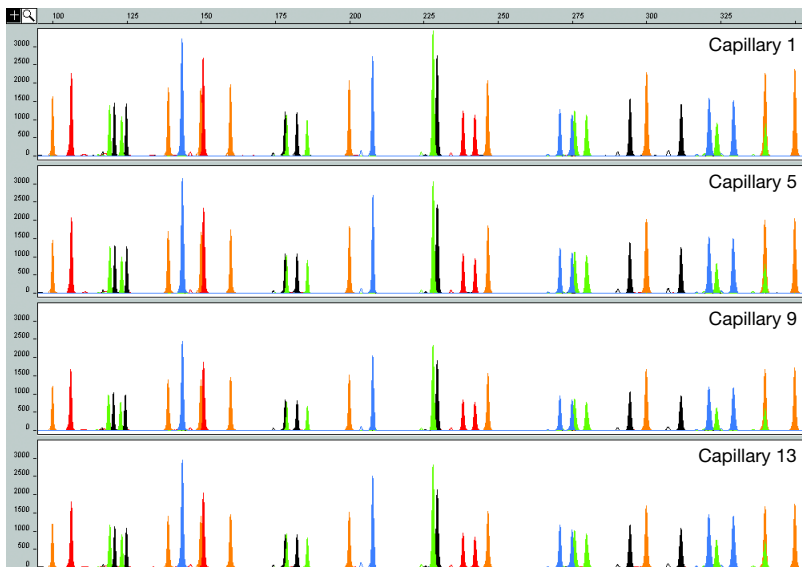
Figure 7-8   A comparison of signal intensity between four (4) capillaries across a capillary array using the AmpF*l*STR Control DNA 9947A (1 ng) amplified with the AmpF*l*STR Identifiler PCR Amplification Kit.
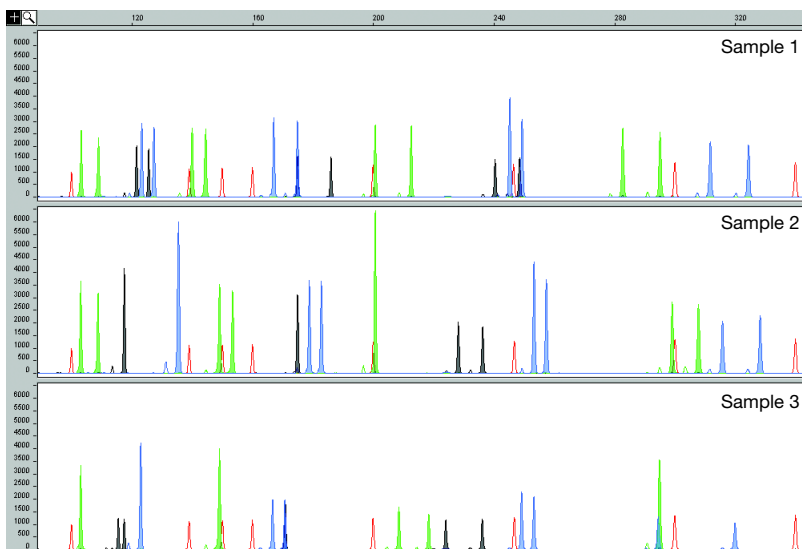
Figure 7-9   Three (3) DNA samples (2 ng) amplified with the AmpF*l*STR SGM Plus PCR Amplification Kit
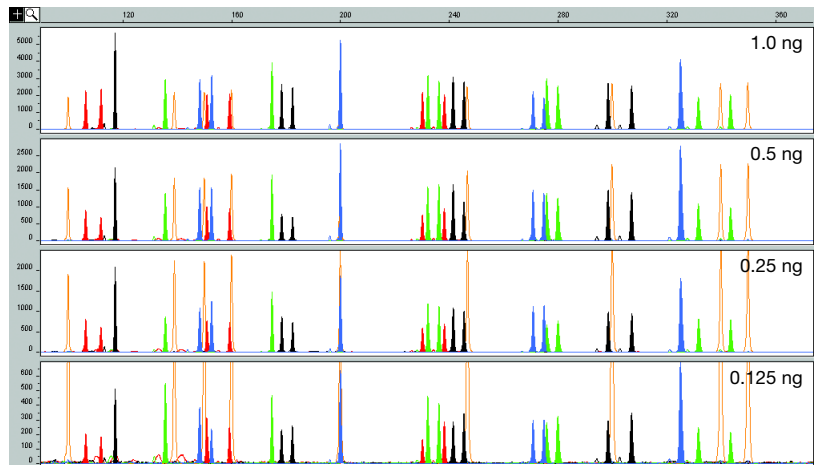
Figure 7-10   Dilutions for a DNA sample amplified with the AmpF*l*STR Identifiler PCR Amplification Kit, 1.0 ng, 0.5 ng, 0.25 ng, and 0.125 ng input DNA. The Y-axis scale is magnified for lower input DNA amounts.
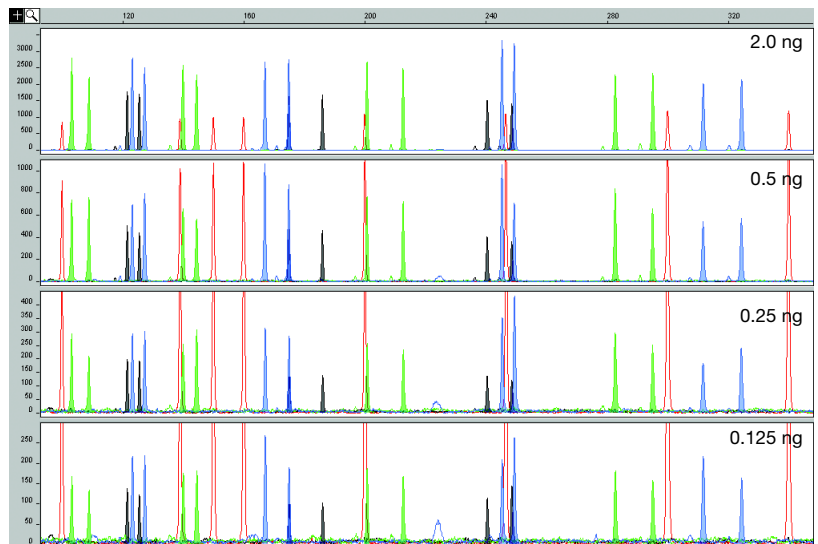
Figure 7-11   Dilutions of a DNA sample amplified with the AmpF*l*STR SGM Plus PCR Amplification Kit, 2.0 ng, 0.5 ng, 0.25 ng, and 0.125 ng input DNA. The Y-axis scale is magnified for lower input DNA amounts.
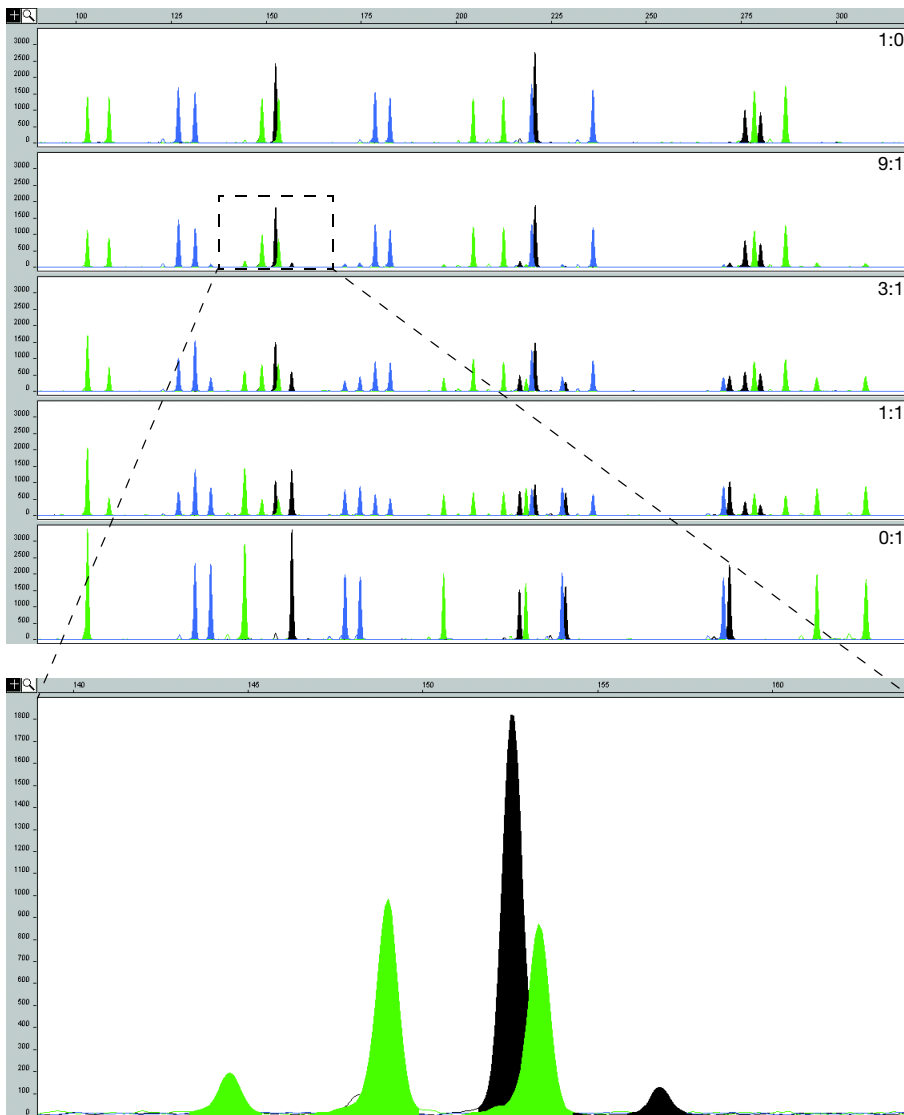
Figure 7-12   The first and last panels display the profiles of each DNA sample amplified individually (the male sample is in the top panel and the female sample is in the bottom panel) with 2 ng DNA with the AmpFℓSTR Profiler Plus PCR Amplification Kit. The other panels display the mixture of these DNA samples mixed at approximate ratios of 9:1, 3:1, and 1:1. The panel inset displays the expanded view of the DNA sample mixed at an approximate ratio of 9:1 at D8S1179 (green) and D5S818 (yellow).

Materials Required

The following tables list the items required to run AmpF*l*STR PCR Amplification Kit PCR products on the 3100 Genetic Analyzer.

Dedicated Equipment and Supplies

Follow the guidelines for dedicated equipment and supplies to ensure that exogenous DNA and PCR products are confined to a designated area:

- Designate an Amplified DNA Work Area for amplified DNA and for dedicated equipment and supplies used to handle amplified DNA.
- Do not remove amplified DNA, equipment, or supplies from the Amplified DNA Work Area.
- Samples that are not amplified should never come into contact with supplies and equipment in the Amplified DNA Work Area.

Accessories

Accessories	Supplier	Part Number
3100 Capillary Array, 36 cm	Applied Biosystems	4315931
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems	N801-0560
96-well plate septa	Applied Biosystems	4315933
Reservoir septa	Applied Biosystems	4315932
Array-fill syringe, 250-μL glass syringe	Applied Biosystems	4304470
Polymer-reserve syringe, 5.0-mL glass syringe	Applied Biosystems	628-3731

Chemicals

Chemicals	Supplier	Part Number
3100 POP-4™ polymer	Applied Biosystems	4316355
Matrix Standard Set DS-32 for 3100 (containing the dyes 5-FAM™, JOE™, NED™, and ROX™)	Applied Biosystems	4323018
Matrix Standard Set DS-33 for 3100 (containing the dyes 6-FAM™, VIC®, NED™, PET™, and LIZ®)	Applied Biosystems	4323016
Hi-Di™ Formamide	Applied Biosystems	4311320
GeneScan™-500 ROX™ Size Standard	Applied Biosystems	401734
GeneScan®-500 LIZ® Size Standard	Applied Biosystems	4322682
10X Genetic Analyzer Buffer	Applied Biosystems	401884
AmpFℓSTR® PCR Amplification Kit, one of the following:		
AmpFℓSTR® COfiler® PCR Amplification Kit	Applied Biosystems	4305246
AmpFℓSTR® Identifiler® PCR Amplification Kit	Applied Biosystems	4322288
AmpFℓSTR® Profiler™ PCR Amplification Kit	Applied Biosystems	403038
AmpFℓSTR® Profiler Plus™ PCR Amplification Kit	Applied Biosystems	4303326
AmpFℓSTR® Profiler Plus™ *ID* PCR Amplification Kit	Applied Biosystems	4330284
AmpFℓSTR® SGM Plus® PCR Amplification Kit	Applied Biosystems	4307133

Software   The following software programs are required to analyze AmpF*l*STR
PCR Amplification Kit PCR products on the 3100 Genetic Analyzer:

- ABI PRISM® GeneScan® Analysis Software Version 3.7.1 or later
  for the Microsoft® Windows NT® operating system
- ABI PRISM® 3100 Data Collection Software Version 1.1 or later

For genotyping, the following software program is required:

- ABI PRISM® Genotyper® Software v 3.7 or later for the
  Microsoft® Windows NT® operating system

User Documentation

When processing AmpF*l*STR PCR Amplification Kit PCR products on the 3100 Genetic Analyzer, it may be helpful to refer to the Applied Biosystems instrument, software, and kit documentation listed below.

Document	Part Number
ABI PRISM® 3100 Genetic Analyzer User Guide	4334785
ABI PRISM® 3100 Genetic Analyzer and ABI PRISM® 3100-Avant Genetic Analyzer User Reference Guide	4335393
ABI PRISM® 3100 Genetic Analyzer Data Collection Software Version 1.1 Upgrade User Bulletin	4333533
ABI PRISM® GeneScan® Analysis Software Version 3.7 for the Windows NT® Platform User Guide	4308923
ABI PRISM® Genotyper® 3.7 NT Software User's Manual	4309947
ABI PRISM® Genotyper® 3.7 NT Software Applications Tutorials	4309961
Overview of the Analysis Parameters and Size Caller User Bulletin	4335617
AmpFlSTR® COfiler® PCR Amplification Kit User Bulletin	4305469
AmpFlSTR® Identifiler™ PCR Amplification Kit User's Manual	4323291
AmpFlSTR® Profiler™ PCR Amplification Kit User's Manual	402945
AmpFlSTR® Profiler Plus™ PCR Amplification Kit User's Manual	4303501
AmpFlSTR® Profiler Plus™ ID PCR Amplification Kit User Bulletin	4330429
AmpFlSTR® SGM Plus® PCR Amplification Kit User's Manual	4309589

Experiments and Results

8

In This Chapter   This chapter describes various experiments performed and results obtained using the AmpFℓSTR® SEfiler™ PCR Amplification Kit.

Experiments Using AmpF*l*STR SEfiler 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 critical for sound data interpretation in casework (Sparkes, Kimpton, Gilbard, *et al*., 1996; Sparkes, Kimpton, Watson, *et al*., 1996; Wallin *et al*., 1998).

Experiments

Experiments to evaluate the performance of AmpF*l*STR SEfiler PCR Amplification Kit were performed at Applied Biosystems. Some of these experiments were performed according to the DNA Advisory Board (DAB) Quality Assurance Standards, effective October 1, 1998 (DNA Advisory Board, 1998). The DNA Advisory Board issued quality assurance standards for forensic DNA testing laboratories in the United States.

These DAB standards describe the quality assurance requirements that a laboratory should follow to ensure the quality and integrity of the data and competency of the laboratory. DAB defines a laboratory as a facility in which forensic DNA testing is performed.

Based on these standards, Applied Biosystems has conducted experiments that comply with Standards 8.1.1 and 8.1.2 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*., 2001).

This chapter discusses many of the experiments performed by Applied Biosystems and examples of results obtained. Conditions that produced maximum PCR product yield were chosen and a window in which reproducible performance characteristics were met. These experiments, while not exhaustive, are appropriate for a manufacturer, in our opinion. Each laboratory using the AmpF*l*STR SEfiler PCR Amplification Kit should perform appropriate validation studies. Forensic validation of the AmpF*l*STR® SGM Plus® Kit has been published (Cotton *et al.,* 2000; Wallin *et al.,* 2002). The AmpF*l*STR SEfiler PCR Amplification Kit contains all the AmpF*l* STR SGM Plus Kit loci and SE33. Refer to the *AmpF*l*STR® SGM Plus® PCR Amplification Kit User's Manual (*P/N 4309589) for more details.

Developmental Validation

DAB Standard 8.1.1 Developmental Validation

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

The DNA Advisory Board has determined critical reagent concentrations and reaction conditions (*e.g.*, magnesium chloride, annealing temperature) to produce reliable, locus-specific amplification and appropriate sensitivity.

PCR Components

One of the critical reagents, concentration of the SE33 primers (forward and reverse) of the AmpFℓSTR SEfiler Primer Set were examined. The concentration for the SE33 primers was established to be in the window that meets the reproducible performance characteristics of specificity and sensitivity. Various magnesium chloride concentrations were also tested on the ABI PRISM 310 Genetic Analyzer. To determine the optimum concentration, a 2 ng amplification of genomic DNA varying the magnesium chloride concentration was analyzed. The results are shown in the figure below.
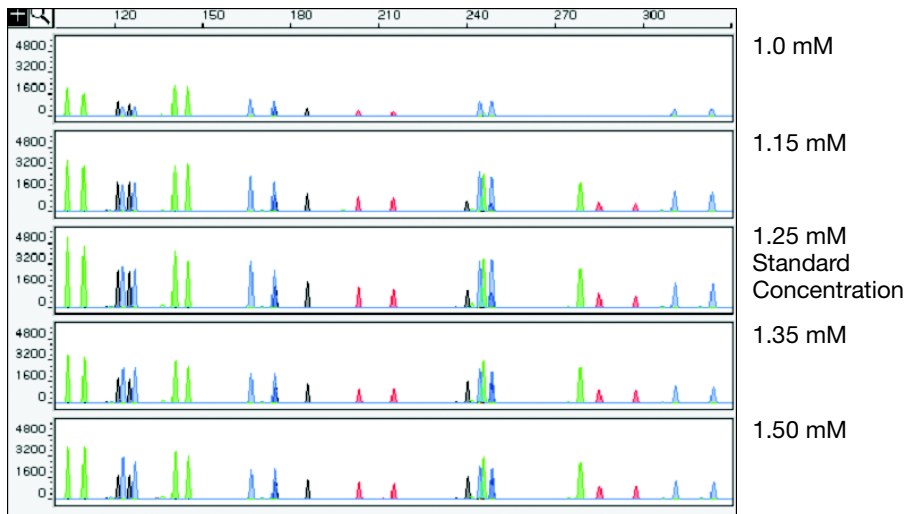
Figure 8-1   A 2 ng amplification of genomic DNA varying the magnesium chloride concentration, analyzed on the ABI PRISM 310 Genetic Analyzer

Thermal Cycler Parameters

Thermal cycling parameters were established for amplification of the AmpF*l*STR SEfiler Kit. Thermal cycling times and temperatures of GeneAmp PCR systems were verified. Varying annealing temperature windows were tested to verify that a ±2.0 °C window produced a specific PCR product with the desired sensitivity of at least 2 ng of AmpF*l*STR Control DNA 007.

The effects of annealing temperatures on the amplification of AmpF*l*STR SEfiler kit loci were examined using AmpF*l*STR Control DNA 007.

The annealing temperatures tested were 55, 57, 59, 61, and 63 °C (see Figure 8-2) in the GeneAmp PCR System 9700. The PCR products were analyzed using the ABI PRISM 310 Genetic Analyzer.

Neither preferential nor differential amplification was observed in the denaturation temperature experiments. Of the tested annealing temperatures, 55, 57, 59, and 61 °C produced robust profiles. At 63 °C, the yield of the majority of loci was significantly reduced. Routine thermal cycler calibration is recommended when following the amplification protocol. Preferential amplification for all loci, including SE33 locus, was not observed at any of the tested annealing temperatures.
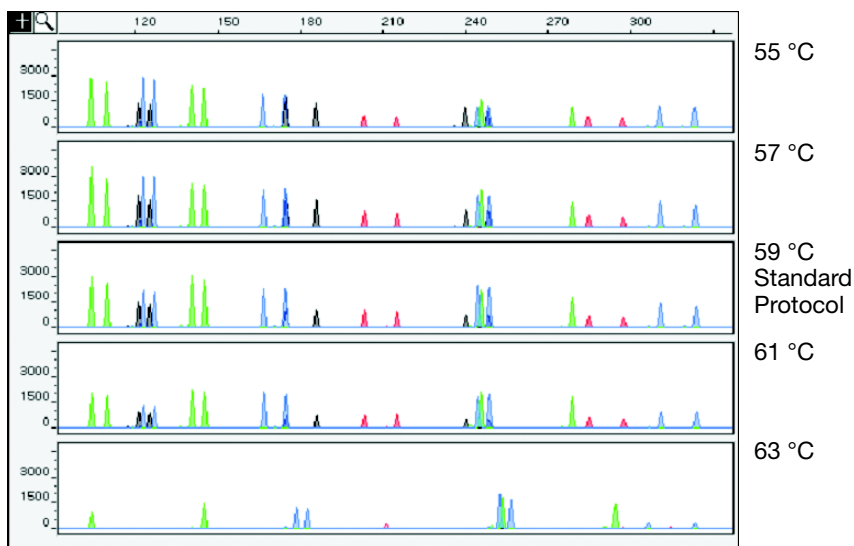
Figure 8-2   Amplification of 2 ng of genomic DNA while varying the annealing temperature, analyzed on the ABI PRISM 310 Genetic Analyzer

Accuracy, Precision, and Reproducibility

DAB Standard 8.1.2 Accuracy

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

Laser-induced fluorescence detection of length polymorphism at short tandem repeat loci is not a novel methodology (Holt *et al*., 2001; and Wallin *et al*., 2002). However, accuracy and reproducibility of AmpFℓSTR SEfiler kit profiles have been determined from various sample types.

Figure 8-3 illustrates the size differences that are typically observed between sample alleles and allelic ladder alleles on the ABI PRISM 3100 Genetic Analyzer with POP-4™ polymer. The x-axis in Figure 8-3 represents the nominal base pair sizes for the AmpFℓSTR SEfiler Allelic Ladder, and the dashed lines parallel to the x-axis represent the ±0.5-bp windows. The y-axis is the deviation of each sample allele size from the corresponding allelic ladder allele size. All sample alleles are within 0.5 bp of a corresponding allele in an allelic ladder.
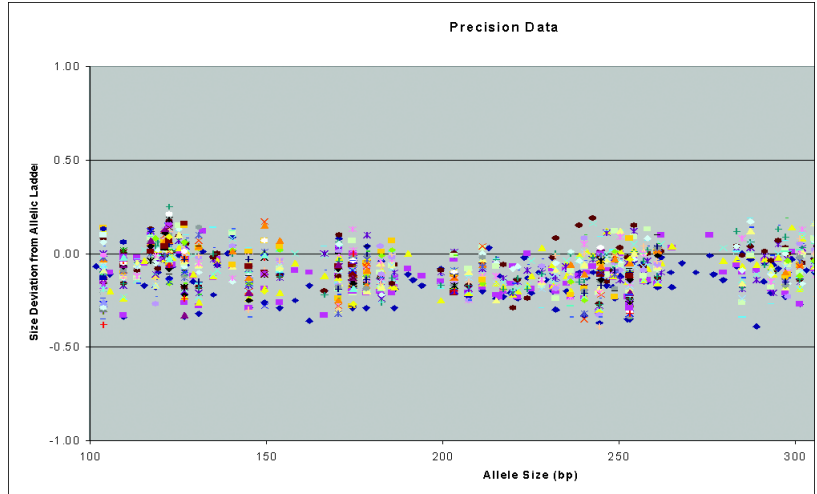
Figure 8-3   Size deviation of 89 samples and two allelic ladders on a single ABI PRISM 3100 Genetic Analyzer run

**Precision and
Size Windows**

Sizing precision allows for determining accurate and reliable genotypes. Sizing precision was measured on the ABI PRISM 310 Genetic Analyzer. As indicated in the Automated Genotyping section, the recommended method for genotyping is to employ a ±0.5-bp "window" around the size obtained for each allele in the AmpF*l*STR SEfiler Allelic Ladder. A ±0.5-bp window allows for the detection and correct assignment of alleles. Any sample allele that sizes outside a window could be either of the following:

- An "off-ladder" allele, *i.e.*, an allele of a size that is not represented in the AmpF*l*STR SEfiler Allelic Ladder
- 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 several injections in a capillary instrument or in several lanes of one gel.

Table 8-1 on page 8-7 indicates typical precision results obtained from the seven injections of the AmpF*l*STR SEfiler Allelic Ladder analyzed on the ABI PRISM 310 Genetic Analyzer (47-cm capillary and POP-4 polymer). The internal lane size standard used was GeneScan®-500 LIZ® Size Standard. These results were obtained within a set of injections on a single capillary.

As indicated above, sample alleles may occasionally size outside of the ±0.5-bp window for a respective allelic ladder allele because of measurement error. The frequency of such an occurrence is lowest in detection systems having the smallest standard deviations in sizing. Figure 8-3 on page 8-5 illustrates the tight clustering of allele sizes obtained on the ABI PRISM 310 Genetic Analyzer, where the standard deviation in sizing is typically less than 0.15 bp. The instance of a sample allele sizing outside of the ±0.5-bp window because of measurement error is relatively rare when the standard deviation in sizing is approximately 0.15 bp or less (Smith, 1995).

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

It is important to note that while the precision within a gel or set of capillary injections is very good, the determined allele sizes vary between platforms. Cross-platform sizing differences arise from a number of parameters, including type and concentration of polymer mixture, run temperature, and electrophoresis conditions. Variations in sizing can also be found between runs on the same instrument and between runs on different instruments because of these parameters.

We strongly recommend that the allele sizes obtained be compared to the sizes obtained for known alleles in the AmpF*l*STR SEfiler Allelic Ladder from the same run and then converted to genotypes (as described in the Automated Genotyping section). Refer to Table 8-1 for the results of injections of the AmpF*l*STR SEfiler Allelic Ladder. For more information on precision and genotyping, see Lazaruk *et al.*, 1998 and Mansfield *et al.*,1998.

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder:

	ABI PRISM 310 Genetic Analyzer	
Allele	**Mean**	**S.D.**
Amelogenin		
X	104.01	0.09
Y	109.68	0.09

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	S.D.
D2S1338		
15	289.52	0.06
16	293.65	0.06
17	297.76	0.07
18	301.97	0.07
19	306.33	0.09
20	310.59	0.10
21	314.90	0.12
22	319.11	0.11
23	323.29	0.14
24	327.45	0.15
25	331.59	0.12
26	335.66	0.10
27	339.69	0.13
28	343.47	0.09

Table 8-1   Precision results of seven injections of the AmpF*l*STR
SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	S.D.
D3S1358		
12	110.77	0.09
13	114.92	0.06
14	118.85	0.05
15	122.79	0.07
16	126.95	0.09
17	131.16	0.04
18	135.21	0.08
19	139.22	0.06

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	**S.D.**
D8S1179		
8	124.16	0.08
9	128.19	0.06
10	132.28	0.08
11	136.38	0.08
12	140.62	0.07
13	145.19	0.05
14	149.60	0.09
15	153.95	0.07
16	158.21	0.07
17	162.35	0.10
18	166.43	0.10
19	170.52	0.09

Table 8-1   Precision results of seven injections of the AmpFℓSTR SEfiler Allelic Ladder: *(continued)*

Allele	ABI Prism 310 Genetic Analyzer	
	Mean	S.D.
D16S539		
5	228.68	0.05
8	240.63	0.08
9	244.68	0.08
10	248.67	0.11
11	252.71	0.08
12	256.70	0.06
13	260.70	0.08
14	264.78	0.05
15	268.86	0.06

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	S.D.
D18S51		
7	264.49	0.09
9	272.62	0.07
10	276.68	0.07
10.2	278.63	0.09
11	280.70	0.09
12	284.75	0.08
13	288.88	0.09
13.2	290.94	0.06
14	293.05	0.08
14.2	295.07	0.12
15	297.18	0.11
16	301.41	0.11
17	305.79	0.11
18	310.15	0.11
19	314.45	0.14
20	318.70	0.12
21	322.98	0.11
22	327.08	0.12
23	331.24	0.15
24	335.41	0.12

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	**S.D.**
D18S51 *(continued)*		
25	339.50	0.15
26	343.30	0.12
27	347.03	0.09
D19S433		
9	101.88	0.06
10	105.80	0.07
11	109.68	0.05
12	113.57	0.06
12.2	115.55	0.04
13	117.51	0.06
13.2	119.52	0.05
14	121.45	0.07
14.2	123.48	0.06
15	125.44	0.06
15.2	127.45	0.04
16	129.43	0.05
16.2	131.44	0.06
17	133.42	0.06
17.2	135.47	0.03

Table 8-1   Precision results of seven injections of the AmpF𝓁STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	**S.D.**
D21S11		
24	187.71	0.07
24.2	189.71	0.09
25	191.63	0.04
26	195.53	0.02
27	199.42	0.06
28	203.29	0.08
28.2	205.23	0.04
29	207.19	0.07
29.2	209.15	0.05
30	211.12	0.09
30.2	213.06	0.06
31	215.05	0.04
31.2	216.98	0.03
32	219.02	0.05
32.2	220.97	0.06
33	222.99	0.06
33.2	224.91	0.07
34	226.97	0.05
34.2	228.85	0.08
35	230.94	0.06

Table 8-1   Precision results of seven injections of the AmpF*l*STR
SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	**S.D.**
D21S11 *(continued)*		
35.2	232.83	0.06
36	234.85	0.05
37	238.83	0.09
38	242.74	0.07

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	S.D.
FGA		
17	211.89	0.11
18	215.91	0.07
19	219.91	0.08
20	223.95	0.06
21	227.96	0.08
22	232.00	0.09
23	236.03	0.11
24	240.06	0.09
25	244.10	0.11
26	248.15	0.10
26.2	250.17	0.08
27	252.21	0.08
28	256.22	0.08
29	260.30	0.06
30	264.41	0.10
30.2	266.20	0.10
31.2	270.32	0.09
32.2	274.43	0.10
33.2	278.48	0.08
42.2	316.56	0.08

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	**S.D.**
FGA *(continued)*		
43.2	320.81	0.12
44.2	325.03	0.13
45.2	329.16	0.13
46.2	333.25	0.14
47.2	337.36	0.12
48.2	341.37	0.10
50.2	348.88	0.08
51.2	352.66	0.09

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	S.D.
SE33		
4.2	198.23	0.04
6.3	206.68	0.05
8	211.55	0.05
9	215.34	0.06
11	222.97	0.05
12	226.81	0.09
13	230.64	0.07
14	234.48	0.06
15	238.34	0.08
16	242.19	0.04
17	246.05	0.06
18	249.92	0.08
19	253.79	0.04
20	257.65	0.06
20.2	259.69	0.07
21	261.59	0.08
21.1	262.48	0.09
21.2	263.57	0.06
22.2	267.44	0.04
23.2	271.32	0.04

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	S.D.
SE33 *(continued)*		
24.2	275.25	0.08
25.2	279.11	0.04
26.2	282.98	0.06
27.2	286.91	0.08
28.2	290.86	0.07
29.2	294.85	0.05
30.2	298.79	0.05
31.2	302.90	0.09
32.2	307.09	0.05
33.2	311.22	0.05
34.2	315.34	0.10
35	317.37	0.08
35.2	319.40	0.08
36	321.44	0.08
37	325.41	0.10

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	S.D.
TH01		
4	162.64	0.07
5	166.70	0.08
6	170.74	0.08
7	174.78	0.06
8	178.80	0.07
9	182.75	0.08
9.3	185.78	0.05
10	186.72	0.07
11	190.66	0.08
13.3	201.39	0.06

Table 8-1   Precision results of seven injections of the AmpF*l*STR SEfiler Allelic Ladder: *(continued)*

Allele	ABI PRISM 310 Genetic Analyzer	
	Mean	**S.D.**
vWA		
11	153.92	0.07
12	158.10	0.07
13	162.32	0.05
14	166.55	0.10
15	170.45	0.05
16	174.44	0.08
17	178.46	0.05
18	182.42	0.10
19	186.35	0.08
20	190.29	0.09
21	194.22	0.06
22	198.05	0.06
23	201.88	0.07
24	206.13	0.07

Extra Peaks in the Electropherogram

Causes of Extra Peaks

To further demonstrate reproducibility, samples have been typed using the AmpF*l*STR SEfiler PCR Amplification Kit. These samples have been previously genotyped with concordant results of the same loci using other AmpF*l*STR kits.

Peaks other than the target alleles may be detected on the electropherogram displays. Causes for the appearance of extra peaks include the stutter product (at the n–4 position), incomplete 3′ A nucleotide addition (at the n–1 position), artifacts and mixed DNA samples (see "DAB Standard 8.1.2.2 Species Specificity" on page 8-34).

Stutter Products

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

The proportion of the stutter product relative to the main allele (percent stutter) is measured by dividing the height of the stutter peak by the height of the main allele peak. Peak heights have been measured for amplified samples at the loci used in the AmpF*l*STR SEfiler kit. All data were generated on the ABI PRISM 310 Genetic Analyzer.

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

- For each AmpF*l*STR SEfiler kit locus, the percent stutter generally increases with allele length, as shown in Figures 8-4 through 8-14 on pages 8-23 through 8-28.
- Data generated for SE33 locus are shown in Figure 8-12.
- Refer to Figures 8-4 through 8-14 on pages 8-23 through 8-28. Smaller alleles display a lower level of stutter relative to the longer alleles within each locus.

- For the alleles within a particular locus, the percent stutter is generally greater for the longer allele in a heterozygous sample. This conclusion is related to the percent stutter increase with allele length.

- Each allele within a locus displays percent stutter that is consistent.

- The highest percent stutter observed for each allele is as follows: D2S1338, 11.8%; D3S1358, 10.4%; D8S1179, 10.0%; D16S539, 10.4%; D18S51, 14.6%; D19S433, 11.7%; D21S11, 13.7%; FGA, 13.9%; SE33, 14.6%; TH01, 4.3%; and vWA, 11.8%.

- The highest observed percent stutter for each locus is included as the filtering step in Genotyper software. Peaks in the stutter position that are above the highest observed percent stutter will not be filtered. Peaks in the stutter position that have not been filtered and remain labeled can be further evaluated. For evaluation of mixed samples, see Figure 8-20 on page 8-44.

- The percent stutter does not change significantly with the recommended quantity of input DNA, for on-scale data. The measurement of percent stutter may be unusually high for main peaks that are off-scale.
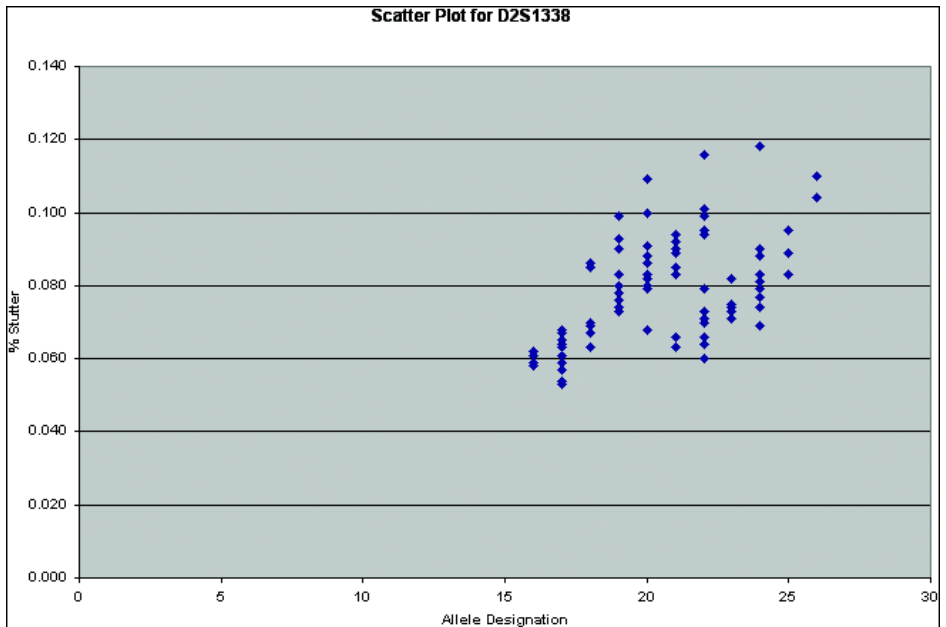
Figure 8-4   D2S1338 scatter plot
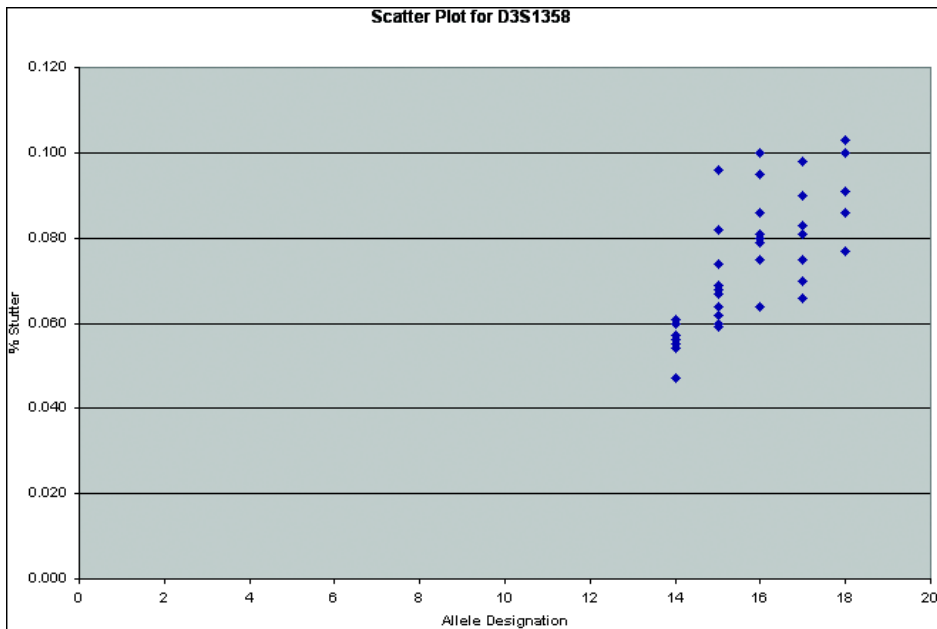
Figure 8-5   D3S1358 scatter plot
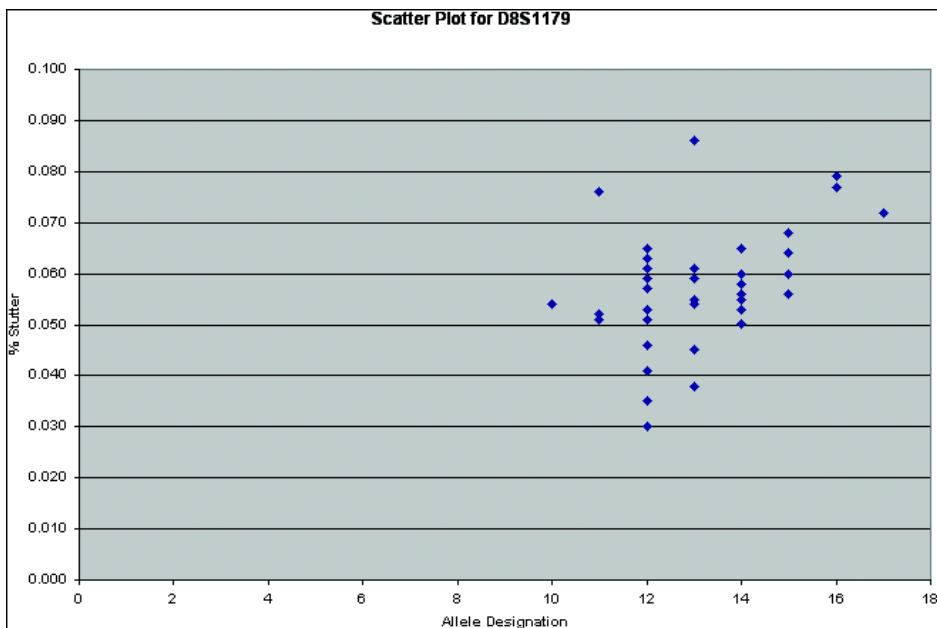
Figure 8-6   D8S1179 scatter plot
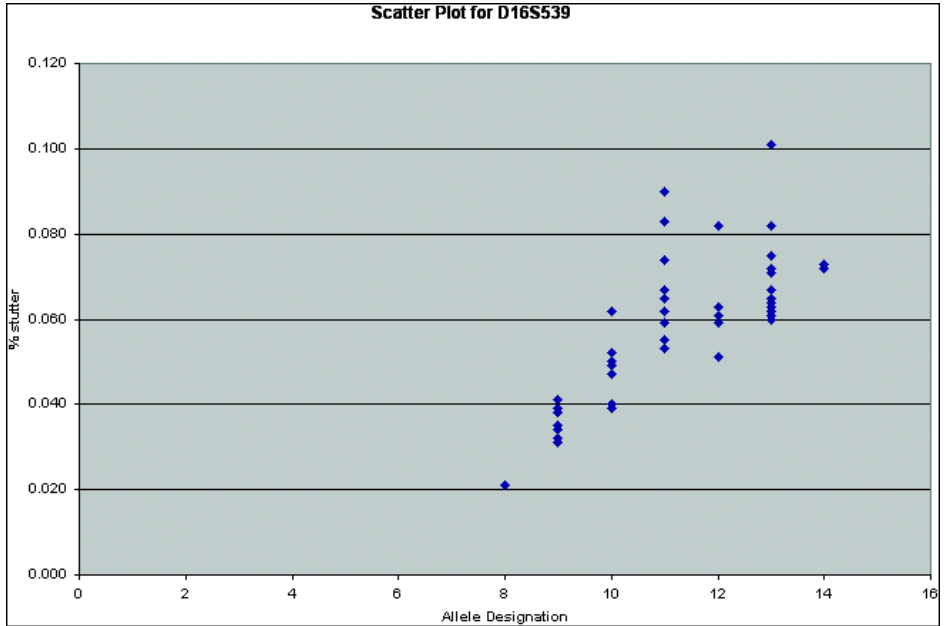
Figure 8-7   D16S539 scatter plot
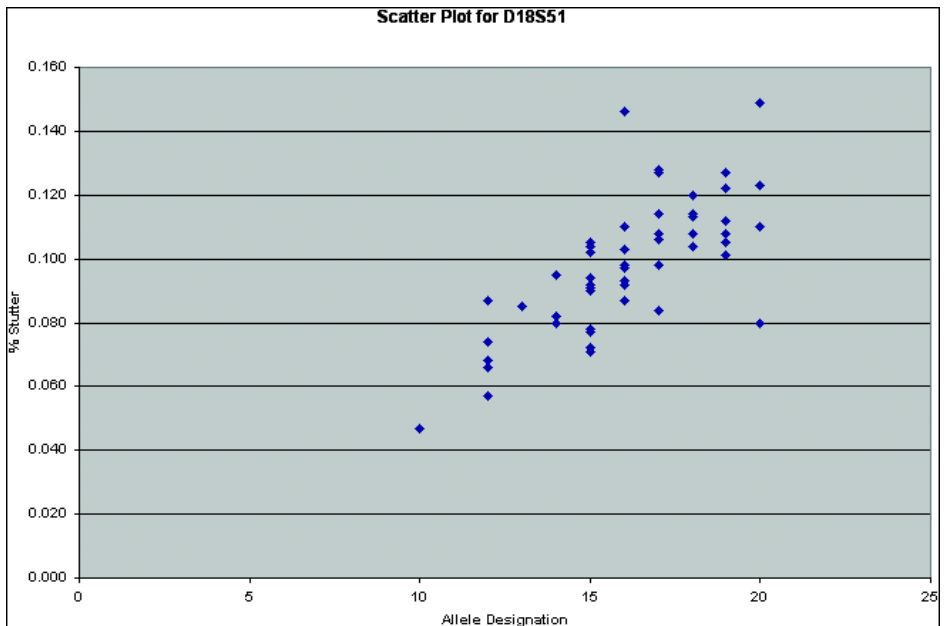
Figure 8-8   D18S51 scatter plot
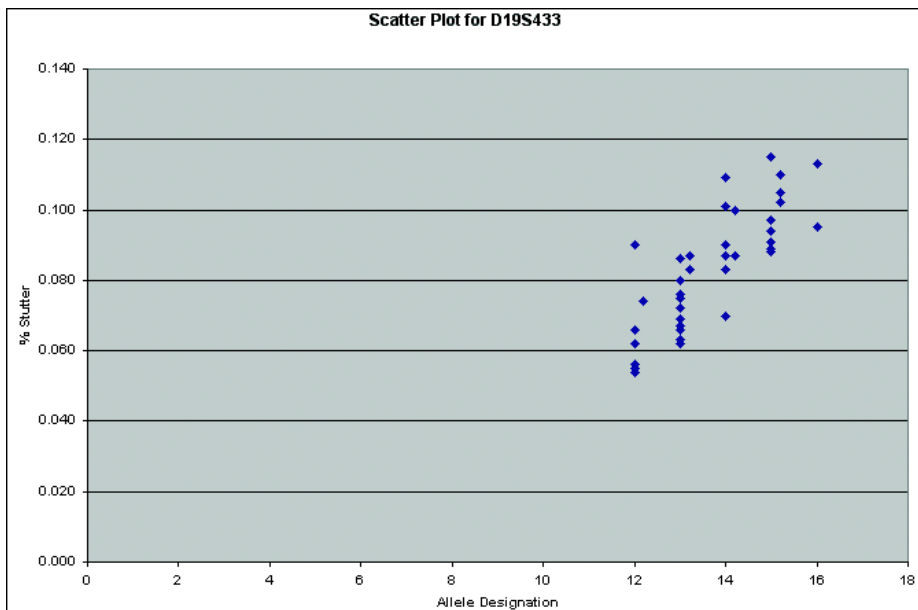
Figure 8-9   D19S433 scatter plot
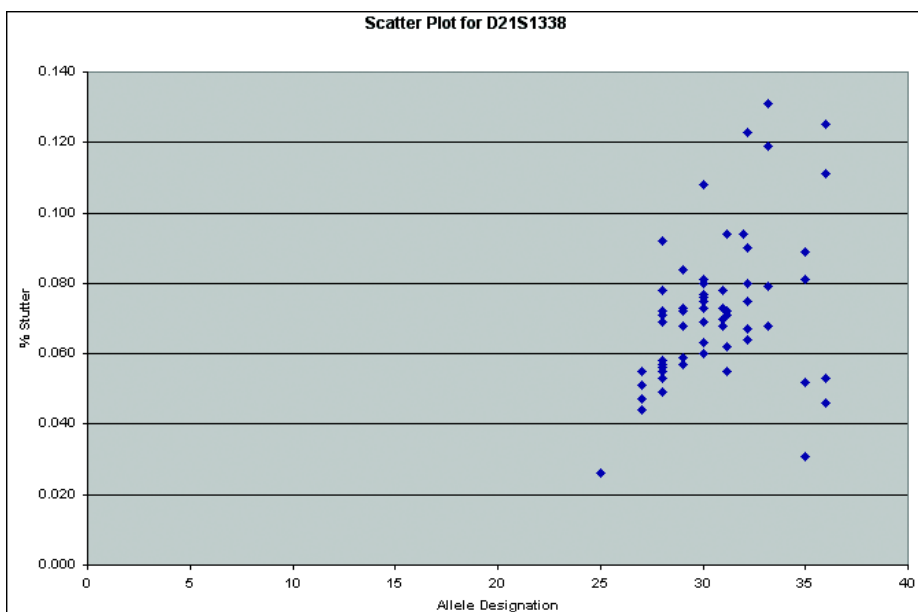
Figure 8-10   D21S11 scatter plot
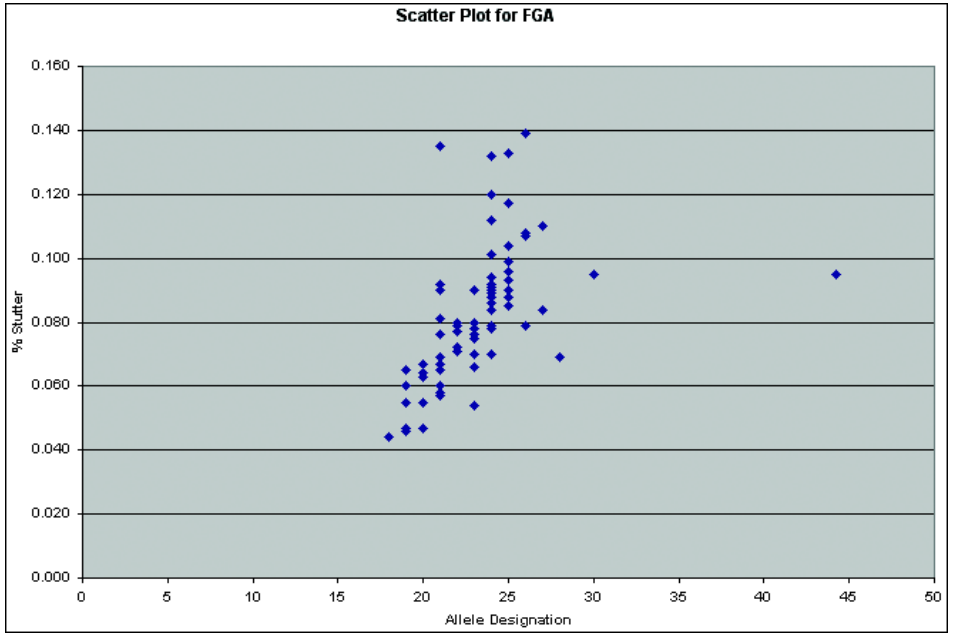
Figure 8-11   FGA scatter plot
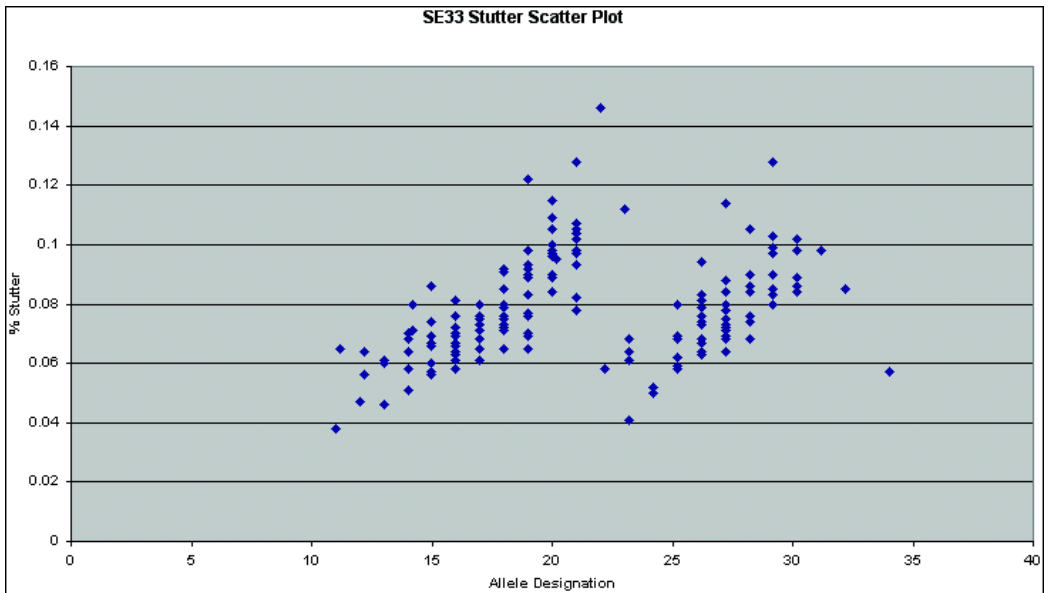
Figure 8-12   SE33 stutter scatter plot
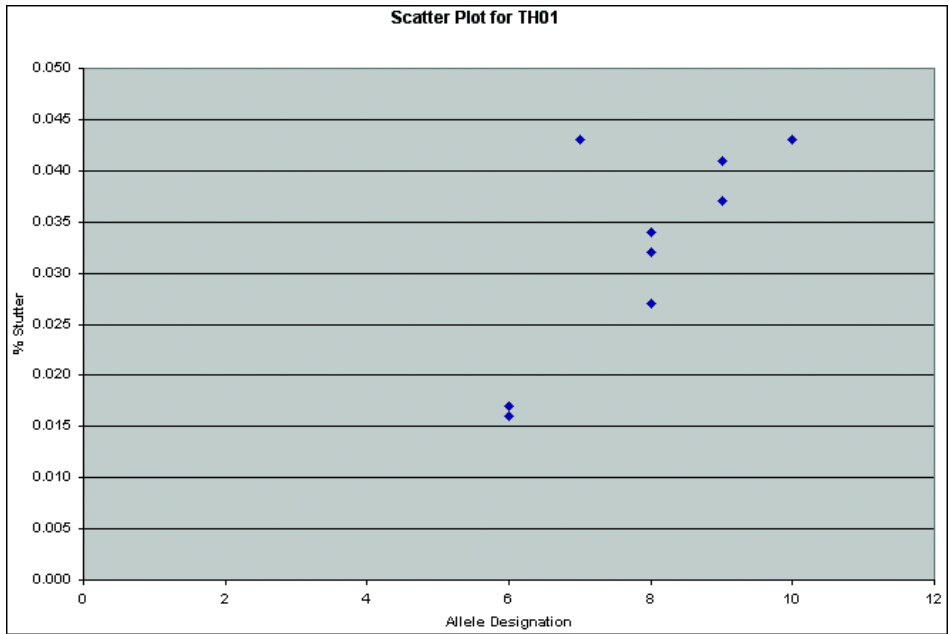
Figure 8-13   TH01 scatter plot
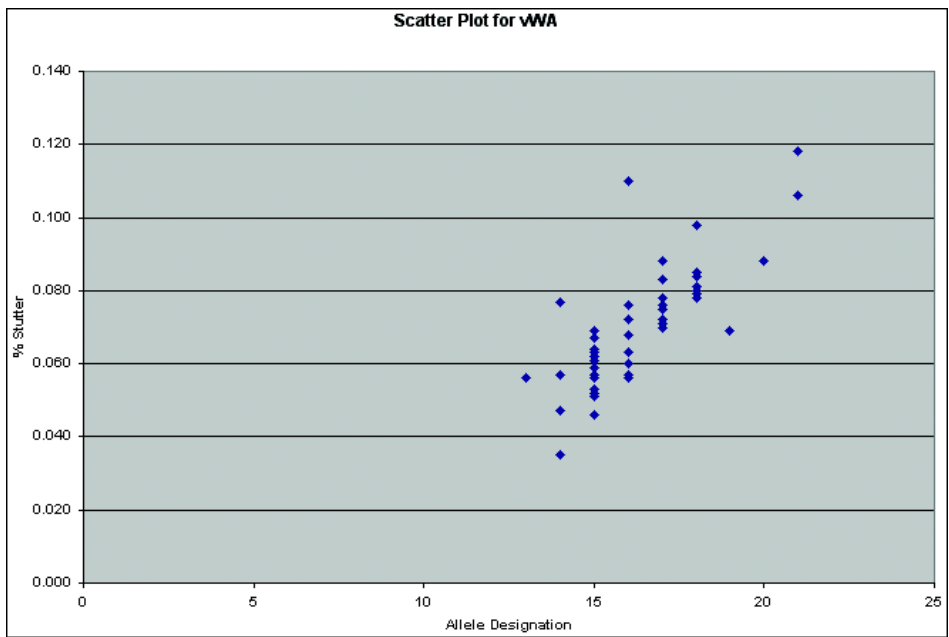
Figure 8-14   vWA scatter plot

Addition of 3′ A Nucleotide

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

The efficiency of "A addition" is related to the particular sequence of the DNA at the 3′ end of the PCR product. The AmpF*ℓ*STR SEfiler kit includes two main design features that promote maximum A addition:

- The primer sequences have been optimized to encourage A addition.
- The final extension step is 60 °C for 45 min.

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

Figure 8-15 shows split peaks that resulted from incomplete A nucleotide addition because of omission of the 45-minute extension step.
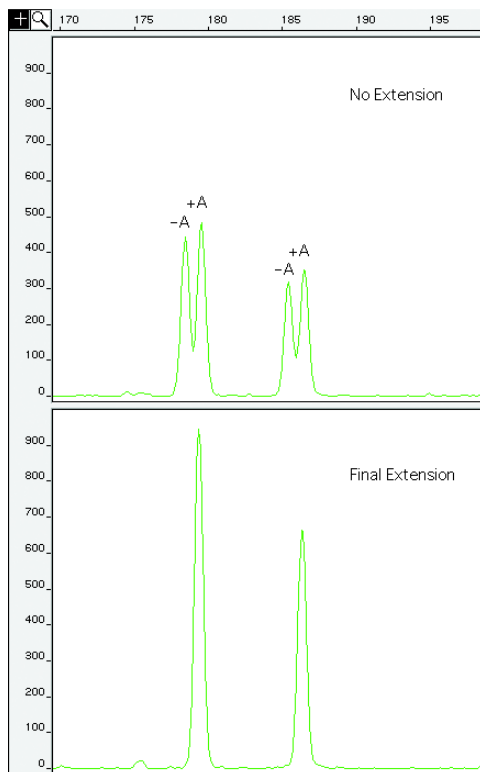
Figure 8-15   Split peaks; these data were generated on the ABI PRISM 310 Genetic Analyzer using another AmpFℓSTR kit

The AmpliTaq Gold DNA Polymerase generally requires extra time to complete the A nucleotide addition at the 3′ end of the PCR products.

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

Artifacts   Artifacts, or anomalies, have been seen in data produced on the ABI PRISM® 310 Genetic Analyzer when using the AmpF𝓁STR SEfiler kit. The shape of these artifacts is not consistent with the shape of labeled DNA fragments as seen on the ABI PRISM 310 Genetic Analyzer. Artifacts may or may not be consistent.

Figure 8-16 demonstrates examples of baseline noise and artifacts in the blue, green, yellow, and red dye electropherograms while using the AmpF𝓁STR SEfiler kit. You should consider possible noise and artifacts when interpreting data from the AmpF𝓁STR SEfiler kit on the ABI PRISM 310 Genetic Analyzer.
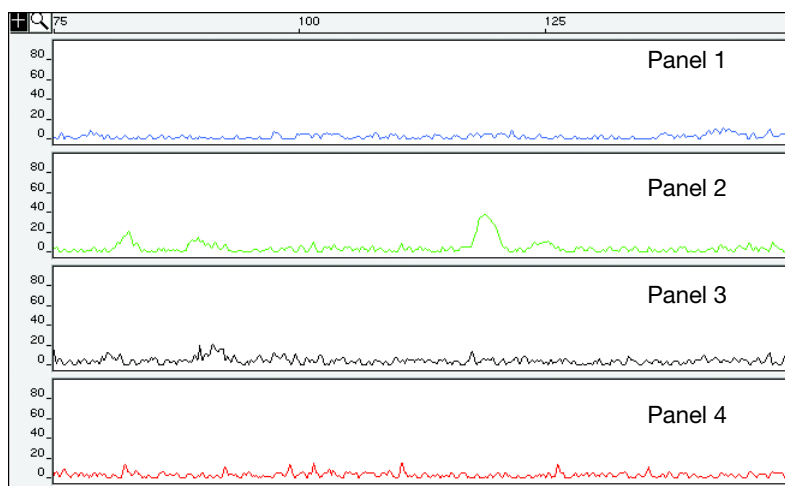
Figure 8-16   Examples of baseline noise and artifacts

Genotyping may result in the detection of these artifacts as off-ladder alleles, or "OL Alleles?".

Note: The degree of magnification (y-axis) is used in this figure to illustrate these artifacts (data produced on the ABI PRISM 310 Genetic Analyzer).

Characterization of Loci

**DAB Standard
8.1.2.1
Documentation**

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

This section describes basic characteristics of the 11 loci that are amplified with the AmpFℓSTR SEfiler kit. These loci have been previously characterized.

**Nature of the
Polymorphisms**

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

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

Some alleles in the AmpFℓSTR SEfiler Allelic Ladder containing partial repeat units in population database and nonhuman primate DNA samples have been subjected to DNA sequencing at Applied Biosystems (Lazaruk, *et al*., 2001). 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 SEfiler 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 39 families of Utah Mormon, French Venezuelan, and Amish descent. These DNA sets have been extensively studied all over the world and are routinely used to characterize the mode of inheritance of various DNA loci. Each family set contains three generations, generally including four grandparents, two parents, and several offspring. Consequently, the CEPH family DNA sets are ideal for studying inheritance patterns (Begovich *et al*.,1992).

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

Mapping   The AmpF*ℓ*STR kit loci Amelogenin, D2S1338, D3S1358, D8S1179, D16S539, D18S51, D19S433, D21S11, FGA, SE33, TH01, and vWA have been mapped and the chromosomal locations have been published (Nakahori *et al*., 1991; Edwards *et al*.,1992; Kimpton *et al*.,1992; Mills *et al*.,1992; Sharma and Litt,1992; Li *et al*.,1993; Straub *et al*.,1993; Barber and Parkin,1996).

Species Specificity

DAB Standard 8.1.2.2 Species Specificity

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

The AmpF*l*STR SEfiler kit provides the required degree of specificity for primates. Other species do not amplify for the loci tested, with the exception of the Amelogenin locus.

Nonhuman Studies

Nonhuman DNA may be present in forensic casework samples. The AmpF*l*STR SEfiler kit provides the required degree of specificity for the species tested (with the exception of the Amelogenin locus).The following experiments were conducted to investigate interpretation of AmpF*l*STR SEfiler kit results from nonhuman DNA sources.
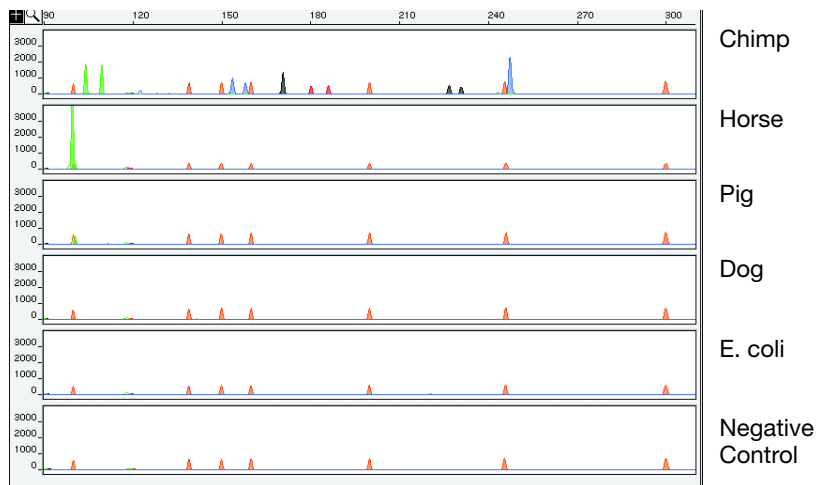
Figure 8-17   Representative electropherograms of a primate, nonprimates, a microorganism, and a negative control

All samples were analyzed on an ABI PRISM 310 Genetic Analyzer. The peaks depicted in orange are the GeneScan-500 LIZ size standard.

The extracted DNA samples were amplified in AmpF*l*STR SEfiler kit reactions and analyzed using the ABI PRISM 310 Genetic Analyzer.

- **Primates**: gorilla, chimpanzee, orangutan, and macaque (1.0 ng each)
- **Non primates**: mouse, dog, pig, cat, horse, chicken and cow (2.5 ng each)
- **Bacteria and yeast**: *Brochothrix*, *Escherichia*, *Neisseria*, *Pseudomonas*, *Bacillus*, *Staphylococcus* (approximately 5 ng each), and *Saccharomyces* (1 ng)

The primate DNA samples all amplified, producing fragments within the 100–400 base pair region (Lazaruk, *et al.*, 2001; Wallin *et al.*,1998).

The microorganisms, chicken, cow, cat, and mouse did not yield detectable product. Horse, pig, and dog produced a 103-bp fragment near the Amelogenin locus in VIC® dye. This fragment is visible on both horse and pig in Figure 8-17 on page 8-34.

Sensitivity

DAB Standard 8.1.2.2 Sensitivity

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

Importance of Quantitation

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

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 the following:

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

  Off-scale data is a problem for two reasons:

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

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

- Incomplete A nucleotide addition

The sample can be re-amplified using less DNA.

When the total number of allele copies added to the PCR is extremely low, unbalanced amplification of the two alleles of a heterozygous individual may occur (Walsh *et al*.,1992; Wallin *et al*.,1998) due to stochastic fluctuation in the ratio of the two different alleles (Sensabaugh *et al*.,1991). The PCR cycle number and amplification conditions have been specified to produce low peak heights for a sample containing 20-pg human genomic DNA. Low peak heights should be interpreted with caution.

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.
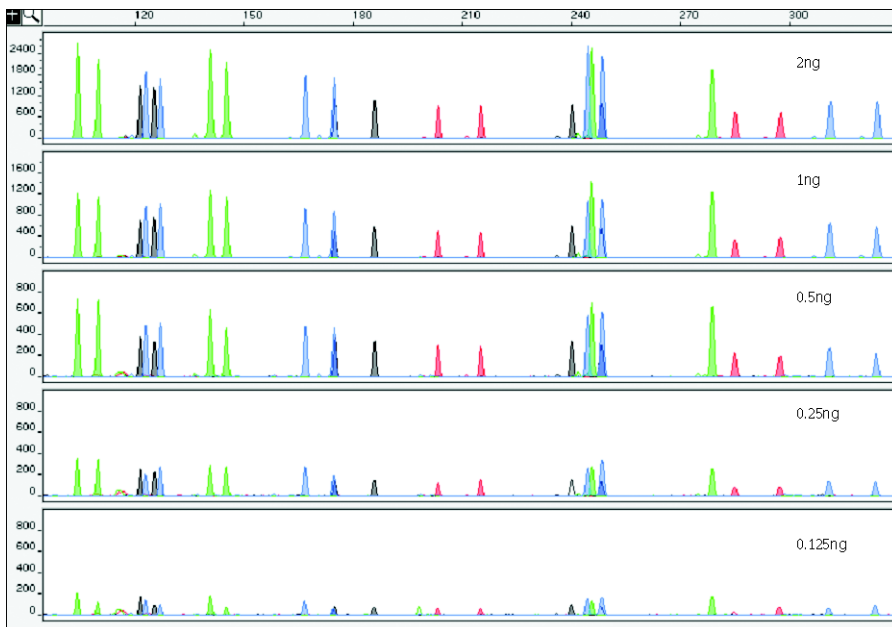
Figure 8-18   Effect of amplifying amounts of DNA ranging from 125 pg to 2 ng

Note that the y-axis scale is magnified for the lower amounts of DNA, analyzed using the ABI PRISM 310 Genetic Analyzer.

Stability

DAB Standard 8.1.2.2 Stability

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

Lack of Amplification of Some Loci

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

Differential and Preferential Amplification

Differential amplification can be defined as the difference in the degree of amplification of each locus within a co-amplified system, such that one or more loci may amplify to a greater extent compared to the other loci. Preferential amplification is used in this manual to describe differences in the amplification efficiency of two alleles at a single locus.

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

Degraded DNA

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

Degraded DNA was prepared to examine the potential for differential amplification of loci. High molecular weight DNA was incubated with the enzyme DNase I for varying amounts of time. The DNA was examined by agarose gel analysis to determine the average size of the DNA fragments at each time point.

Four nanograms of degraded DNA (or 1 ng undegraded DNA) were amplified using the AmpF*l*STR SEfiler kit. As the DNA became increasingly degraded, the loci became undetectable according to size. Preferential amplification was not observed. The loci failed to robustly amplify in the order of decreasing size as the extent of degradation progressed.

Figure 8-19 Multiplex amplifications of a DNA sample in the absence of DNase I and the sample incubated for 30 sec, 4 min, and 8 min with DNase I, analyzed using the ABI PRISM 310 Genetic Analyzer

Mixture Studies

DAB Standard 8.1.2.2 Mixture Studies

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

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

Mixed Specimen 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. In the case of STRs, stutter peaks may be informative in the interpretation of mixed samples. Furthermore, alleles amplified with the AmpF*l*STR SEfiler kit have similar peak height values for a heterozygous genotype within a locus. This balance can be used as an aid in detecting and interpreting mixtures.

Detection of Mixed Samples

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

- The presence of greater than two alleles at a locus
- The presence of a peak at a stutter position that is significantly greater in percentage than what is typically observed in a single-source sample
- 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 AmpF*l*STR SEfiler kit loci in unmixed population database samples are shown in Table 8-2.

Table 8-2   Peak height ratios

Locus	Number of Observations (n)	Mean[a]	Median[a]	Minimum[a]	Maximum[a]
D2S1338	102	92	92	68.4	99.2
D3S1358	118	97	95	75.2	100
D8S1179	104	95	95	77.2	100
D16S539	112	94	91	72.9	99.8
D18S51	83	89	89	74.4	99
D19S433	102	94	93	71.3	100
D21S11	92	93	92	71.4	99.9
FGA	130	91	91	62.4	100
SE33	103	86	86	60.6	99.8
TH01	91	96	95	75.9	99.9
vWA	105	94	93	73.9	99.7

[a]Peak height ratios were determined for those heterozygous samples with peak heights > 200 RFU.

For all 12 loci, the mean peak height ratios indicate that the two alleles of a heterozygous individual are generally very well balanced.

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, or the presence of an allele containing a rare sequence that does not amplify as efficiently as the other allele.

Resolution of Genotypes in Mixed Samples

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

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

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

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

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

Limit of Detection of the Minor Component

Mixtures of two DNA samples were examined at various ratios (1:1 to 1:10). The total amount of genomic input DNA mixed at each ratio was 1 ng.

The samples were amplified in a GeneAmp® PCR System 9700 and were electrophoresed and detected using an ABI PRISM 310 Genetic Analyzer.

The results of the mixed DNA samples are shown in Figure 8-20 on page 8-44, where sample A and sample B were mixed according to the ratios provided. Table 8-3 shows profiles of samples in Figure 8-20 on page 8-44.

Table 8-3   Profiles of Samples

Allele	Profile	
	Sample A	Sample B
Amelogenin	X, Y	X
D2S1338	20, 23	17, 25
D3S1358	15,16	15, 18
D8S1179	12,13	13
D16S539	9, 10	11, 12
D18S51	12, 15	17, 19
D19S433	14,15	13
D21S11	28, 31	30, 30.2
FGA	24, 26	23.2, 24
SE33	17, 25.2	27.2, 29.2
TH01	7, 9.3	7, 9
vWA	14,16	17, 19

For these 1-ng total DNA mixture studies, the limit of detection is when the minor component is present at approximately one-tenth of the concentration of the major component and a threshold of 50 RFU. The limit of detection for the minor component is influenced by the combination of genotypes in the mixture.
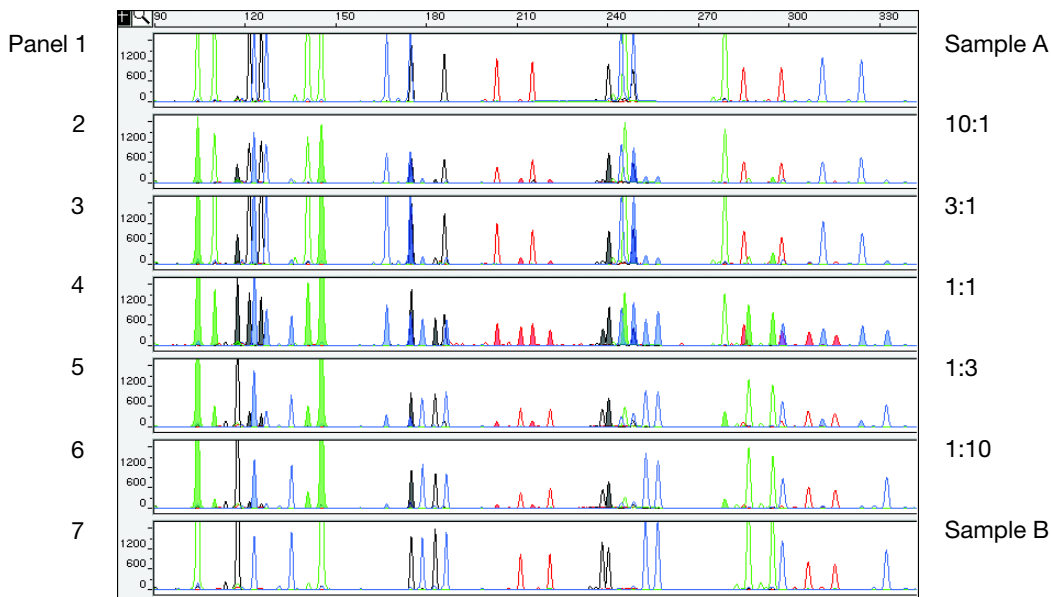
Figure 8-20   Results of the two DNA samples mixed together at defined ratios and amplified with the AmpFℓSTR SEfiler PCR Amplification Kit

Sample A and Sample B are a female and male sample, respectively. The ratios of Sample A to Sample B (A:B ratios) shown are 10:1, 3:1, 1:1, 1:3, and 1:10, respectively. The alleles attributable to the minor component, even when the major component shares an allele, are highlighted in panels 2, 3, 5, and 6. All alleles are highlighted in panel 4.

Data Interpretation

Minimum Sample Requirement

The AmpF*l*STR SEfiler PCR Amplification Kit has been optimized to amplify and type approximately 1.0–2.5 ng of sample DNA reliably.

The PCR cycle number and amplification conditions have been specified to produce low peak heights for a sample containing 20 pg human genomic DNA. Thus, the overall sensitivity of the assay has been adjusted to avoid or minimize stochastic effects. Applied Biosystems has successfully typed samples containing less than 0.5 ng DNA.

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

Population Data

**8.1.2.3
Population Data**

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

Overview

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

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

**Population
Samples Used in
These Studies**

The AmpFℓSTR SEfiler PCR Amplification Kit was used to generate the population data provided in this section. Samples were collected from individuals throughout the United States with no geographical preference.

African-American

104 samples were provided.

U.S. Caucasian

69 samples were provided.

**AmpF*l*STR
SEfiler Kit Allele
Frequencies**

Table 8-4 shows the AmpF*l*STR SEfiler kit allele frequencies in two populations, listed as percentages.

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
D3S1358		
12	*	*
13	*	*
14	12.98	11.59
15	25.96	28.99
15.2	*	*
16	33.65	19.57
17	20.19	21.74
18	7.21	18.12
19	*	*

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
vWA		
11	1.92	*
12	*	*
13	0.96	*
14	4.33	9.42
15	24.52	15.94
16	23.56	12.32
17	21.63	26.81
18	11.54	22.46
19	7.21	11.59
20	3.37	1.45
21	0.96	*
22	*	*
23	*	*
24	*	*

Table 8-4　AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
D16S539		
5	*	*
8	1.92	1.45
9	19.23	13.77
10	14.90	6.52
11	29.33	29.71
12	16.35	28.99
13	14.90	17.39
14	3.37	2.17
15	*	*

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
D2S1338		
15	*	0.72
16	6.25	6.52
17	10.10	20.29
18	8.65	8.70
19	13.46	15.22
20	11.06	7.97
21	9.62	2.17
22	14.90	2.90
23	10.58	10.14
24	7.69	15.22
25	4.81	7.97
26	2.88	2.17
27	*	*
28	*	*

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
D8S1179		
8	*	2.90
9	0.48	1.45
10	2.88	7.25
11	4.81	9.42
12	19.71	12.32
13	22.60	31.88
14	30.29	19.57
15	14.42	11.59
16	3.85	2.17
17	0.96	1.45
18	*	*
19	*	*

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
SE33		*
4.2	*	*
6.3	*	*
8	*	*
9	*	*
9.2	0.48	*
11	0.48	*
11.2	0.48	*
12	0.48	2.17
12.2	0.96	*
13	1.92	2.90
13.2	0.48	*
14	2.88	3.62
14.2	0.96	*
15	4.81	3.62
15.2	0.00	*
16	6.25	5.07
16.2	0.48	*
17	8.17	10.14
17.2	*	*
18	11.54	7.25
19	8.17	7.97

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
SE33 *(continued)*		
20	8.65	2.90
20.2	0.96	*
21	4.33	5.07
21.1	*	*
21.2	0.48	0.72
22	0.48	*
22.2	0.96	4.35
23	0.48	*
23.2	0.96	3.62
24.2	0.48	*
25.2	3.37	5.07
26.2	5.77	4.35
27.2	11.54	7.97
28.2	4.33	9.42
29.2	4.33	4.35
30.2	2.88	6.52
31.2	1.44	2.17
32.2	*	0.72
33.2	*	*
34.2	*	*
35	*	*

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
SE33 *(continued)*		
35.2	*	*
36	*	*
37	*	*
D19S433		
9	0.48	*
10	1.44	*
11	4.33	1.45
11.2	0.48	*
12	15.87	3.62
12.2	2.40	*
13	25.96	27.54
13.2	7.21	1.45
14	21.15	39.13
14.2	4.81	*
15	7.69	15.94
15.2	3.85	3.62
16	0.96	5.07
16.2	2.88	1.45
17	*	0.72
17.2	0.48	*

Table 8-4 AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
TH01		
4	*	*
5	0.96	*
6	12.98	23.19
7	37.98	22.46
8	19.71	9.42
9	15.87	10.87
9.3	11.54	33.33
10	0.96	0.72
11	*	*
13.3	*	*

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
FGA		
17	*	*
18	0.96	3.62
18.2	0.96	0.72
19	6.25	5.07
19.2	0.48	*
20	5.29	12.32
21	12.02	17.39
22	16.83	15.22
23	16.35	13.77
23.2	*	1.45
24	15.87	18.12
25	11.54	7.25
26	5.29	4.35
26.2	*	*
27	5.29	0.72
28	1.44	*
29	0.48	*
30	0.48	*
30.2	*	*
31.2	*	*
32.2	*	*

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
FGA *(continued)*		
33.2	*	*
42.2	*	*
43.2	*	*
44.2	0.48	*
45.2	*	*
46.2	*	*
47.2	*	*
48.2	*	*
50.2	*	*
51.2	*	*

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
D21S11		
24	*	*
24.2	*	*
25	*	*
26	*	*
27	4.81	2.17
28	25.48	15.94
29	14.42	26.09
30	22.12	23.19
30.2	1.92	2.17
31	11.06	7.25
31.2	3.37	7.97
32	0.96	3.62
32.2	5.29	7.97
33	*	*
33.2	2.40	3.62
34	*	*
34.2	0.48	*
35	4.81	*
35.2	*	*
36	2.88	*

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
D21S11 *(continued)*		
37	*	*
38	*	*

Table 8-4   AmpF*l*STR SEfiler kit allele frequencies *(continued)*

Allele	African American (n = 104)	U.S. Caucasian (n = 69)
D18S51		
7	*	*
9	*	*
10	*	0.72
10.2	*	*
11	*	1.45
12	8.17	17.39
13	4.33	18.12
14	8.65	11.59
15	21.15	15.94
16	16.35	13.77
17	15.87	9.42
18	11.54	7.25
19	10.58	2.90
20	2.88	*
21	*	1.45
22	0.48	*
23	*	*
24	*	*
25	*	*
26	*	*
27	*	*

Mutation Rate

Estimating Germline Mutations

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

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

Additional 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 AmpF*l*STR SEfiler kit loci.
• Methods for modifications of these mutation rates (to infer mutation rates indirectly for those loci where these rates are not large enough to be measured directly and/or to account for those events undetectable as Mendelian errors).

Probability of Identity

Table of Probability of Identity

Table 8-5 shows the Probability of Identity (P_I) values of the AmpFℓSTR SEfiler kit loci individually and combined.

Table 8-5   Probability of Identity values for the AmpFℓSTR SEfiler kit STR loci

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

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

Probability of Paternity Exclusion

Table of Probability of Paternity Exclusion

Table 8-6 shows the Probability of Paternity Exclusion (P_E) values of the AmpF*l*STR SEfiler kit STR loci individually and combined.

Table 8-6   Probability of paternity exclusion for the AmpF*l*STR SEfiler kit STR loci

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

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

Genotyping Using Windows NT OS

9

In This Chapter   This chapter describes the use of ABI PRISM® Genotyper® Software v3.7 in conjunction with the AmpF*l*STR® SEfiler™ Kit Template and the Microsoft® Windows NT® operating system to automatically genotype samples.

Using Genotyper Software for Automated Genotyping

About the Software

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

A Genotyper software template file that contains macros specifically written for use with the AmpFℓSTR® SEfiler™ PCR Amplification Kit is provided with this manual. Use this template with the AmpFℓSTR SEfiler kit data. Install the template onto your computer following the instructions in the "READ_ME" file.

Note: You must have Genotyper Software v3.7 or higher and Windows NT 4.0 with Service Pack 4 or 5 operating system to run the AmpFℓSTR SEfiler Kit Template. Refer to the *ABI PRISM® Genotyper® 3.7 NT Software User's Manual* (P/N #4309947C) and *ABI PRISM® Genotyper® 3.7 NT Software Applications Tutorials* (P/N #4309961C) for more detailed information about the Genotyper software. The Human Identification Tutorial and HID template file included with the Genotyper Software v3.7 package are for tutorial purposes only.

Before Running Genotyper Software

GeneScan Analysis Software sample data (particularly the allelic ladder) must meet a few specific requirements before you can use the macros in the AmpFℓSTR SEfiler Kit Template. These requirements are described in this section.

Sample Info Column

All samples must have a unique sample description in the Sample Info column of the GeneScan software sample sheet so that the macros in the AmpFℓSTR SEfiler Kit Template can build a table. Samples with an empty Sample Info column will not be incorporated into the table of genotypes. Also, lanes or injections that contain the AmpFℓSTR SEfiler Allelic Ladder must have the word "ladder" in the Sample Info column. The first lane or injection of ladder is the one used by the Kazam macro in the AmpFℓSTR SEfiler Kit Template to determine the sizes in the allele categories that will be used for genotyping.

It is possible to skip the first lane or injection of allelic ladder and use the second lane or injection of allelic ladder for genotyping instead. After importing the sample files, but before running the Kazam macro, remove the word "ladder" from the Sample Info column in all four sample dye colors for the first lane or injection of allelic ladder in the Dye/lanes window. Make sure that the word "ladder" is entered for Sample Info in the second lane or injection of allelic ladder. See for a description of how to access the Sample Info column in the Dye/lanes window.

GeneScan Analysis Software Peak Recognition

All allele peaks in the allelic ladder for each locus must be "recognized" (labeled) in the GeneScan Analysis Software (*i.e.*, each allele peak must have an entry in the GeneScan table). Thus, all allele peaks in each allelic ladder must have a peak height value in relative fluorescence units (RFU) greater than the Peak Amplitude Threshold (PAT) specified in the GeneScan software Analysis Parameters. Also, all allele peaks in each allelic ladder must be resolved. For example, the FGA 26, 26.2, and 27 alleles must be resolved so that each peak has an entry in the GeneScan software table.

Sample allele peak heights must also be greater than the GeneScan Software PAT in order to be recognized (labeled) by Genotyper software. Note that the PAT value specified in the GeneScan software Analysis Parameters is not necessarily the same as the RFU value that may be used by the forensic analyst as the "interpretational threshold." The "Low Signal" column of the appropriate Genotyper software table (see) can be used to identify peaks that are greater than the GeneScan software PAT, but less than a specified minimum threshold (default 150 RFU in the table macro).

AmpF*l*STR SEfiler Kit Template

The AmpF*l*STR SEfiler Kit Template contains macros that perform the following steps automatically:

- Finds the lane or injection containing the allelic ladder
- Creates allele size categories that are centered on the sizes obtained for the allelic ladder alleles
- Assigns the appropriate allele label to sample alleles that size within the allele size categories
- Removes labels from stutter peaks by applying a filter
- Builds a table containing genotypes for all samples

Using the AmpF*l*STR SEfiler Kit Template File

Use the following procedure to assign genotypes to AmpF*l*STR SEfiler kit alleles automatically.

To use the AmpF*l*STR SEfiler Kit Template:

1.	Double-click the **SEfiler** icon to launch the Genotyper software application and open the template file simultaneously. **Note:** The AmpF*l*STR SEfiler Kit Template is a read-only file, which means that a new file must be saved as a different name to ensure that the original template file is not overwritten.
2.	Set preferences to import raw data, and Blue, Green, Yellow, Red, and Orange data.
3.	To import the GeneScan sample files: a. Select **File** > **Import GeneScan File**(s). b. Select the project file and click **Import**.
4.	If each sample does not already have Sample Info completed in the sample sheet: a. Select **Views** > **Show Dye/lanes**. b. Click the first sample row to select it. c. Click the **Sample Info** box at the top of the window and type the sample designation or description. d. Repeat steps b and c to enter a sample description for every dye/lane in the list. Enter the same sample description for all dye colors of a single sample.
5.	From the **Macro** list at the bottom left of the Main window, select **Check GS500**.
6.	Select **Macro** > **Run Macro**. In the plot window that opens, scroll through each sample to verify that each GeneScan-500 peak (from 75–450 bp) was assigned the correct size in the GeneScan Analysis Software.
7.	From the **Macro** list at the bottom left of the Main window, select **Kazam**.

To use the AmpF*ℓ*STR SEfiler Kit Template: *(continued)*

8.	Select **Macro** > **Run Macro**. This macro may take a few minutes to run. When it is finished, a plot window opens with the blue allelic ladder (D3S1358, vWA, D16S539, and D2S133) and sample allele peaks labeled.
9.	Examine data and edit peaks.
10.	Print the electropherograms in the plot window by selecting **File** > **Print**.
11.	a. In the Main Window, click the green **G** button at the top left. b. Select **Views** > **Show Plot Window**. c. Repeat steps 8 and 9.
12.	a. In the Main Window, click the yellow **Y** button at the top left. b. Select **Views** > **Show Plot Window**. c. Repeat steps 8 and 9.
13.	a. In the Main Window, click the red **R** button at the top left. b. Select **Views** > **Show Plot Window**. c. Repeat steps 8 and 9.

Examining Data

Check that the peaks in the allelic ladder are labeled correctly. Scroll through the samples below the allelic ladder to examine the peak labels in each electropherogram.

Peak Labeling

- Allele categories (which appear as dark gray bars in the Plot window) are defined to be ±0.5 bp wide. Peaks that size within ±0.5 bp of an allele category has a label indicating the allele designation.

Note: The categories for TH01 alleles 9.3 and 10 are ± 0.4 bp wide.

- Peaks that do not size within an allele category will have a label indicating "OL Allele?" (off-ladder allele).
- The Kazam macro includes a step that removes labels from stutter peaks by applying a percentage filter. Labels are removed from peaks that are followed by a (specified percent difference) higher, labeled peak within 3.25 to 4.75 bp.

  The specified filter percentages for these loci are 809% for TH01, 809% for D3S1358, 809% for vWA, 809% for FGA, 733% for D8S1179, 669% for D16S539, 669% for D21S11, 567% for D2S1338, 488% for D19S433, 525% for D18S51, and 488% for SE33.

Note: The label "OVLR" refers to the overlap region between rare TH01 and rare FGA alleles. The reported rare alleles that may be observed in the OVLR region are as follows: TH01 13.3 (204 bp), TH01 14 (205 bp), FGA 12.2 (197 bp), and FGA 13 (199 bp). The peak labels for any alleles that are detected in this overlap region size range will include the OVLR designation (including the TH01 13.3 allele in the AmpF*l*STR SGM Plus Allelic Ladder.

- A sample allele peak must have been recognized by GeneScan software before it can be recognized by Genotyper software. Thus, sample allele peaks that are below the PAT that was specified in the GeneScan software Analysis Parameters cannot be labeled by Genotyper software.

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

Peak Editing

Clicking a labeled peak removes the label. Clicking the same peak again defaults to the placement of bp size of that peak. A dialog box with a field to enter the requested text may be accessed by selecting the **Analysis** > **Set Click Options**. Type the allele designation and/or desired text, then click **OK**.

Plot Window Viewing Options

To zoom in and out on regions of the plot window:

1.	In the Plot window, click and drag in a region of an electropherogram to draw a box around the desired size range (the vertical size of the box is not important).
2.	Type **Ctrl+R** (hold down the Ctrl key and type the letter R) to zoom in.
3.	Type **Ctrl+H** to zoom out completely.

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

1.	Select **Views** > **Show Dye/Lanes Window**.
2.	Click the desired Dye/lane rows. **Note:** Hold down the Shift key on the keyboard to select multiple adjacent Dye/lane rows. Hold down the Ctrl key to select Dye/lane rows that are not adjacent.
3.	Select **Views** > **Show Plot Window**.

Making Tables

Three macros for making tables are included in the AmpF*l*STR SEfiler Kit Template. They are:

- Make Allele Table
- 310: Make Table
- 377: Make Table

The Make Allele Table macro contains only Sample Info and genotype data. The other table, 310: Make Table contains additional information.

All four of the tables have two features in common:

- A locus that has no labeled peaks has zeros in the cells of the table for that locus.
- Loci that have homozygous alleles have the allele designation indicated twice in the table.

Make Allele Table

This table has Sample Info in the first column, and allele designations for each locus in columns 2–23. The first two labeled peaks within each locus appear in the table.

310: Make Table

This table can be used if the data was generated on the ABI PRISM 310 Genetic Analyzer. This table has Sample Info in the first column, Sample Comment in the second column, locus name in the third column, and allele designations in columns 4–7. Four columns are provided for allele designations to accommodate mixed samples. The first four labeled peaks within each locus appear in the table. The remaining five table columns are as follows:

- **Overflow**: If more than two peaks are labeled at one locus, the text "> two labels" appears in this column.
- **Low Signal**: If the height of any peak at a locus is greater than the PAT specified in the GeneScan Analysis Parameters but less than 150 RFU, the text "< 150 RFU" appears in this column.
- **Saturation**: If the raw data signal for any peak at a locus is greater than 8191 RFU, the text "310: off-scale" appears in this column.

- **Edited Label**: The text "Edited" appears in this column for any loci where the peak labels were edited manually. For example, clicking an unlabeled peak in the Plot window to add a label constitutes an edit.

- **Edited Row**: The text "Edited" appears in this column for any rows in the table that contain table cells that have been edited after initial creation of the table.

377: Make Table

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

IMPORTANT! Before making a table, examine all electropherograms and edit their peaks as described in the previous section.

To create and use tables:

1.	From the **Macro** list at the bottom of the Genotyper software Main Window, click one of the three table macros.
2.	Select **Macro** > **Run Macro**.
3.	Select **Views** > **Show Table Window** to view the table in full screen mode.
4.	Open and view the plot: **Note:** For all tables except the Make Allele Table, clicking in a cell of the table causes the corresponding sample electropherogram to appear in the plot window: • Click any cell in the table to display the locus region of the corresponding electropherogram in the Plot window for that sample. • Zoom out (**Ctrl+H**) to view all loci for a particular dye color for the corresponding sample.
5.	To edit the cells of the table: a. Click a cell of the table that contains an allele designation. b. Select **Edit** > **Edit Cell**. c. Type the desired information in the box and click **OK**.

To create and use tables: *(continued)*

6.	Print the table by selecting **File** > **Print**.
7.	This step is optional. Select **Table** > **Export to File** to save the table as a file that can be opened in Microsoft Excel.
8.	Select **File** > **Save** to save the template file with data.

Understanding the AmpFℓSTR SEfiler Kit Template

Troubleshooting Automated Genotyping

To Troubleshoot Automated Genotyping:

Observation	Probable Cause	Recommended Action
Warning message: "Could not complete 'Run Macro' command because no dye/lanes are selected."	The word "ladder" is not in Sample Info for the lane or injection of allelic ladder.	Type the word **ladder** in the Sample Info column for each dye color (Blue, Green, Yellow, and Red) for the AmpFℓSTR SEfiler Allelic Ladder sample.
Warning message: "Could not complete 'Run Macro' command because the labeled peak could not be found."	One or more peaks in the allelic ladder are below the Peak Amplitude Threshold that was specified in the GeneScan software Analysis Parameters.	Use another allelic ladder in the project, or 1. In the GeneScan Analysis Software, lower the Peak Amplitude Threshold values for Blue, Green, Yellow, and Red dye colors in the Analysis Parameters. 2. Reanalyze the sample file(s) containing the allelic ladder. 3. Import all sample files into a new Genotyper software project, and run the Kazam macro again.

About This Kit Template

This section describes the organization and functionality of the AmpFℓSTR SEfiler Kit Template. Read this section for a greater understanding of the macros and steps that are used in the AmpFℓSTR SEfiler Kit Template.

Categories

In the Genotyper software, each allele is defined by a category. Each category contains information about the allele size, size range, and dye color. To view the list of categories in the AmpFℓSTR SEfiler Template, select **View** > **Show Categories**. The categories for each locus are listed together under the locus name. The locus is called a group.

In the Categories window, each locus actually has two sets of categories. For example, the D3S1358 locus has one list of categories under the group "D3S1358" and another list of categories under the group "D3S1358.os." The categories in the D3S1358 group are allele categories and are used for allele assignment.

Offset Categories

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

Finding and Recognizing the Leftmost (first) Allele Peak in Each Allelic Ladder

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

Calculating the Offset Values

Categories with the ".os" suffix contain offset categories.

The base pair size indicated in each category is a "reference size." One main function of the macros in the AmpFℓSTR SEfiler Template is to offset the reference sizes relative to the sizes obtained for the alleles in the allelic ladder. These offset steps are performed by the Calculate [locus] Offsets macros, located in the Macro list of the Genotyper software Main window. After the macros are run, the calculated offset values are indicated in parentheses near the end of each category line in the Categories window.

An example of how to interpret the offset values is given here for D3S1358 allele 14. The reference size for this allele is 122 bp. On a particular ABI PRISM 310 injection, the size obtained for D3S1358 allele 14 was 119.06 bp. The offset value is calculated as $119.06 - 122 = -2.94$.

In this example, the actual category size used for allele assignment is 119.06 (equals 122–2.94), which is the size of the D3S1358 allele 14 in this particular injection of the allelic ladder. In other words, the category sizes used for genotyping are equivalent to the allele sizes obtained in the lane or injection of allelic ladder.

Applying the Appropriate Offset Value to Each Allele in Succession

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

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

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

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

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

Off-Ladder Alleles and Virtual Alleles

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

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

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

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

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

Many of the loci in the Categories window contain virtual allele categories. For example, the FGA locus contains a virtual category for many 2-bp length variants.

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

Filtering Stutter Peaks

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

To filter stutter peaks:

1.	In the Step Window for the Kazam macro, scroll down to the line that reads "Select category: D3S1358."
2.	Five rows below, select the line that reads, "Remove labels from peaks followed by an 809% higher, labeled peak within 3.25 to 4.75 bp."
3.	Select **Macro** > **Edit Step** to open the Filter Labels window.
	In the Filter Labels window, there are four options (check boxes) for filtering. In this example, the filtering option for D3S1358 is denoted in the last check box. This filtering option includes another check box that reads "(higher by at least 809%)."
	For each labeled peak (*e.g.* peak A) in the locus size range, this filtering option examines the very next (*i.e.* greater in bp size) labeled peak (peak B). The label will be removed from peak A if peak B meets both of the specified criteria:
	• Peak B is higher by at least 809%
	• Peak B is within 3.25 to 4.75 bp
	The percentage value in this filtering option is calculated as follows:
	[(peak B – peak A) / peak A] x 100 = percentage value

To filter stutter peaks: *(continued)*

	3. *(continued)*
	For example, if peak A = 175 RFU and peak B = 2500 RFU, then the percentage value is calculated as follows:
	[(2500 – 175) / 175] x 100 = 1329%
	In this example, the label will be removed from peak A, provided that the filter option specifies a threshold of 809%, and that peak B is within 3.25 to 4.75 bp of peak A.
	Conventionally, percent stutter is calculated:
	(peak A / peak B) x 100 = percent stutter
	The percentage value that is used in the Genotyper software filtering option (F) can be derived from the conventional percent stutter expression (S):
	F = (10,000 / S) – 100
	For example, if the desired stutter percent threshold for D3S1358 is 11%, then the percentage value that should be used in the Genotyper software filtering option is:
	F = (10,000 / 11) – 100 = 809%
4.	To use a filter value different than 809% for D3S1358, enter another value, then click **Replace**.

The peak filtering included in the Kazam macro is intended only as a tool and guideline. Final conclusions should be based on careful examination of the STR profiles.

Kazam (20% Filter)
The standard Kazam macro is written so that a different filter threshold can be used for each locus (the steps for each locus are written separately in the macro). The Kazam macro thus provides maximum flexibility and the opportunity to customize the filter that is used for each locus.

A different version of the Kazam macro called "Kazam (20% filter)" is also provided in the Macro list. This macro is simpler than the Kazam macro in that a 20% stutter filtering step is specified for all loci.

To view the various steps in the Kazam (20% filter) macro:

1.	Click the Kazam (20% filter) line in the Macro list.
2.	Select **Views** > **Show Step Window**. The first filter step for this macro (which applies to the sample alleles) reads, "Remove labels from peaks whose height is less than 20% of the highest peak in a category's range."

Note: This particular option does not include any condition regarding the bp size of the filtered peak relative to a higher peak. Indeed, this second filtering option removes labels from all peaks that are less than a specified percentage of the highest peak observed anywhere in the locus range.

To edit the filter value:

1.	Click this step in the Step window. Refer to step 2 of the procedure, "To view the various steps in the Kazam (20% filter) macro," on page 9-16.
2.	Select **Macro** > **Edit Step**. **Note:** This macro uses the second filter option in the Filter Labels window.
3.	If desired, change the value from 20% to some other value, then click **Replace**.

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

Modifying the Template

The original AmpFℓSTR SEfiler Template File can be modified so that the changes made to the macros or settings are used as the default:

To modify the template:

1.	Close all Genotyper windows, but do not quit the application.
2.	Right-click the **AmpFℓSTR SEfiler Kit Template** icon.
3.	In the template, select **Properties**.
4.	Deselect the check box for **Read-only** at the bottom of the window, then close the Properties window.
5.	Double-click the **AmpFℓSTR SEfiler Kit Template** icon.
6.	Make any desired changes.
7.	Save the template file by selecting **File** > **Save**.
8.	Right-click the **AmpFℓSTR SEfiler Kit Template** icon.
9.	In the template, select **Properties**.
10.	Select the check box for **Read-only**, then close the Properties window to save your modifications.

Determining Genotypes

**AmpF*l*STR
SEfiler Allelic
Ladder**

The AmpF*l*STR® SEfiler™ Allelic Ladder contains the most common alleles for each locus. Genotypes are assigned by comparing the sizes obtained for the unknown samples with the sizes obtained for the alleles in the allelic ladder.

In addition to the alleles included in the AmpF*l* STR SGM Plus Kit, alleles for SE33 are also included.

The macro size ranges include the actual number of nucleotides contained in the smallest and largest allelic ladder alleles for each locus. The size range also includes 3´. The AmpF*l*STR SEfiler PCR Amplification Kit is designed so that a majority of the PCR products contain the non-templated 3´ A nucleotide. The alleles have been named in accordance with the recommendations of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) (DNA Recommendations, 1994; Bar *et al*., 1997).

The number of complete four base pair repeat units observed is designated by an integer. Variant alleles that contain a partial repeat are designated by a decimal followed by the number of bases in the partial repeat. For example, an FGA 26.2 allele contains 26 complete repeat units and a partial repeat unit of two base pairs.

Additional variation has been seen at some loci where alleles exist that differ from integer allele lengths by one or three base pairs. For example, D21S11 allele 33.1 contains 33 complete repeat units and one nonconsensus base pair. Likewise, a D21S11 29.3 allele contains 29 complete repeat units and a partial 3-bp unit (Moller *et al*., 1994; Gill *et al*., 1997).

A Genotyper software electropherogram of the AmpF*l*STR SEfiler Allelic Ladder listing the designation for each allele is shown in Figure 9-1. This electropherogram indicates the designation for each allele. Results were obtained on an ABI PRISM 310 Genetic Analyzer.
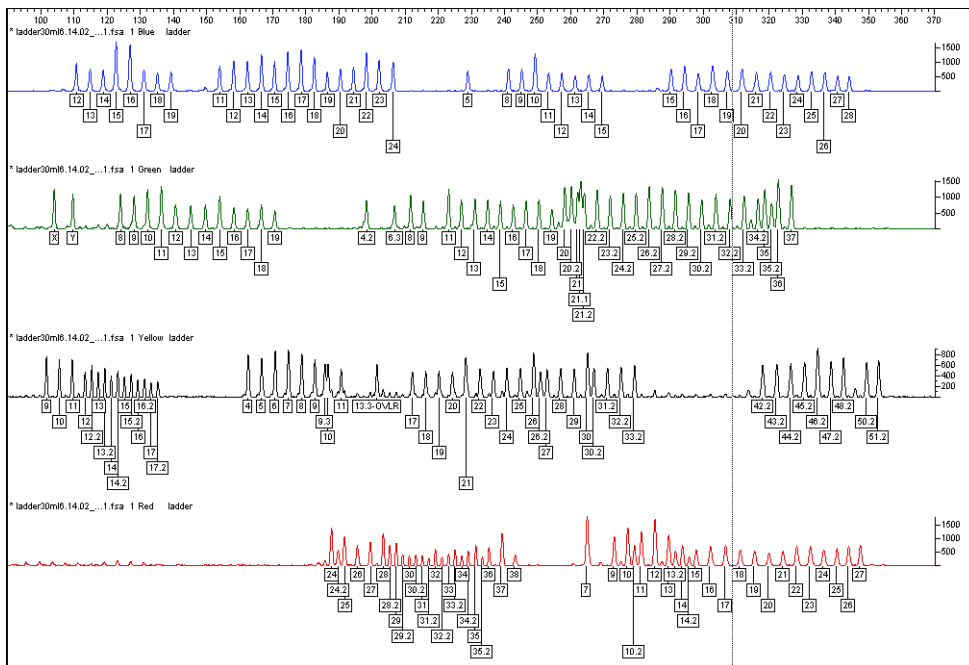

Figure 9-1   Genotyper® software plot of the AmpF*l*STR SEfiler Allelic Ladder

Genotyping Using the AmpF*l*STR SEfiler Allelic Ladder

When you interpret AmpF*l*STR SEfiler kit results, be aware that the system assigns genotypes to sample alleles by comparing their sizes to those obtained for the known alleles in the AmpF*l*STR SEfiler Allelic Ladder. Genotypes, not sizes, are used for comparison of data between runs, instruments, and laboratories.

We strongly recommend that laboratories use an AmpF*l*STR SEfiler Allelic Ladder from each project to convert the allele sizes to genotypes because:

- The size values obtained for the same sample can differ between instrument platforms because of differences in the type and concentration of the gel/polymer matrices and in electrophoretic conditions.
- Sizes may differ between protocols for the same instrument platform because of differences in gel or polymer concentration, run temperature, gel or capillary thickness, and well-to-read length.
- Slight procedural and reagent variations between gels or between single and multiple capillaries result in greater size variation than that found between samples on the same gel or between samples injected in the same capillary in a single run.

Size Standard

Use the GeneScan-500 LIZ® Size Standard with the AmpF*l*STR SEfiler kit. Common alleles for all AmpF*l*STR SEfiler kit loci are less than 400 base pairs. The recommended sizing method, Local Southern, uses two internal lane size standard peaks larger than each allele and two smaller than each allele to be sized. When size standard peaks are defined in routine analyses, inclusion of the 400 base pair and 450 base pair peaks in the GeneScan-500 LIZ Size Standard is recommended.

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

Analyzing AmpF*l***STR SEfiler Allelic Ladder**

To size the AmpF*l*STR SEfiler Allelic Ladder alleles, analyze the lanes/injections containing allelic ladder using the same parameters used for samples.

To compare the results of lanes or injections of AmpF*l***STR SEfiler Allelic Ladder:**

1.	Compare the base pair sizes of one lane or injection of allelic ladder to those obtained for the other lanes or injections of allelic ladder. All corresponding peaks (peaks at the same position in the allelic ladder) should be within ±0.5 bp of each other.
2.	If one or more corresponding peaks are not within ±0.5 bp of each other, check the GeneScan-500 LIZ Size Standard peaks in all allelic ladder lanes or injections to confirm that all GeneScan-500 LIZ Size Standard peaks have been assigned the correct size and/or that all peaks are clearly resolved.

To manually genotype samples:

1.	Select one lane or injection of allelic ladder to use for genotyping. **Note:** Our studies have shown that it does not matter which lane or injection of allelic ladder is selected if the alleles in the allelic ladder samples are within ±0.5 bp of each other.
2.	Compare the base pair size obtained for each sample allele peak to the sizes obtained for the allelic ladder peaks.
3.	Assign genotypes to those sample allele peaks falling within ±0.5 bp of the corresponding allelic ladder peak. The allele designation for each allelic ladder peak is given in Figure 9-1 on page 9-19.

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

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

Genotyping Using the Macintosh OS

10

In This Chapter   This chapter describes the use of ABI PRISM® Genotyper® Software v2.5.2 in conjunction with the AmpFℓSTR® SEfiler™ Kit Template and the Macintosh® OS to automatically genotype samples.

Using Genotyper Software for Automated Genotyping

About the Software

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

A Genotyper software template file that contains macros specifically written for use with the AmpF*l*STR SEfiler PCR Amplification Kit is provided with this manual. Use this template with AmpF*l*STR SEfiler kit data. Install the template onto your computer following the instructions in the "READ_ME" file.

Note: You must have Genotyper Software v2.5.2 or higher to run the AmpF*l*STR SEfiler Kit Template. The minimum system requirement for this version of Genotyper software is a Power Macintosh computer with Macintosh OS 8.x or 9.1. Refer to the *ABI PRISM® Genotyper® 2.5 Software User's Manual* (P/N 904648) and *ABI PRISM® Genotyper® 2.0 Software Applications Tutorials* (P/N 904649) for more detailed information about the Genotyper software. The Human Identification Tutorial and HID template file included with the Genotyper Software v2.5.2 package are for tutorial purposes only.

Before Running Genotyper Software

GeneScan Analysis Software sample data (particularly the allelic ladder) must meet a few specific requirements before you can use the macros in the AmpF*l*STR SEfiler Kit Template.

Sample Info Column

All samples must have a unique sample description in the Sample Info column of the GeneScan software sample sheet so that the macros in the AmpF*l*STR SEfiler Kit Template can build a table. Samples with an empty Sample Info column will not be incorporated into the table of genotypes. Also, lanes or injections that contain the AmpF*l*STR SEfiler Allelic Ladder must have the word "ladder" in the Sample Info column. The first lane or injection of ladder is the one used by the Kazam macro in the AmpF*l*STR SEfiler Kit Template to determine the sizes in the allele categories that will be used for genotyping.

It is possible to skip the first lane or injection of allelic ladder and use the second lane or injection of allelic ladder for genotyping instead. After importing the sample files, but before running the Kazam macro, remove the word "ladder" from the Sample Info column in all four sample dye colors for the first lane or injection of allelic ladder in the Dye/lanes window. Make sure that the word "ladder" is entered for Sample Info in the second lane or injection of allelic ladder. See for a description of how to access the Sample Info column in the Dye/lanes window.

GeneScan Analysis Software Peak Recognition

All allele peaks in the allelic ladder for each locus must be "recognized" (labeled) in the GeneScan Analysis Software (*i.e.*, each allele peak must have an entry in the GeneScan table). Thus, all allele peaks in each allelic ladder must have a peak height value in relative fluorescence units (RFU) greater than the Peak Amplitude Threshold (PAT) specified in the GeneScan software Analysis Parameters. Also, all allele peaks in each allelic ladder must be resolved. For example, the FGA 26, 26.2, and 27 alleles must be resolved so that each peak has an entry in the GeneScan software table.

Sample allele peak heights must also be greater than the GeneScan Software PAT in order to be recognized (labeled) by Genotyper software. Note that the PAT value specified in the GeneScan software Analysis Parameters is not necessarily the same as the RFU value that may be used by the forensic analyst as the "interpretational threshold." The "Low Signal" column of the appropriate Genotyper software table (see) can be used to identify peaks that are greater than the GeneScan software PAT, but less than a specified minimum threshold (default 150 RFU in the table macro).

AmpFℓSTR SEfiler Kit Template

The AmpFℓSTR SEfiler Kit Template contains macros that perform the following steps automatically:

• Finds the lane or injection containing the allelic ladder
• Creates allele size categories that are centered on the sizes obtained for the allelic ladder alleles
• Assigns the appropriate allele label to sample alleles that size within the allele size categories
• Removes labels from stutter peaks by applying a filter
• Builds a table containing genotypes for all samples

Using the AmpF*l*STR SEfiler Kit Template File

Use the following procedure to assign genotypes to AmpF*l*STR SEfiler kit alleles automatically.

To use the AmpF*l*STR SEfiler Kit Template:

1.	Double-click the **SEfiler** icon to launch the Genotyper software application and open the template file simultaneously. **Note:** The AmpF*l*STR SEfiler Kit Template is a Stationery pad, which means that a new document is created when the template file is opened. The original template file is not overwritten.
2.	Set preferences to import raw data, and Blue, Green, Yellow, Red, and Orange dyes.
3.	To import the GeneScan sample files: a. Select **File** > **Import GeneScan File**(s). b. Select the project file and click **Import**.
4.	If each sample does not already have Sample Info completed in the sample sheet: a. Select **Views** > **Show Dye/lanes**. b. Click the first sample row to select it. c. Click in the **Sample Info** box at the top of the window, and type the sample designation or description. d. Repeat steps b and c to enter a sample description for every dye/lane in the list. Enter the same sample description for all dye colors of a single sample.
5.	From the **Macro** list at the bottom left of the **Main** window, select **Check GS500**.
6.	Select **Macro** > **Run Macro**. In the plot window that opens, scroll through each sample to verify that each GeneScan-500 peak (from 75–450 bp) was assigned the correct size in the GeneScan Analysis Software.
7.	From the **Macro** list at the bottom left of the Main window, select Kazam.

To use the AmpF*ℓ***STR SEfiler Kit Template:** *(continued)*

8.	Select **Macro** > **Run Macro**.
	This macro may take a few minutes to run. When it is finished, a plot window opens with the blue allelic ladder (D3S1358, vWA, D16S539, D2S1338) and sample allele peaks labeled.
9.	Examine data and edit peaks.
10.	Print the electropherograms in the plot window by selecting **File** > **Print**.
11.	a. In the Main Window, click the green **G** button at the top left. b. Select **Views** > **Show Plot Window**. c. Repeat steps 8 and 9.
12.	a. In the Main Window, click the yellow **Y** button at the top left. b. Select **Views** > **Show Plot Window**. c. Repeat steps 8 and 9.
13.	a. In the Main Window, click the red **R** button at the top left. b. Select **Views** > **Show Plot Window**. c. Repeat steps 8 and 9.

Examining Data

Check that the peaks in the allelic ladder are labeled correctly. Scroll through the samples below the allelic ladder to examine the peak labels in each electropherogram.

Peak Labeling

- Allele categories (which appear as dark gray bars in the Plot window) are defined to be ±0.5 bp wide. Peaks that size within ±0.5 bp of an allele category will have a label indicating the allele designation.

Note: The categories for TH01 alleles 9.3 and 10 are ± 0.4 bp wide.

- Peaks that do not size within an allele category will have a label indicating "OL Allele?" (off-ladder allele).
- The Kazam macro includes a step that removes labels from stutter peaks by applying a percentage filter. Labels are removed from peaks that are followed by a (specified percent difference) higher, labeled peak within 3.25 to 4.75 bp.

  The specified filter percentages for these loci are 809% for TH01, 809% for D3S1358, 809% for vWA, 809% for FGA, 733% for D8S1179, 669% for D16S539, 669% for D21S11, 567% for D2S1338, 488% for D19S433, 525% for D18S51, and 488% for SE33.

Note: The label "OVLR" refers to the overlap region between rare TH01 and rare FGA alleles. The reported rare alleles that may be observed in the OVLR region are as follows: TH01 13.3 (204 bp), TH01 14 (205 bp), FGA 12.2 (197 bp), and FGA 13 (199 bp). The peak labels for any alleles that are detected in this overlap region size range will include the OVLR designation (including the TH01 13.3 allele in the AmpF*l*STR SGM Plus Allelic Ladder.

- A sample allele peak must have been recognized by GeneScan software before it can be recognized by Genotyper software. Thus, sample allele peaks that are below the PAT that was specified in the GeneScan software Analysis Parameters cannot be labeled by Genotyper software.

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

Peak Editing

Clicking a labeled peak removes the label. Clicking the same peak again defaults to the placement of bp size of that peak. To access a dialog box and enter the requested text, select **Analysis** > **Set Click Options**. Type the allele designation and/or desired text in the field, then click **OK**.

Plot Window Viewing Options

To zoom in and out on regions of the plot window:

1.	In the Plot window, click and drag in a region of an electropherogram to draw a box around the desired size range (the vertical size of the box is not important).
2.	Type ⌘ **R** (hold down the command key and type the letter R) to zoom in.
3.	Type ⌘ **H** to zoom out completely.

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

1.	Select **Views** > **Show Dye/Lanes Window**.
2.	Click the desired Dye/lane rows. **Note:** Hold down the Shift key on the keyboard to select multiple adjacent Dye/lane rows. Hold down the Command (⌘) key to select Dye/lane rows that are not adjacent.
3.	Select **Views** > **Show Plot Window**.

Making Tables   Two macros for making tables are included in the AmpFℓSTR SEfiler Kit Template. They are:

- Make Allele Table
- 310: Make Table

The Make Allele Table macro contains Sample Info and genotype data fields. The other table, 310: Make Table contains additional information.

All four of the tables have two features in common:

- A locus that has no labeled peaks contains zeros in the cells of the table for that locus.
- Loci that have homozygous alleles contain the allele designation indicated twice in the table.

Make Allele Table

This table has Sample Info in the first column, and allele designations for each locus in columns 2–23. The first two labeled peaks within each locus appear in the table.

310: Make Table

This table can be used if the data was generated on the ABI PRISM 310 Genetic Analyzer. This table has Sample Info in the first column, Sample Comment in the second column, locus name in the third column, and allele designations in columns 4–7. Four columns are provided for allele designations to accommodate mixed samples. The first four labeled peaks within each locus appear in the table. The remaining five table columns are as follows:

- **Overflow**: If more than two peaks are labeled at one locus, the text "> two labels" appears in this column.
- **Low Signal**: If the height of any peak at a locus is greater than the PAT specified in the GeneScan Analysis Parameters but less than 150 RFU, the text "< 150 RFU" appears in this column.
- **Saturation**: If the raw data signal for any peak at a locus is greater than 8191 RFU, the text "310: off-scale" appears in this column.

- **Edited Label**: The text "Edited" appears in this column for any loci where the peak labels were edited manually. For example, clicking an unlabeled peak in the Plot window to add a label constitutes an edit.
- **Edited Row**: The text "Edited" appears in this column for any rows in the table that contain table cells that have been edited after initial creation of the table.

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

To create and use tables:

1.	In the **Macro** list at the bottom of the Genotyper software Main Window, click one of the two table macros.
2.	Select **Macro** > **Run Macro**.
3.	Select **Views** > **Show Table Window** to view the table in full screen mode.
4.	Open and view the plot: **Note:** For all tables except the Make Allele Table, clicking in a cell of the table causes the corresponding sample electropherogram to appear in the plot window: a. Click any cell in the table to display this locus region of the corresponding electropherogram for that sample in the Plot window. b. Zoom out (⌘ **H**) to view all loci for a particular dye color for the corresponding sample.
5.	To edit the cells of the table: a. Click a cell of the table that contains an allele designation. b. Select **Edit** > **Edit Cell**. c. Type the desired information in the box and click **OK**.
6.	To print the table, select **File** > **Print**.

To create and use tables: *(continued)*

7.	This step is optional. Select **Table** > **Export to File** to save the table as a Microsoft Excel-readable document.
8.	Select **File** > **Save** to save the template file with data.

Understanding the AmpF*l*STR SEfiler Kit Template

Troubleshooting Automated Genotyping

To Troubleshoot Automated Genotyping:

Observation	Probable Cause	Recommended Action
Warning message: "Could not complete 'Run Macro' command because no dye/lanes are selected."	The word "ladder" is not in Sample Info for the lane or injection of allelic ladder.	Type the word **ladder** in the Sample Info column. You must enter the word "ladder" for each dye color (Blue, Green, Yellow, and Red) in the Sample Info column for the AmpF*l*STR SEfiler Allelic Ladder sample.
Warning message: "Could not complete 'Run Macro' command because the labeled peak could not be found."	One or more peaks in the allelic ladder are below the Peak Amplitude Threshold that was specified in the GeneScan software Analysis Parameters.	Use another allelic ladder in the project, or: 1. In the GeneScan Analysis Software, lower the Peak Amplitude Threshold values for blue, green, yellow, and red dye colors in the Analysis Parameters. 2. Reanalyze the sample file(s) containing the allelic ladder. 3. Import all sample files into a new Genotyper software project, and run the Kazam macro again.

About This Kit Template

This section describes the organization and functionality of the AmpFℓSTR SEfiler Kit Template. Read this section for a greater understanding of the macros and steps used in the AmpFℓSTR SEfiler Kit Template.

Categories

In the Genotyper software, each allele is defined by a category. Each category contains information about the allele size, size range, and dye color. To view the list of categories in the AmpFℓSTR SEfiler Template, select **View** > **Show Categories**. The categories for each locus are listed together under the locus name. The locus is called a group.

In the Categories window, each locus actually has two sets of categories. For example, the D3S1358 locus has one list of categories under the group "D3S1358" and another list of categories under the group "D3S1358.os." The categories in the D3S1358 group are allele categories used for allele assignment.

Offset Categories

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

Finding and Recognizing the Leftmost (first) Allele Peak in Each Allelic Ladder

• Identification of the leftmost peak is accomplished through the specifications of the first ".os" category listed within each group of offset categories. This first ".os" category (12.os in the case of D3S1358) is specified to find all peaks in a range of ±7 bp around the reference size for the indicated allele.

• Each Calculate [locus] Offsets macro applies a percentage filter to all peaks in the ±7-bp range in the allelic ladder, avoiding the first stutter peak in each allelic ladder and thus identifying the first allele peak as the leftmost peak.

Calculating the Offset Values

Categories with the ".os" suffix contain offset categories.

The base pair size indicated in each category is a "reference size." One main function of the macros in the AmpF*l*STR SEfiler Template is to offset the reference sizes relative to the sizes obtained for the alleles in the allelic ladder. These offset steps are performed by the Calculate [locus] Offsets macros, located in the Macro list of the Genotyper software Main window. After the macros are run, the calculated offset values are indicated in parentheses near the end of each category line in the Categories window.

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

Applying the Appropriate Offset Value to Each Allele in Succession

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

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

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

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

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

Off-Ladder Alleles and Virtual Alleles

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

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

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

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

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

Many of the loci in the Categories window contain virtual allele categories. For example, the FGA locus contains a virtual category for many 2-bp length variants.

Kazam Macro

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

Filtering Stutter Peaks

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

To filter stutter peaks:

1.	In the Step Window for the Kazam macro, scroll down to the line that reads "Select category: D3S1358."
2.	Five rows below, select the line that reads, "Remove labels from peaks followed by an 835% higher, labeled peak within 3.25 to 4.75 bp."
3.	Select **Macro** > **Edit Step** to open the Filter Labels window.
	In the Filter Labels window, there are four options (check boxes) for filtering. In this example, the filtering option for D3S1358 is denoted in the last check box. This filtering option includes another check box that reads "(higher by at least 835%)."
	For each labeled peak (*e.g.* peak A) in the locus size range, this filtering option examines the very next (*i.e.* greater in bp size) labeled peak (peak B). The label will be removed from peak A if peak B meets both of the specified criteria:
	• Peak B is higher by at least 835%
	• Peak B is within 3.25 to 4.75 bp
	The percentage value in this filtering option is calculated as follows:
	[(peak B – peak A) / peak A] x 100 = percentage value
	For example, if peak A = 175 RFU and peak B = 2500 RFU, then the percentage value is calculated as follows:
	[(2500 – 175) / 175] x 100 = 1329%

To filter stutter peaks: *(continued)*

	3. *(continued)*
	In this example, the label will be removed from peak A, provided that the filter option specifies a threshold of 809%, and that peak B is within 3.25 to 4.75 bp of peak A.
	Conventionally, percent stutter is calculated:
	(peak A / peak B) x 100 = percent stutter
	The percentage value that is used in the Genotyper software filtering option (F) can be derived from the conventional percent stutter expression (S) as follows:
	$F = (10,000 / S) - 100$
	For example, if the desired stutter percent threshold for D3S1358 is 11%, then the percentage value that should be used in the Genotyper software filtering option is:
	$F = (10,000 / 11) - 100 = 809\%$
4.	To use a filter value different from 809% for D3S1358, enter another value, then click **Replace**.

The peak filtering included in the Kazam macro is intended only as a tool and guideline. Final conclusions should be based on careful examination of the STR profiles.

Kazam (20% Filter)

The standard Kazam macro is written so that a different filter threshold can be used for each locus (the steps for each locus are written separately in the macro). The Kazam macro thus provides maximum flexibility and the opportunity to customize the filter used for each locus.

A different version of the Kazam macro called "Kazam (20% filter)" is also provided in the Macro list. This macro is simpler than the Kazam macro in that a 20% stutter filtering step is specified for all loci.

To view the various steps in the Kazam (20% filter) macro:

1.	Click the Kazam (20% filter) line in the Macro list
2.	Select **Views** > **Show Step Window**.
	The first filter step for this macro (which applies to the sample alleles) reads, "Remove labels from peaks whose height is less than 20% of the highest peak in a category's range."

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

To edit the filter value:

1.	Click the first filter step in the **Step** window.
	Refer to step 2 in the previous procedure, "To view the various steps in the Kazam (20% filter) macro."
2.	Select **Macro** > **Edit Step**.
	Note: This macro uses the second filter option in the Filter Labels window.
3.	If desired, change the value from 20% to some other value, then click **Replace**.

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

Modifying the Template

The original AmpFℓSTR SEfiler Template File can be modified so that the changes made to the macros or settings are used as the default:

To modify the template:

1.	Close all Genotyper windows, but do not quit the application.
2.	Right-click the **AmpFℓSTR SEfiler Kit Template** icon.
3.	In the template, select **Properties**.
4.	Deselect the check box for **Read-only** at the bottom of the window, then close the Properties window.
5.	Double-click the **AmpFℓSTR SEfiler Kit Template** icon.
6.	Make any desired changes.
7.	Save the template file by selecting **File** > **Save**.
8.	Right-click the **AmpFℓSTR SEfiler Kit Template** icon.
9.	In the template, select **Properties**.
10.	Select the check box for **Read-only**, then close the Properties window.

Determining Genotypes

AmpF*l*STR SEfiler Allelic Ladder

The AmpF*l*STR® SEfiler™ Allelic Ladder contains the most common alleles for each locus. Genotypes are assigned by comparing the sizes obtained for the unknown samples with the sizes obtained for the alleles in the allelic ladder.

In addition to the alleles included in the AmpF*l* STR SGM Plus Kit, alleles for SE33 have also been included.

The macro size ranges include the actual number of nucleotides contained in the smallest and largest allelic ladder alleles for each locus, as well as those alleles reported in STRBase (www.cstl.nist.gov/div831/strbase) as of September 2000. The size range also includes 3′. The AmpF*l*STR SEfiler PCR Amplification Kit is designed so that a majority of the PCR products contain the non-templated 3′ A nucleotide. The alleles have been named in accordance with the recommendations of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) (DNA Recommendations, 1994; Bar *et al*., 1997).

The number of complete four base pair repeat units observed is designated by an integer. Variant alleles that contain a partial repeat are designated by a decimal followed by the number of bases in the partial repeat. For example, an FGA 26.2 allele contains 26 complete repeat units and a partial repeat unit of two base pairs.

Additional variation has been seen at some loci where alleles exist that differ from integer allele lengths by one or three base pairs. For example, D21S11 allele 33.1 contains 33 complete repeat units and one nonconsensus base pair. Likewise, a D21S11 29.3 allele contains 29 complete repeat units and a partial 3-bp unit (Moller *et al*., 1994; Gill *et al*., 1997).

A Genotyper software electropherogram of the AmpF*l*STR SEfiler Allelic Ladder listing the designation for each allele is shown in Figure 10-1. These results were obtained on an ABI PRISM 310 Genetic Analyzer. The electropherogram indicates the designation for each allele.
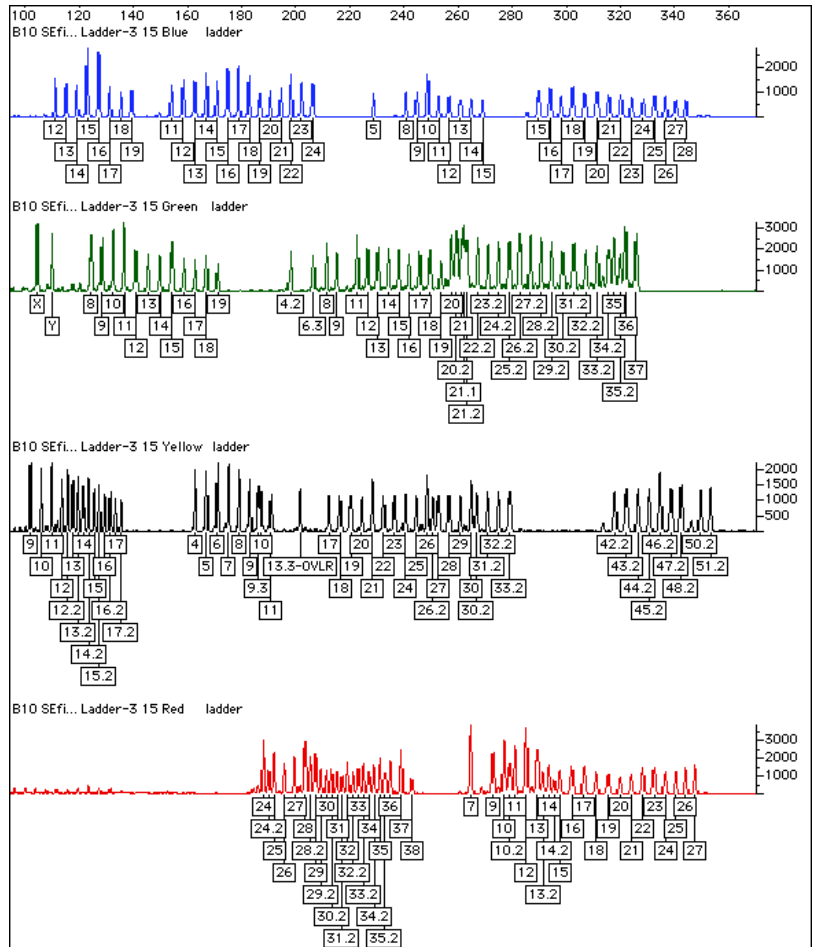

Figure 10-1   Genotyper® software plot of the AmpF*l*STR SEfiler Allelic Ladder

Genotyping Using the AmpF*l*STR SEfiler Allelic Ladder

When you interpret AmpF*l*STR SEfiler kit results, be aware that the system assigns genotypes to sample alleles by comparing their sizes to those obtained for the known alleles in the AmpF*l*STR SEfiler Allelic Ladder. Genotypes, not sizes, are used for comparison of data between runs, instruments, and laboratories.

We strongly recommend that laboratories use an AmpF*l*STR SEfiler Allelic Ladder from each project to convert the allele sizes to genotypes.

- The size values obtained for the same sample can differ between instrument platforms because of differences in the type and concentration of the gel/polymer matrices and in electrophoretic conditions.
- Sizes may differ between protocols for the same instrument platform because of differences in gel or polymer concentration, run temperature, gel or capillary thickness, and well-to-read length.
- Slight procedural and reagent variations between gels or between single and multiple capillaries result in greater size variation than that found between samples on the same gel or between samples injected in the same capillary in a single run.

Size Standard

Use the GeneScan-500 LIZ® Size Standard with the AmpF*l*STR SEfiler kit. Common alleles for all AmpF*l*STR SEfiler kit loci are less than 400 base pairs. The recommended sizing method, Local Southern, uses two internal lane size standard peaks larger than each allele and two smaller than each allele to be sized. When size standard peaks are defined in routine analyses, inclusion of the 400 base pair and 450 base pair peaks in the GeneScan-500 LIZ Size Standard is recommended.

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

Analyzing AmpF*l*STR SEfiler Allelic Ladder

To size the AmpF*l*STR SEfiler Allelic Ladder alleles, analyze the lanes/injections containing allelic ladder with the same parameters used for samples.

To compare the results of lanes or injections of AmpF*l*STR SEfiler Allelic Ladder:

1.	Compare the base pair sizes of one lane or injection of allelic ladder to those obtained for the other lanes or injections of allelic ladder. All corresponding peaks (peaks at the same position in the allelic ladder) should be within ±0.5 bp of each other.
2.	If one or more corresponding peaks are not within ±0.5 bp of each other, check the GeneScan-500 LIZ Size Standard peaks in all allelic ladder lanes or injections to confirm that all GeneScan-500 LIZ Size Standard peaks have been assigned the correct size and/or that all peaks are clearly resolved.

To manually genotype samples:

1.	Select one lane or injection of allelic ladder to use for genotyping. **Note:** Our studies have shown that it does not matter which lane or injection of allelic ladder is selected if the alleles in the allelic ladder samples are within ±0.5 bp of each other.
2.	Compare the base pair size obtained for each sample allele peak to the sizes obtained for the allelic ladder peaks.
3.	Assign genotypes to those sample allele peaks falling within ±0.5 bp of the corresponding allelic ladder peak. The allele designation for each allelic ladder peak is given in .

The AmpF*l*STR SEfiler Allelic Ladder contains most alleles for the Amelogenin, D2S1338, D3S1358, D8S1179, D16S539, D18S51, D19S433, D21S11, FGA, SE33, TH01, and vWA loci. However, alleles not found in the AmpF*l*STR SEfiler Allelic Ladder do exist. These off-ladder alleles may contain full and/or partial repeat units. An off-ladder allele should flag itself by not falling inside the ±0.5 bp window of any known allelic ladder allele.

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

Troubleshooting

A

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

Troubleshooting

Table A-1   Troubleshooting causes and recommended actions

Observation	Possible Causes	Recommended Actions
Faint or no signal from both the AmpF*l*STR® Control DNA 007 and the DNA test samples at all loci	Incorrect volume or absence of either AmpF*l*STR® PCR Reaction Mix, AmpF*l*STR SEfiler™ Primer Set, or AmpliTaq Gold® DNA Polymerase	Repeat amplification.
	No activation of AmpliTaq Gold DNA Polymerase	Repeat amplification, making sure to hold reactions initially at 95 °C for 11 min.
	PCR Master Mix not vortexed thoroughly before aliquoting	Vortex PCR Master Mix thoroughly.
	AmpF*l*STR SEfiler Primer Set exposed to too much light	Store Primer Set protected from light.
	GeneAmp® PCR System malfunction	Refer to the thermal cycler user's manual and check instrument calibration.
	Incorrect thermal cycler parameters	Check the protocol for correct thermal cycler parameters.
	Tubes not seated tightly in the thermal cycler during amplification	Push reaction tubes firmly into contact with block after first cycle. Repeat test.
	GeneAmp PCR System 9600 heated cover misaligned	Align GeneAmp 9600 heated cover properly so that white stripes align after twisting the top portion clockwise.
	Wrong PCR reaction tube	Use Applied Biosystems MicroAmp Reaction Tubes with Caps for the GeneAmp 9600 and 9700.
	MicroAmp® Base used with tray/retainer set and tubes in GeneAmp 9600 and 9700	Remove MicroAmp Base from tray/retainer set and repeat test.

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

Observation	Possible Causes	Recommended Actions
Faint or no signal from both the AmpF*l*STR® Control DNA 007 and the DNA test samples at all loci. *(continued)*	Insufficient PCR product electrokinetically injected	**For ABI PRISM® 310 runs:** Mix 1.5 µL of PCR product and 24.5 µL of Hi-Di™ Formamide/GeneScan®-500 LIZ® solution. ⚠ **WARNING** **CHEMICAL HAZARD. Formamide** causes eye, skin, and respiratory tract irritation. It is a possible reproductive and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
	Degraded formamide	Check the storage of formamide; do not thaw and refreeze multiple times. Try Hi-Di™ Formamide. ⚠ **WARNING** **CHEMICAL HAZARD. Formamide** causes eye, skin, and respiratory tract irritation. It is a possible reproductive and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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

Observation	Possible Causes	Recommended Actions
Positive signal from AmpF*l*STR Control DNA 007 but no signal from DNA test samples	Quantity of test DNA sample is below assay sensitivity	Quantitate DNA and add 1.0–2.5 ng of DNA. Repeat test.
	Test sample contains PCR inhibitor (*e.g.*, heme compounds, certain dyes)	Quantitate DNA and add minimum necessary volume. Repeat test.
		Wash the sample in a Centricon®-100. Repeat test.
	Test sample DNA is degraded	If possible, evaluate the quality of DNA sample by running an agarose gel. If DNA is degraded, re-amplify with an increased amount of DNA.
	Dilution of test sample DNA in H_2O or wrong buffer (*e.g.*, wrong EDTA concentration)	Re-dilute DNA using 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.
	Too much DNA in reaction	Use recommended amount of template DNA (1.0–2.5 ng).
	Mixed sample	See Chapter 8, "Experiments and Results," "Stutter Products."
	Amplification of stutter product (n-4 bp position)	
	Incomplete 3′ A base addition (n-1 bp position)	See Chapter 8, "Experiments and Results," "Addition of 3′ A Nucleotide." Be sure to include the final extension step of 60 °C for 45 min in the PCR.
	Signal exceeds dynamic range of instrument (off-scale data)	Quantitate DNA and re-amplify sample, adding 1.0–2.5 ng of DNA.
	Poor spectral separation (bad matrix)	Follow the steps for creating a matrix file.
		Confirm that Filter Set G5 modules are installed and used for analysis.

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

Observation	Possible Causes	Recommended Actions
Some but not all loci visible on electropherogram	Test sample DNA is degraded	If possible, evaluate the quality of DNA sample by running an agarose gel. If DNA is degraded, re-amplify with an increased amount of DNA.
	Test sample contains PCR inhibitor (*e.g.*, heme compounds, certain dyes)	Quantitate DNA and add minimum necessary volume. Repeat test.
		Wash the sample in a Centricon-100. Repeat test.
ABI PRISM 310 Genetic Analyzer		
Data was not automatically analyzed	Sample sheet not completed	Complete sample sheet as described.
	Injection list not completed	Complete injection list as described.
	Preferences not set correctly in ABI PRISM® 310 Data Collection Software	Select **Window > Preferences**, then select **Injection List Defaults** and the **Autoanalyze** check box.
Extra peaks visible when sample is known to contain DNA from a single source	Incomplete denaturation before loading onto detection instrument	Heat samples to 95 °C for 3 min in deionized formamide solution. Snap cool on ice. Use Genetic Analyzer 0.5-mL Sample Tubes and a thermal cycler.
Current too high	Decomposition of urea in the POP-4™ polymer solution	Add fresh POP-4 polymer solution to the syringe. ⚠ **WARNING** **CHEMICAL HAZARD**. **POP-4 polymer** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Use for research and development purposes only.
	Incorrect buffer concentration	Replace buffer with 1X Genetic Analyzer Buffer.

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

Observation	Possible Causes	Recommended Actions
No current	No 1X Genetic Analyzer buffer	Refill buffer vials with 1X Genetic Analyzer buffer.
	Pump block channel blockage	Remove and clean block. Refer to the *ABI PRISM® 310 Genetic Analyzer User Guide.*
	Loose valve fittings or syringe	Tighten valve fittings and syringe.
	Capillary not flush with electrode	Tape capillary securely to heat plate. Refer to the *ABI PRISM® 310 Genetic Analyzer User Guide* (P/N 903565).
	Electrode bent	Check calibration of autosampler.
No signal	Capillary misaligned with electrode	Align capillary and electrode.
	No PCR product added	Add 1.5-μL PCR product to formamide/GeneScan-500 LIZ mixture.
	Capillary bent out of sample tube	Align capillary and electrode. Recalibrate autosampler.
	Autosampler not calibrated correctly	Calibrate autosampler in X, Y, and Z directions.
	PCR product not at bottom of tube	Spin sample tube in microcentrifuge.
	Air bubble at bottom of sample tube	Spin tube in microcentrifuge to remove air bubbles.
	Sealed sample tube septum	Replace septum.
Low signal	PCR product added to non-deionized formamide	Always use deionized formamide for sample preparation. Verify conductivity is < 30-μ siemens.
	PCR product not mixed well with formamide/GeneScan-500 LIZ mixture	Mix PCR product with formamide/GeneScan-500 LIZ mixture by pipetting up and down several times.

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

Observation	Possible Causes	Recommended Actions
Loss of resolution after 100 bp	Excess salt in sample	Do not concentrate PCR product by evaporation. Use Centricon-100 if necessary.
	Too much DNA in sample	Treat and dilute the PCR product.
	Bad water	Use autoclaved or freshly prepared deionized water.
	Incorrectly prepared and/or old solutions	Replace buffer and polymer with fresh solutions.
Runs get progressively slower, *i.e.*, size standard peaks come off at higher and higher scan numbers	Leaking syringe: polymer is not filling capillary before every injection	Clean syringe thoroughly.
		Replace syringe.
Runs get progressively faster, *i.e.*, size standard peaks come off at lower and lower scan numbers	Water in syringe	Prime syringe with small volume of polymer and discard. Fill syringe with polymer.
High baseline	Dirty capillary window	Clean capillary window with 95% ethanol. ⚠️ **WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
	Capillary moved out of position in laser window	Position capillary in front of laser window.
	Cracked capillary	Replace the capillary

Laboratory Setup

B

In This Appendix   This appendix provides some references for laboratories preparing to implement PCR technology. Careful planning and design of the laboratory, and training of all laboratory personnel are necessary to ensure that exogenous DNA and PCR products are confined to designated areas.

Lab Design

Sensitivity of PCR   Many resources are available for the appropriate design of a PCR laboratory. If you are using the SEfiler kit for forensic DNA testing, refer to http://www.ojp.usdoj.gov/nij/scidocs.htm, "Forensic Laboratories: Handbook for Facility Planning, Design, Construction and Moving." If you are using the SEfiler kit for parentage DNA testing, refer to the "Standards for Parentage Testing Laboratories." The sensitivity of the AmpF*l*STR® SEfiler™ PCR Amplification Kit (and other PCR-based tests) permits amplification of minute quantities of DNA, necessitating precautions to avoid contamination of samples yet to be amplified (Kwok and Higuchi, 1989).

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

Care should be taken while handling and processing samples to prevent chance contamination by human DNA. Gloves should be worn at all times and changed frequently. Sample tubes should be closed when not in use. Dispersal of aerosols should be limited through careful handling of sample tubes and reagents.

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

Extra precautions and care should be taken during DNA extraction and PCR setup to prevent transfer of DNA from one sample to another. Use a new, filter-plugged pipet tip for each sample, open tubes carefully, and keep sample tubes closed when not in use. Applied Biosystems does not intend these references for laboratory design to constitute all precautions and care necessary when using PCR technology.

DNA Extraction Protocols

C

In This Appendix   Appendix C describes some extraction methods for various DNA samples.

Introduction

Overview of DNA Sample Types

Both manual and automated extraction procedures can be divided into organic and nonorganic procedures. Depending on the material received, the scientist should determine which procedure is appropriate for each piece of evidence.

DNA for PCR amplification and analysis using the AmpF*l*STR® SEfiler™ PCR Amplification Kit may be extracted from fresh or frozen whole blood, peripheral blood lymphocytes, blood stains, sperm cells, paraffin blocks, teeth, hair, tissue, bone, and other biological samples.

DNA Extraction Methods

Numerous procedures are currently used for DNA extraction. Extraction procedures include Chelex®, phenol-chloroform and FTA™ paper. In DNA extraction method, all samples must be handled carefully to prevent sample-to-sample contamination or contamination by extraneous DNA. Also, when possible, we recommend that the samples be processed at a separate time from reference samples.

Phenol-Chloroform Method

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

Chelex Method

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

FTA Paper Extraction

The FTA paper extraction begins immediately when blood is spotted on FTA paper. The cells are lysed and the DNA is immobilized within the matrix of the paper. The DNA is purified by performing a series of washes, after which the DNA is ready for PCR amplification.

Material Safety Data Sheets

For information and ordering instructions for Material Safety Data Sheets (MSDSs), refer to "About MSDSs" on page xvi.

Storage of Samples for DNA Extraction

Proper Storage

Storage of various DNA specimens is an essential step to ensuring that the DNA profiles obtained are accurate and meaningful. Proper chain of custody is vital to maintaining the integrity of each particular specimen.

DNA Quantification

D

In This Appendix   This appendix discusses the importance of quantifying DNA samples prior to amplification. The QuantiBlot® Human DNA Quantitation Kit described in this appendix can be used for the quantification of samples.

Importance of Quantification

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

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

Quantification of samples shows if there is a sufficient amount of DNA present for amplification. You can minimize PCR inhibition by adding the smallest volume of DNA extract necessary for successful amplification (volume containing approximately 1–2.5 ng). Using the minimal volume of extracted DNA for PCR maximizes the number of different genetic marker tests or repeat analyses that can be performed. Likewise, informed decision(s) can be made regarding typing of samples present in extremely limiting quantities.

DNA quantification is particularly important for amplifications using the AmpF*ℓ*STR® SEfiler™ kit, where optimal results are obtained using a range of 1–2.5 ng of input DNA. Adding greater than 2.5 ng of DNA can result in too much PCR product, such that the dynamic range of the instrument used to detect and analyze the PCR product is exceeded.

Using the QuantiBlot Kit

How the Kit Works

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

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

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

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

The colorimetric method allows for detection and quantification down to 150 pg. The chemiluminescent method can detect 150 pg with a 15-minute exposure to film and can detect as little as 20 pg with longer film exposures (3 hours to overnight). Results obtained from various biological samples using the QuantiBlot Kit are shown in .

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

Specificity for Primate DNA

One significant advantage offered by the QuantiBlot kit is that the probe is highly specific for human/primate DNA. When tested, 300-ng quantities of several non-primate DNA samples (*E. coli*, yeast, dog, cat, mouse, rat, pig, cow, chicken, fish, and turkey) were found to give either no signals or signals that were less than or equal to that obtained for 0.15 ng of human DNA. This high degree of specificity for human/primate DNA allows for the accurate quantification of target human DNA in samples that also contain significant amounts of microbial or other non-primate DNA.
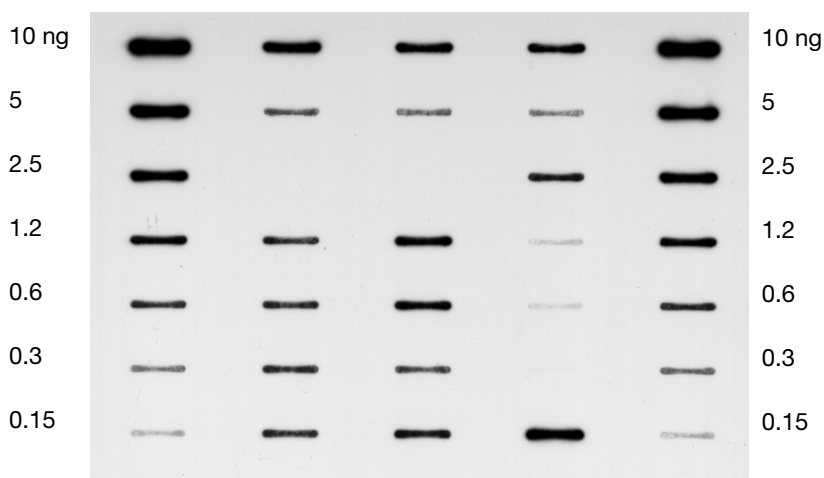
Figure D-1   QuantiBlot Human DNA Quantitation Kit results (ECL detection)

Single-Stranded and Degraded DNA

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

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

Extremely degraded DNA usually amplifies less efficiently than intact DNA, so a greater quantity of degraded DNA may be required to give the same results as intact DNA.

Commonly Asked Questions about the QuantiBlot Kit

How Much DNA

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

As an example, assume that 5 µL of the DNA extract is spotted, and the lowest DNA standard tested is 150 pg. So, the DNA concentration in the sample must be less than 150 pg/5 µL or 30 pg/µL. The quantity of DNA in 10 µL of extract, which is the maximum that can be added to an AmpF*l*STR SEfiler kit amplification, would therefore be less than 0.3 ng.

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

- Attempt amplification using 10 µL of the extract.
- Concentrate the sample to a smaller volume using a Centricon®-100 before amplification.

Multiple Film Exposures

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

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

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

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

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

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

To repeat the assay:

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

Performing Hybridization and Detection at a Later Time	**Is it possible to spot the samples onto the membrane and then perform the hybridization and detection steps at a later time? Yes.**

To stop and resume hybridization and detection:

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

ABI PRISM GeneScan Analysis Software for the Windows NT OS   E

In This Appendix   This appendix is a review of the analysis parameters and size caller for the Microsoft® Windows NT® platform using the ABI PRISM® GeneScan® Analysis Software.

Overview of Analysis Parameters and Size Caller

Purpose This appendix supplements the *ABI PRISM® GeneScan® Analysis Software Version 3.7 for the Windows NT® Platform User Guide* (P/N 4308923). It explains the analysis parameters and size caller available in the Windows NT version of the software.

The *GeneScan Analysis Software v3.7.1 Updater CD* (P/N 4336026) includes new analysis parameter default values. For additional information and installation instructions, refer to the GeneScan v3.7.1 About file.

Intended Audience This appendix is intended for users familiar with the GeneScan analysis software for the Macintosh® operating system who are now using the software on the Windows NT operating system.

GeneScan Analysis Software Process

Overview   The ABI PRISM® GeneScan Analysis Software is available in versions for both the Windows NT operating system and the Macintosh operating system. The Windows NT version of the software uses different algorithms and has additional analysis parameters that give users more control with data analysis.

Flowchart   The following flowchart shows how GeneScan analysis software analyzes data.

Note: For multicapillary instruments, multicomponenting is performed by the data collection software.
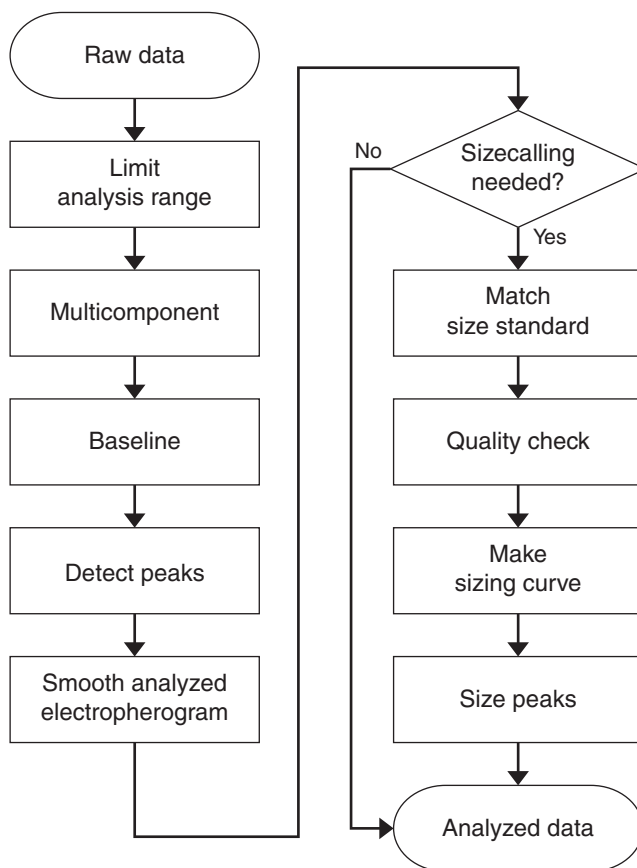
Figure E-2   Simplified GeneScan analysis software flowchart

Analysis Parameters

Table of Parameters

Table E-2   The table below lists the analysis parameters.

Parameter Status	Parameter	Discussed in...
Unchanged from Macintosh versions	• Analysis Range • Size Call Range • Size Calling Method • Peak Amplitude Thresholds	ABI PRISM® GeneScan Analysis Software Version 3.7 User Guide
Changed from Macintosh versions	• Smooth Options • Min. Peak Half Width	This user bulletin and the *ABI PRISM® GeneScan Analysis Software Version 3.7 NT and 3.1 Macintosh User Guides*
Added for the Windows NT version	• Smooth Options • Min. Peak Half Width • Polynomial Degree • Peak Window Size • Slope Threshold for Peak Start • Slope Threshold for Peak End • Window Size	This appendix and the *ABI PRISM® GeneScan Analysis Software Version 3.7 User Guide*
Removed options from the Windows NT version	Baseline Multicomponent	*ABI PRISM® GeneScan Analysis Software Version 3.1 User's Manual*

Analysis Parameters Dialog Box

About the Analysis Parameters Dialog Box

Use the Analysis Parameters dialog box to set analysis parameter values for data processing.

The default analysis parameter values are analysis guidelines. However, we encourage you to use this appendix as a guide for modifying these values as appropriate for each laboratory.

Example

Figure E-3 shows the Analysis Parameters dialog box with default values for GeneScan analysis software v3.7.1 on the Windows NT operating system.
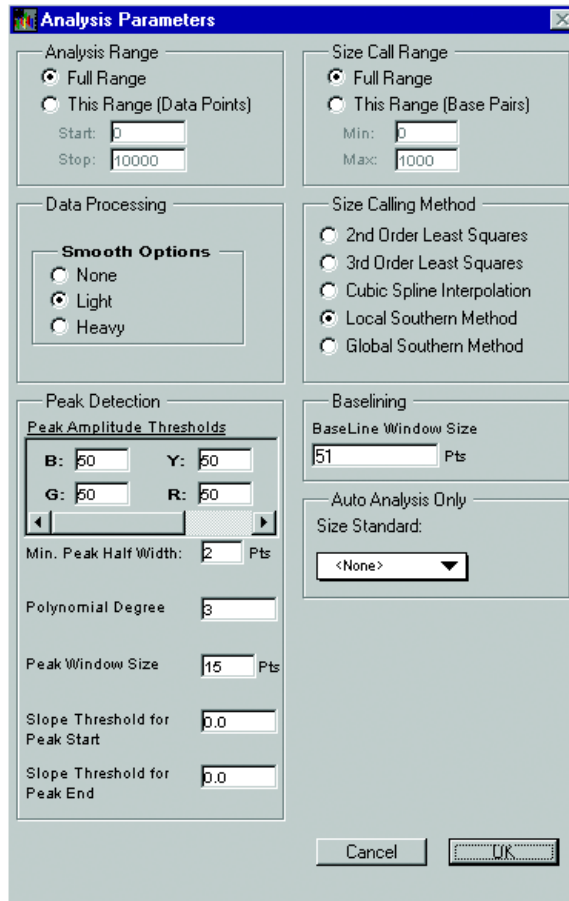
Figure E-3   Analysis Parameters dialog box displaying default values

Data Processing: Smooth Options Parameter

About the Parameter

The Smooth Options parameter sets the degree of smoothing applied to the display of the analyzed electropherogram. Smoothing may aid in data interpretation.

How the Parameter Works

The Smooth Options parameter is applied after peak detection and affects only the display of analyzed electropherograms. The peak heights and areas are calculated and displayed in the tabular data display based on the "none" smoothing option. Selecting light or heavy smoothing will not affect the calculation of these values.

Smoothing Example

Figure E-4 is an electropherogram showing the peaks from the same sample file after analysis using no smoothing (black); light smoothing (green); and heavy smoothing (red). All tabular data, including peak height and area, remain unchanged.
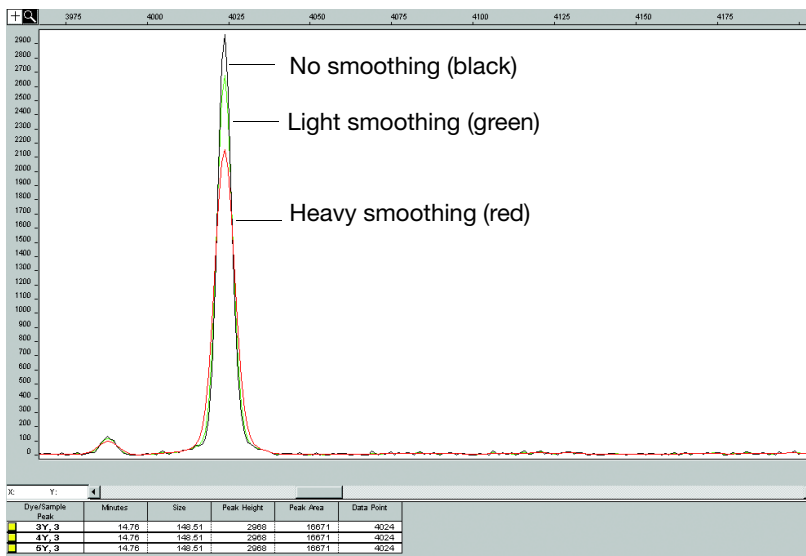
Figure E-4   Effects of smoothing on peaks from the same sample file

Figure E-5 is an electropherogram showing the effects of smoothing on the smaller peak and baseline when the y scale is changed from Figure E-4.
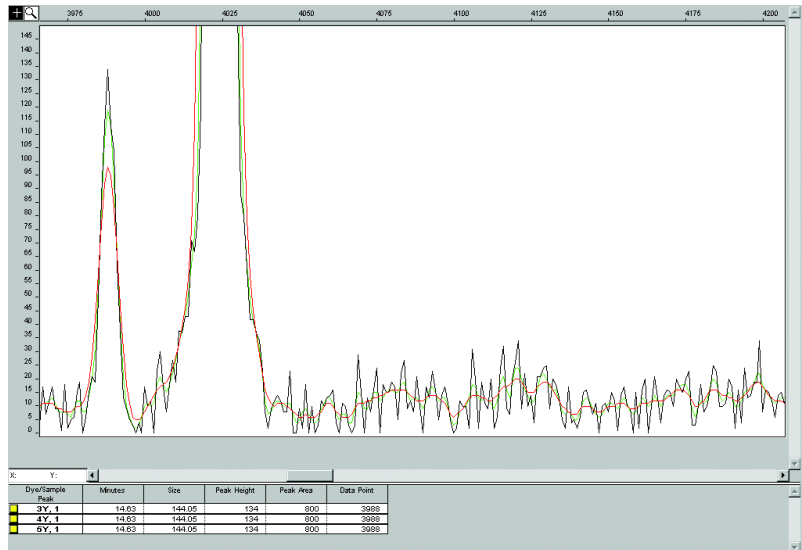
Figure E-5   Effects of smoothing on the smaller peak and baseline

Peak Detection: Min. Peak Half Width Parameter

About This Parameter

Use the Min. Peak Half Width parameter to specify the smallest full width at half maximum height for peak detection. This parameter can be used to ignore noise spikes.

How This Parameter Works

The Min. Peak Half Width parameter defines what constitutes a peak. The software ignores peak half widths smaller than the specified value.

The way in which this version of the software defines the minimum peak half width is different than in previous versions.

Old Versions	Current Version
Half width — Half width of the peak measured from peak start	Full width / Half height — Full width of the peak measured at half its height
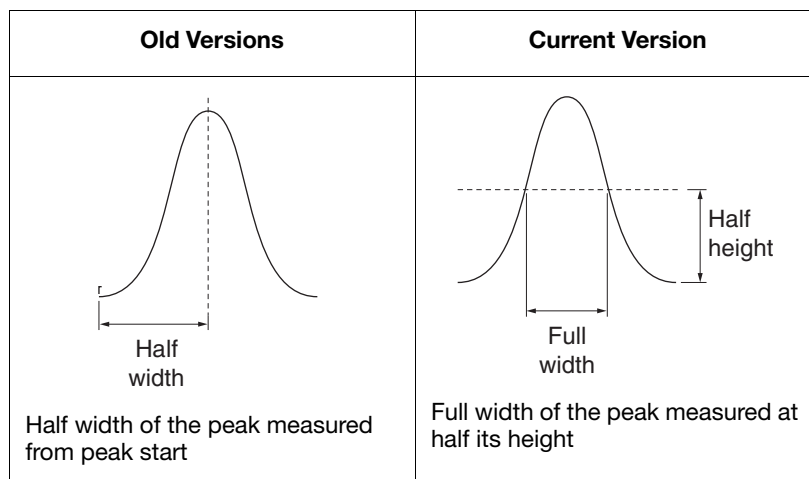
Figure E-6   Defining the Min. Peak Half Width

Polynomial Degree and Peak Window Size

About These Parameters

Use the Polynomial Degree and the Peak Window Size settings to adjust the sensitivity of the peak detection. You can adjust these parameters to detect a single base pair difference while minimizing the detection of shoulder effects or noise.

Sensitivity increases with larger polynomial degree values and smaller window size values. Conversely, sensitivity decreases with smaller polynomial degree values and larger window size values.

How These Parameters Work

The peak window size functions with the polynomial degree to set the sensitivity of peak detection.

The peak detector computes the first derivative of a polynomial curve fitted to the data within a window that is centered on each data point in the analysis range.

Using curves with larger polynomial degree values allows the curve to more closely approximate the signal and, therefore, the peak detector captures more peak structure in the electropherogram.

The peak window size sets the width (in data points) of the window to which the polynomial curve is fitted to data. Higher peak window size values smooth out the polynomial curve, which limits the structure being detected. Smaller window size values allow a curve to better fit the underlying data.

How to Use These Parameters

Use the table below to adjust the sensitivity of detection.

Table E-3   Sensitivity of Detection

To...	Polynomial Degree Value	Window Size Value
Increase Sensitivity Use...	Higher	Lower
Decrease Sensitivity Use...	Lower	Higher

Guidelines for Using These Parameters

To detect well-isolated, baseline-resolved peaks, use polynomial degree values of 2 or 3. For finer control, use a degree value of 4 or greater.

As a guideline, set the peak window size (in data points) to be about 1 to 2 times the full width at half maximum height of the peaks that you want to detect.

Examining Peak Definitions

To examine how GeneScan analysis software defines a peak, select **View** > **Show Peak Positions**. The peak positions, including the beginning, apex, and end of each peak, are tick-marked in the electropherogram.

Effects of Varying the Polynomial Degree

Figure E-7 is an electropherogram showing peaks detected with a window size of 15 data points and a polynomial curve of degree 2 (green); 3 (red); and 4 (black). The diamonds represent a detected peak using the respective polynomial curves.

Note that the smaller trailing peak is not detected using a degree of 2 (green). As the peak detection window is applied to each data point across the displayed region, a polynomial curve of degree 2 could not be fitted to the underlying data to detect its structure.
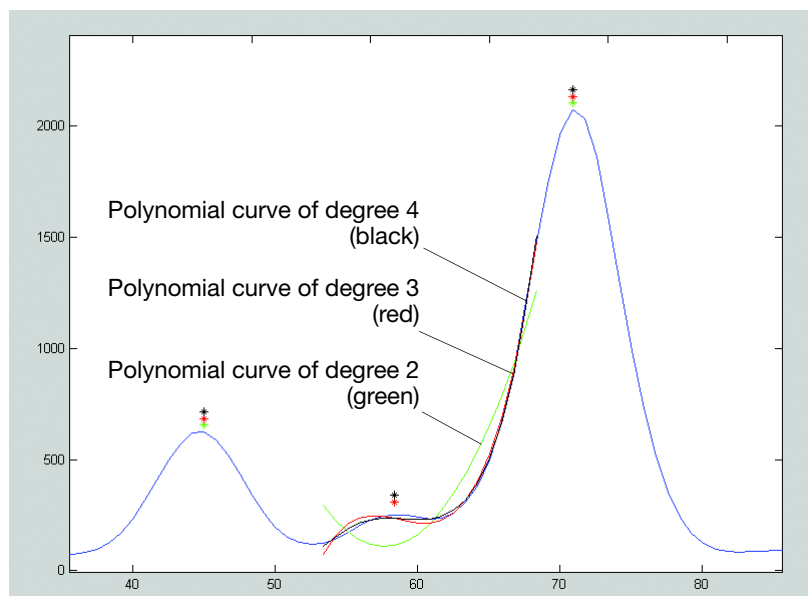
Figure E-7   Peaks detected with the same window size and three different polynomial degrees

Effects of Increasing the Window Size Value

Figure E-8 is an electropherogram showing the same peaks that are shown in Figure E-7. However, in this depiction both polynomial curves have a degree of 3 and the window size value was increased from 15 (red) to 31(black) data points. The polynomial curve is the same as that shown in Figure E-7.

As the cubic polynomial is stretched to fit the data in the larger window size, the polynomial curve becomes smoother. Note that the structure of the smaller trailing peak is no longer detected as a distinct peak from the adjacent larger peak to the right.
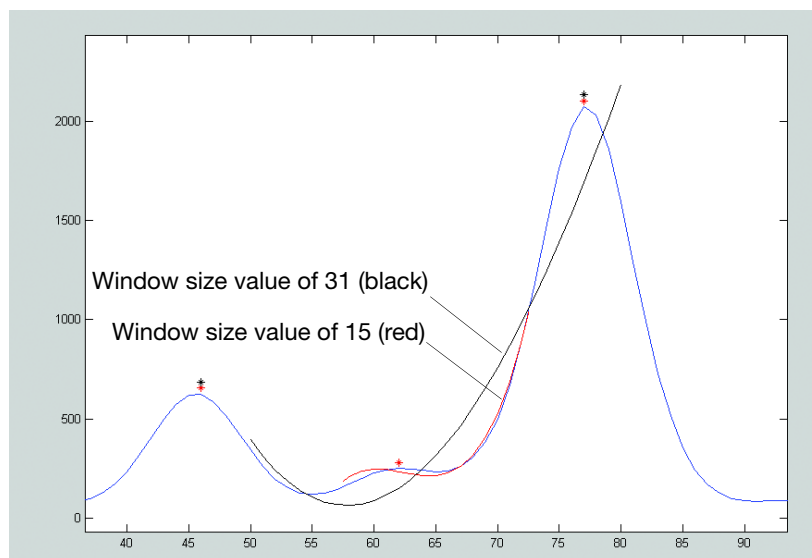
Figure E-8   The effect of increasing window size value

Optimizing Peak Detection Sensitivity: Example 1

Initial Electropherogram

Figure E-9 is an electropherogram showing two resolved alleles of known fragment lengths (that differ by one nucleotide) detected as a single peak. The analysis was performed using a polynomial degree of 3 and a peak window size of 19 data points.
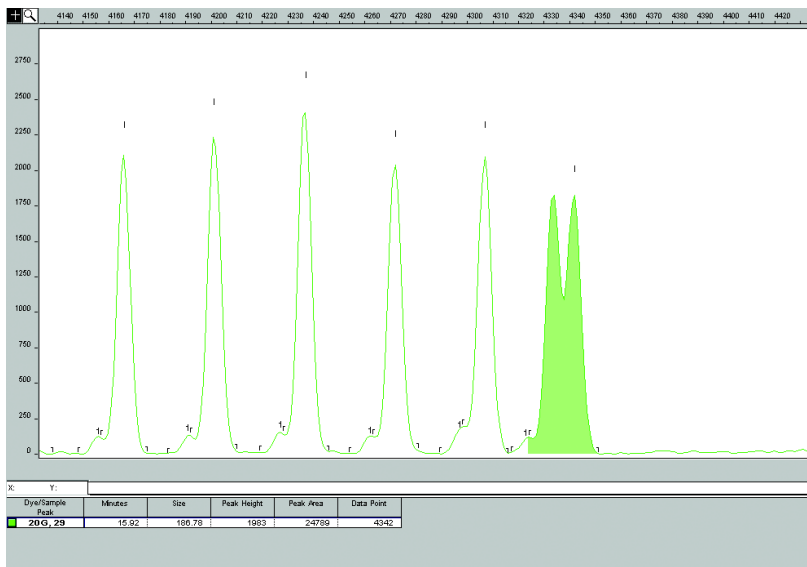
Figure E-9   Two resolved alleles detected as a single peak

Note: For information on the tick marks displayed in the electropherogram, see "Examining Peak Definitions" on page E-10.

Effects of Decreasing the Window Size Value

Figure E-10 is an electropherogram showing that both alleles are detected after re-analyzing with the polynomial degree set to 3 while decreasing the window size value to 15 (from 19) data points.
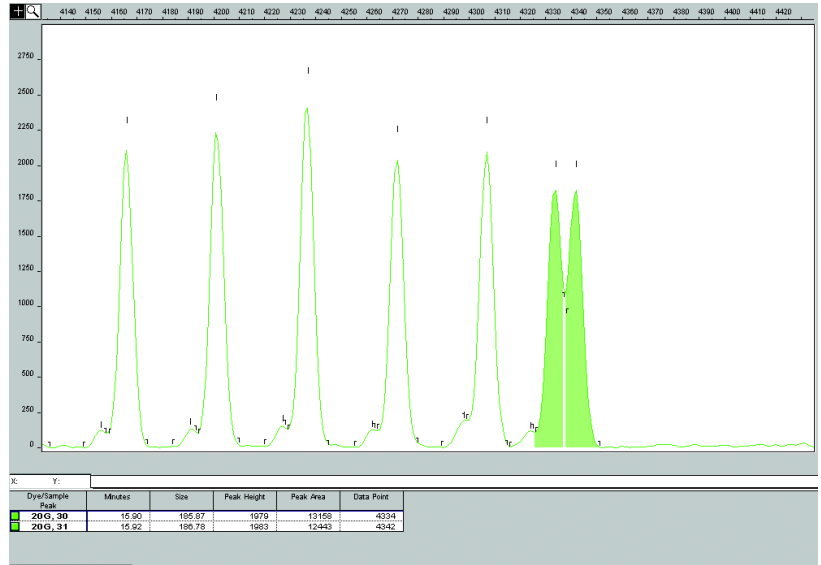
Figure E-10   Alleles detected as two peaks after decreasing the window size value

Optimizing Peak Detection Sensitivity: Example 2

Initial Electropherogram

Figure E-11 is an electropherogram showing an analysis performed using a polynomial degree of 3 and a peak window size of 19 data points.
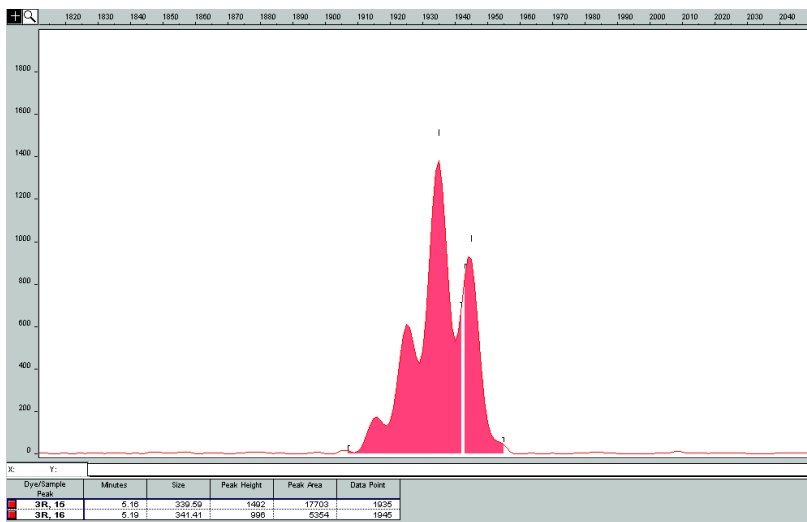
Figure E-11   Four resolved peaks detected as two peaks

Effects of Reducing the Window Size Value and Increasing the Polynomial Degree Value

Figure E-12 is an electropherogram showing the data presented in Figure E-11 re-analyzed with a window size value of 10 and polynomial degree value of 5.
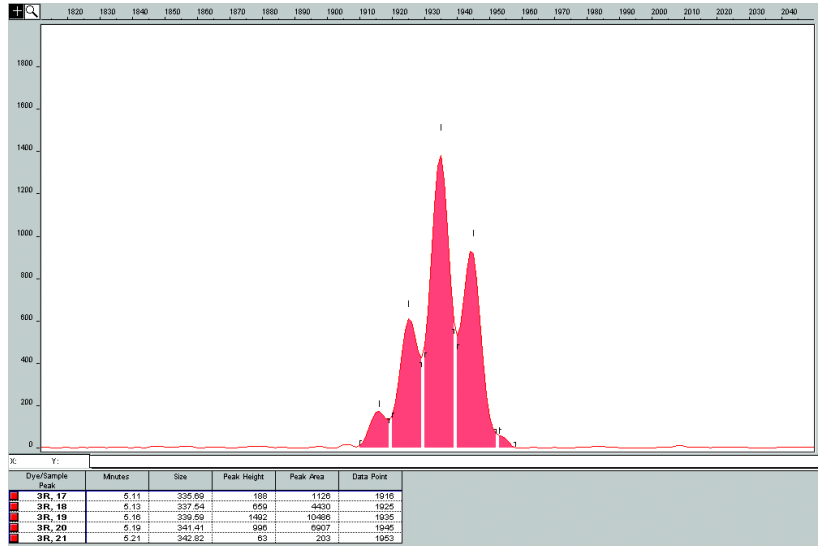
Figure E-12   All four peaks detected after reducing window size value and increasing polynomial degree value

Optimizing Peak Detection Sensitivity: Example 3

Effects of Extreme Settings

Figure E-13 is an electropherogram showing the result of an analysis using a peak window size value set to 10 and a polynomial degree set to 9. This extreme setting for peak detection led to several peaks being split and detected as two separate peaks.

Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
24B, 2	46.93	122.27	1075	5217	1760
24B, 3	47.17	123.20	53	100	1769
24B, 4	47.25	123.51	62	255	1772
24B, 16	108.77	328.35	100	403	4079
24B, 17	108.85	328.60	97	467	4082
24B, 18	110.11	332.55	1028	9136	4129
24B, 19	110.45	333.65	62	214	4142
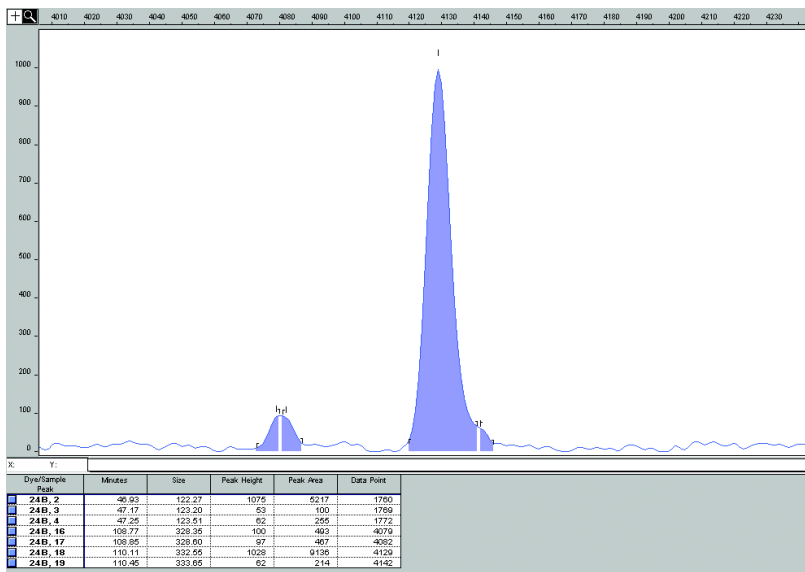
Figure E-13   Analysis using extreme settings for peak detection

Parameters for Peak Detection of Slope Threshold

About These Parameters

Use the Slope Threshold for Peak Start and Slope Threshold for Peak End parameters to adjust the start and end points of a peak. Use this parameter to position the start and end points of an asymmetrical peak, or a poorly resolved shouldering peak, to more accurately reflect the peak position and area.

How These Parameters Work

In general, from left to right, the slope of a peak increases from the baseline to the apex. From the apex down to the baseline, the slope becomes decreasingly negative until it returns to zero at the baseline.
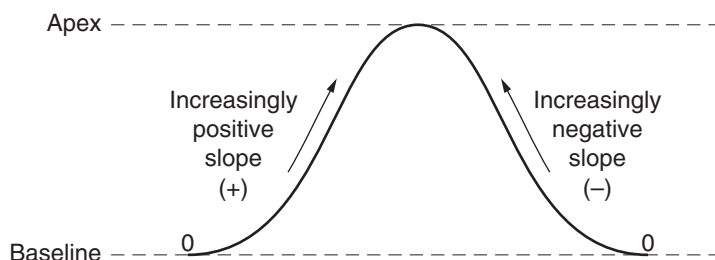
Figure E-14   Sample of slope of a peak

If either of the slope values you have entered exceeds the slope of the peak being detected, the software overrides your value and reverts to zero.
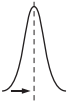
Guidelines for Using These Parameters

As a guideline, use a value of zero for typical or symmetrical peaks. Select values other than zero to better reflect the beginning and end points of asymmetrical peaks.

A value of zero will not affect the sizing accuracy or precision for an asymmetrical peak.

Using These Parameters

Use the table below to move the start or end point of a peak.

If you want to move the...	Then change the...
start point of a peak closer to its apex	**Slope Threshold for Peak Start** value from zero to a positive number
end point of a peak closer to its apex	**Slope Threshold for Peak End** value to an increasingly negative number

Note: The size of a detected peak is the calculated apex between the start and end points of a peak and will not change based on your settings.

Slope Threshold Example

Initial Electropherogram

In the electropherogram in Figure E-15, the initial analysis with a value of 0 for both the Slope Threshold for Peak Start and the Slope Threshold for Peak End value produced an asymmetrical peak with a noticeable tail on the right side.
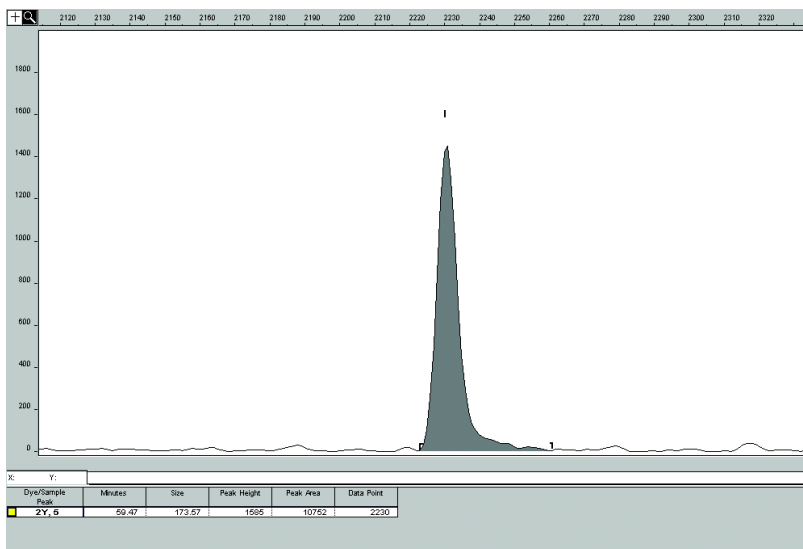
Figure E-15   Asymmetrical peak

Electropherogram After Adjustments

After re-analyzing with a value of -35.0 for the Slope Threshold for Peak End, the end point that defines the peak moves closer to its apex, thereby removing the tailing feature. In the electropherogram shown in Figure E-16, note that the only change to tabular data was the area (peak size and height are unchanged).
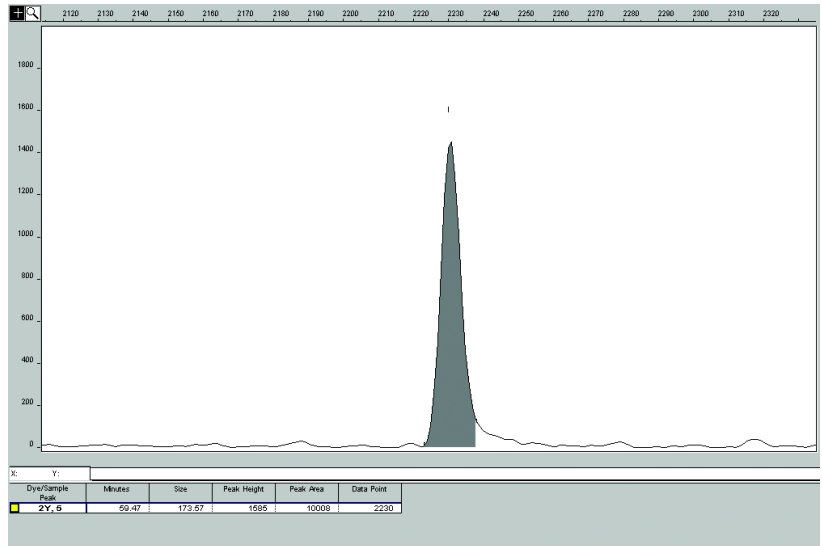
Figure E-16   Effect of changing the slope threshold for peak end

Baseline Window Size Parameter

About This Parameter

Use the Baseline Window Size parameter to control the baseline for a group of peaks.

How This Parameter Works

The software determines a reference baseline value for each data point. In general, the software sets the reference baseline to be the lowest value that it detects in a specified window size (in data points) centered on each data point.

A small baseline window relative to the width of a cluster, or grouping of peaks spatially close to each other, can cause shorter peak heights.

Larger baseline windows relative to the peaks being detected can create an elevated baseline, resulting in peaks that are elevated or not baseline resolved.

Guidelines for Using This Parameter

As a guideline, choose a value that encompasses the width in data points of the peaks being detected while preserving a qualitatively smooth baseline. The trade-off for a smoother baseline that touches all peaks is a reduction in peak height.

Baselining Example

Figure E-17 depicts an allelic ladder containing clusters of alleles. The alleles have been labeled with green dye and the data displayed has been multicomponented, but not baselined. The electropherogram spans approximately 2800 data points.

The red, blue, and black traces depict various reference baselines (zero in the analyzed electropherogram) that result from different baseline window size settings. These reference baselines are subtracted from the sample data during baselining. In Figure E-17:

- The red trace depicts the reference baseline that results from an extreme baseline window size value of 2801. At this setting, the reference baseline does not touch all peaks, resulting in elevated peak heights.
- The blue trace depicts the reference baseline that results from the default value of 51 data points.

- The black trace depicts the reference baseline that results from an extreme baseline window size value of 5 data points. At this setting, the peaks are tracked too closely by the reference baseline, resulting in significantly reduced peak height.
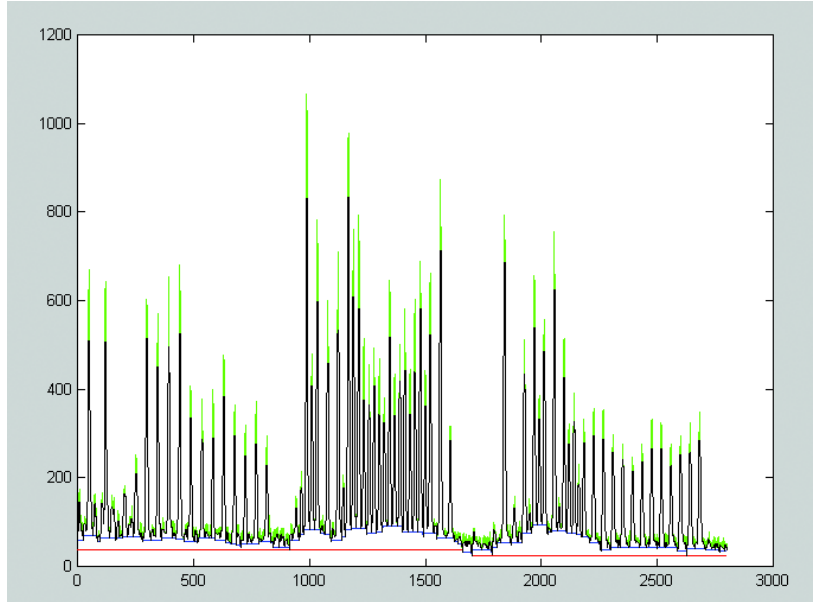
Figure E-17   Baselining of an electropherogram

Baselining Example 1

Initial Electropherogram

Figure E-18 shows a portion of the electropherogram shown in Figure E-17, which depicts various window sizes. The electropherogram shows the default Baseline Window Size value of 51 that appears in Figure E-17 as the blue trace. Note that all peaks in this cluster have been baselined.

Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
21G, 30	9.71	180.85	99	918	3640
21G, 32	9.83	184.70	984	7399	3686
21G, 35	10.07	192.73	530	4229	3777
21G, 38	10.31	200.66	895	7330	3868
21G, 40	10.43	204.84	707	5463	3910
21G, 46	10.78	217.58	556	4285	4044
21G, 50	11.02	225.98	367	2817	4132
21G, 55	11.37	238.54	807	6635	4263
21G, 56	11.48	242.58	252	2129	4305
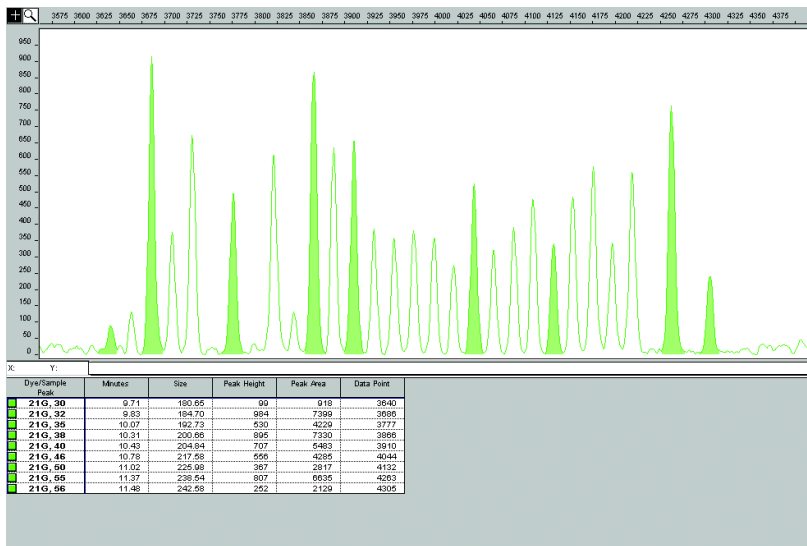
Figure E-18   An allelic ladder with a cluster of peaks

Effects of Extreme Increase of the Baseline Window Size

Figure E-19 is an electropherogram showing an extreme Baseline Window Size value of 2801 that appears in Figure E-17 as the red trace (2801 is approximately the width in data points of all the peaks shown). This increase resulted in an overall raised baseline and many elevated peaks within the cluster.
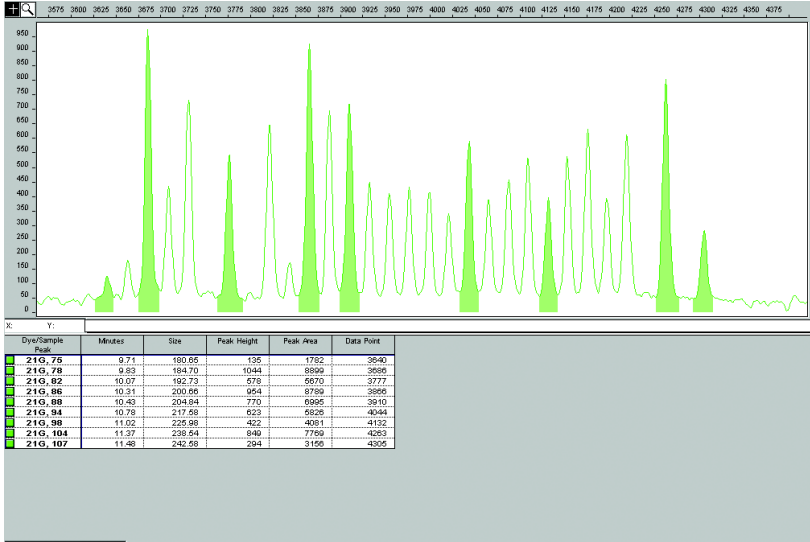
Figure E-19   Raised baseline caused by an increase in the baseline window size value

Effects of Extreme Decrease of the Baseline Window Size

Figure E-20 is an electropherogram showing an extreme Baseline Window Size value of 5 that appears in Figure E-17 as the black trace (5 is much smaller than the width in data points for any of the peaks prior to baselining). This decrease resulted in a significant decrease in the peak heights.
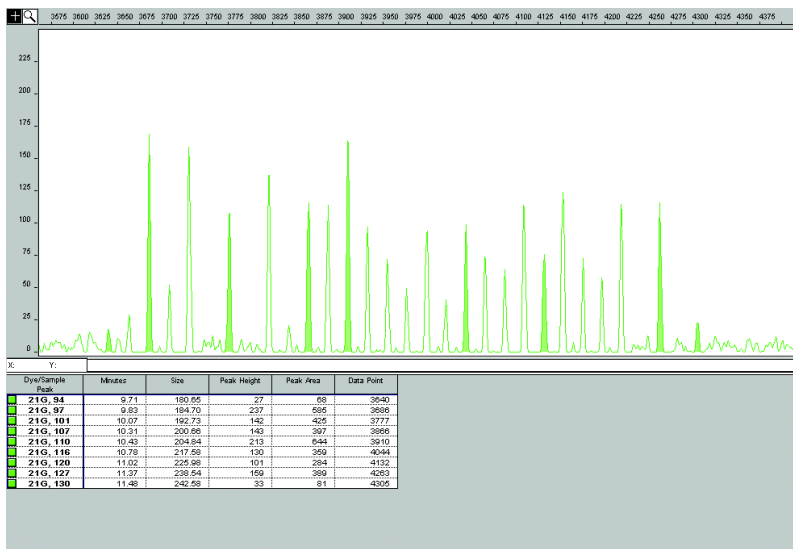
Figure E-20   Significantly reduced peak heights caused by a reduction in the baseline window size value

Baselining Example 2

Initial Electropherogram

Figure E-21 is an electropherogram showing an analysis of a cluster of peaks using the default Baseline Window Size value of 51 data points.

Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
3R, 17	5.11	335.69	188	1126	1916
3R, 18	5.13	337.54	659	4430	1925
3R, 19	5.16	339.59	1492	10498	1935
3R, 20	5.19	341.41	996	6907	1945
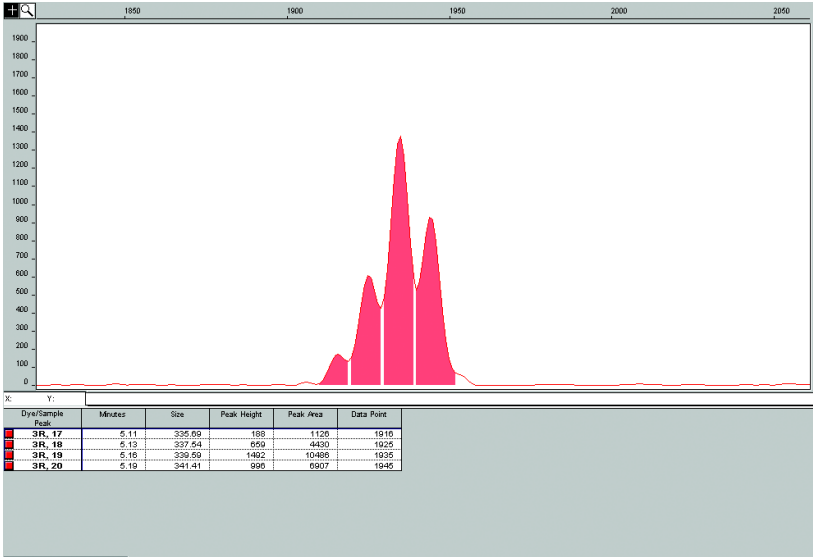
Figure E-21   Typical result using the default baseline window size value

Effects of Extreme Decrease of the Baseline Window Size

Figure E-22 is an electropherogram showing the re-analysis of the electropherogram shown in Figure E-21 with an extreme Baseline Window Size value of 5. All peaks within the cluster have been baselined and have dramatically reduced peak heights.
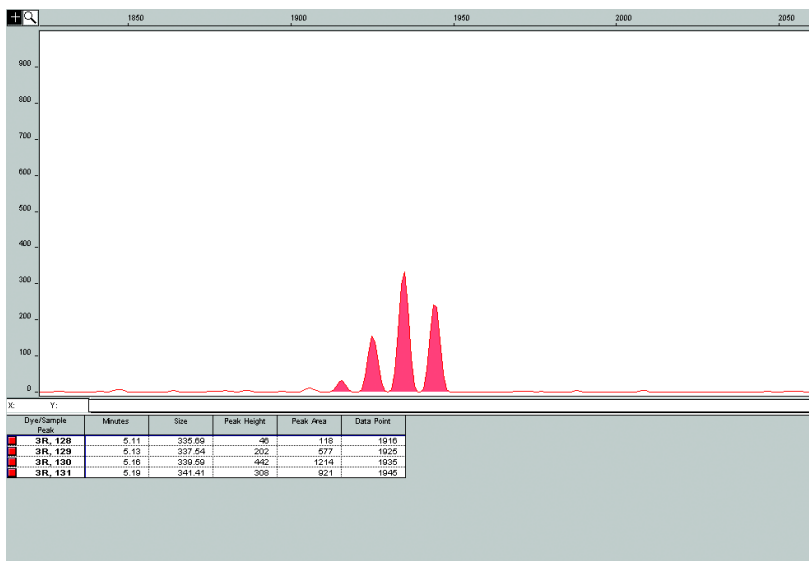
Figure E-22   Reduction in the baseline window size value

Baselining Example 3

Raw Data   The data in the electropherogram in Figure E-23 have been multicomponented but not baselined. There are two pull-down peaks in the blue trace below the two major green peaks (see arrows).
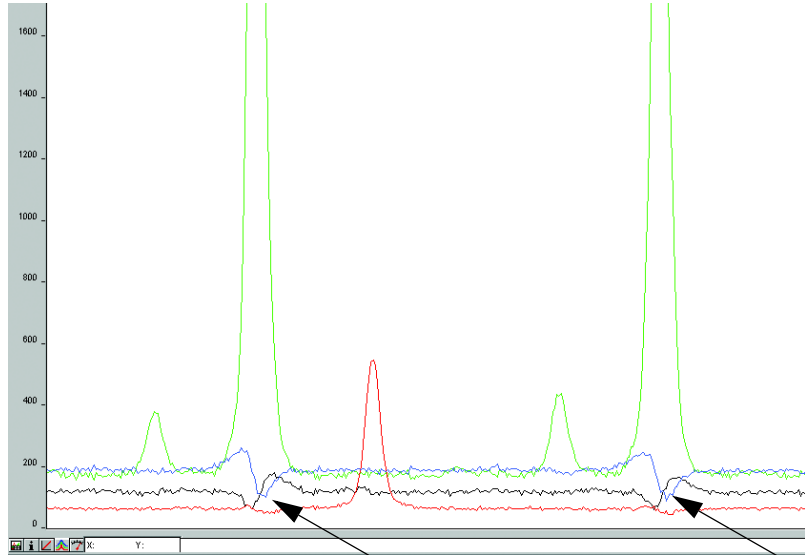
Figure E-23   Raw data multicomponented but not baselined

Raised Baseline   After analyzing with a baseline window size of 251 data points, the low points represented in the blue trace (within this 251 data point window) are set to zero. Setting the pull-down traces to zero results in a raised baseline between these points, as shown in the electropherogram in Figure E-24.
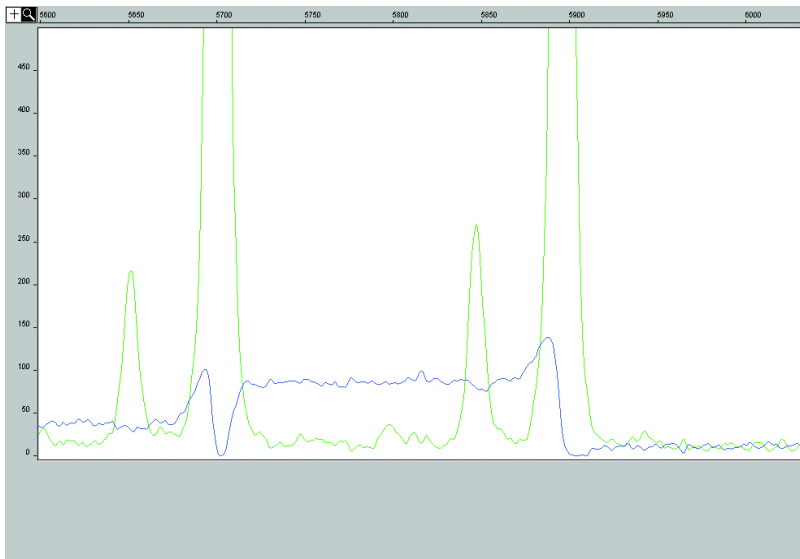
Figure E-24   A raised baseline

Eliminating Raised Baseline

After re-analyzing with a baseline window size of 51 data points (a window size range between the pull-down peaks), the raised baseline is eliminated. This results in a more accurate baseline.
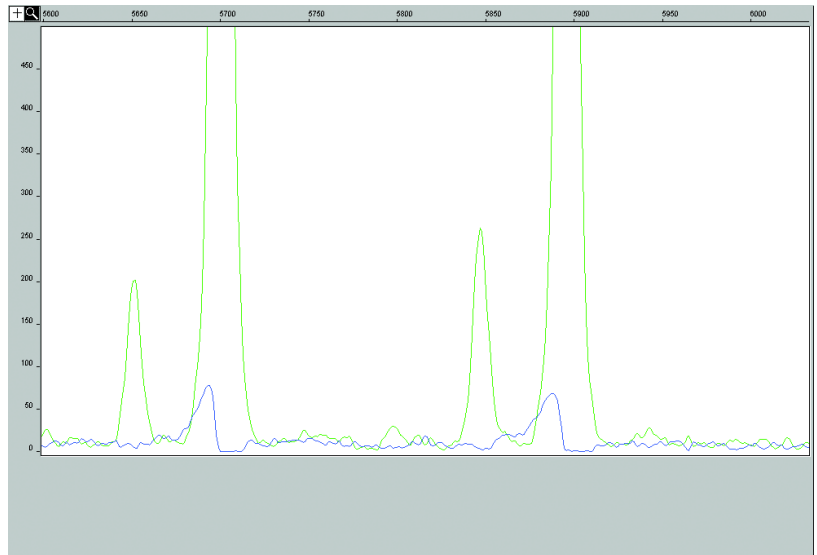
Figure E-25   Re-analyzed baseline with window size of 51 data points

Size Caller

About the Size Caller

The size caller matches size-standard peaks with a quality check.

How the Size Caller Works

The way in which the fragment sizes are calculated has not changed from previous versions of the software (*e.g.,* local southern). However, the way in which the Windows NT version of the software identifies the size standard is different from previous versions.

Method for Identifying the Size Standard

Macintosh Versions	Windows NT Version
User assigns fragment sizes to particular peaks based on scan number	Software matches the size standard fragments by ratio matching based on relative distance between neighboring peaks

Macintosh Version

In GeneScan analysis software for the Macintosh operating system, the size standard peaks are identified based on their mobility and assignment within a run or a previous run.

Anomalous peaks outside of a ±10 data point bin are ignored, but those within the bins can be incorrectly called resulting in an incorrect size curve. In that case, you must redefine a new size standard for that particular sample.
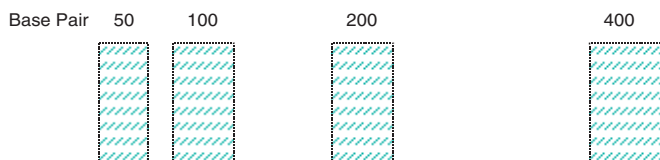
Defining the Size Standard The boxes show a ±30 data point range used to identify size standard peaks in subsequent runs.
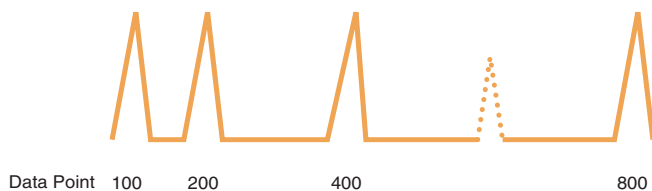
Data (100, 200, 400, and 800) shown with anomalous peak dotted.
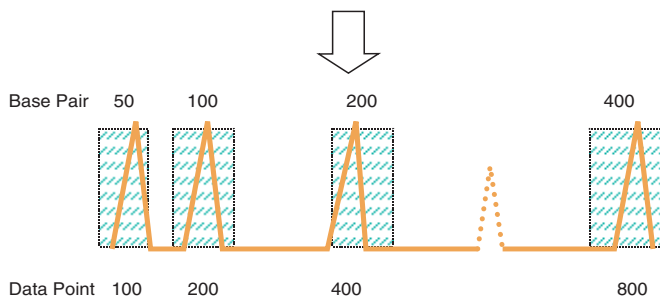
Assigning Peaks that fall into the correct range. The anomalous peak is ignored.
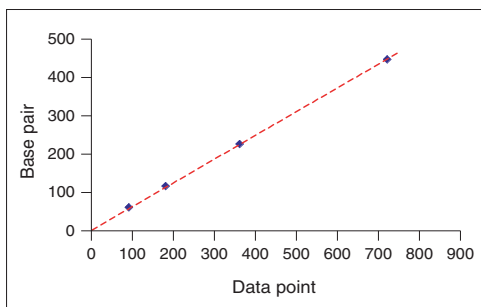
Constructing Size Standard Curve for sample file using specified sizing method, *e.g.,* Local Southern.

Figure E-26   Peak identification with GeneScan analysis software for the Macintosh operating system

Windows NT Version   GeneScan analysis software for the Windows NT operating system uses ratio matching to identify the size standard fragments.

Ratio matching does not rely on the manual assignment of size standard definitions (in base pairs) to their associated data points within a run or a previous run. Selecting a peak in the electropherogram to enter an associated value in the Size column now serves only as a guide. Simply listing the values to be used for sizing as an array of numbers without regard to the highlighted peak is sufficient.
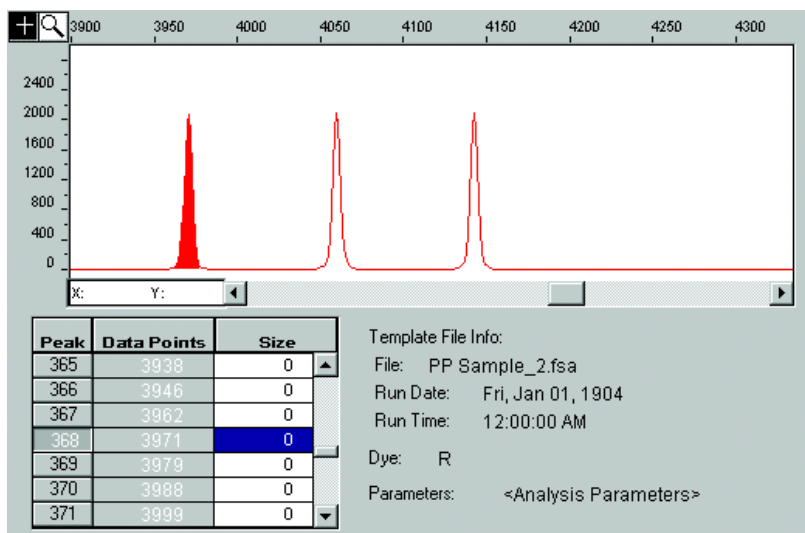
Figure E-27   Electropherogram showing a selected peak and the associated value in the Size column

The size caller ignores anomalous peaks that do not match the expected ratio. The size caller constructs a best-fit curve using the data points of each size standard fragment detected. A comparison between the sizes calculated from the best-fit curve and the matched peaks from the size standard definition using the array of numbers is performed. Size calling will fail if significant differences are found or if no match can be made based on the expected ratios. (In Figure E-28, that is x, 2x, and 4x.)

Additionally, you may find that one of the size fragments has not been identified, even though it was listed as part of the definition. The size caller has been designed to allow the exclusion of one of the listed values to obtain a better match. To use an excluded fragment, try the steps outlined in Figure E-29.

Defining the Size Standard from a list of sizes (50, 100, 200, and 400) used to calculate the expected ratios (in red).

Data (100, 200, 400, 800) shown with anomalous peak dotted.

Generating the Size Standard Curve when a good ratio match is found.

Figure E-28   Peak identification with GeneScan analysis software for the Windows NT operating system

**GeneScan
Macintosh version**

All peaks present in size standard are detected.

↓

Information in the size standard definition is used to select the peaks of the size standard (±10 data points from the position defined in the Size Standard file).

Fail →

New standard for failed sample is defined, regardless of migration or quality.

Pass ↓

Size standard curve is constructed using the method selected by the user (*e.g.,* local southern).

↓

Size standard curve used to size all peaks in sample.

**GeneScan
Windows NT version**

All peaks present in size standard are detected.

↓

Information in the size standard definition is used to select the peaks of the size standard (ratio matching is used based on the list of sizes defined in the Size Standard file).

↓

Best fit curve of the detected sized standard fragments is constructed.

↓

For each peak in the size standard, the matched size of the peak is compared to the calculated size using the best fit curve previously constructed.

If the sizes differ significantly or the peaks cannot be found, the sizing fails.

Fail →

Sample is not sized.

↓

The user should:

1. Make sure all fragments listed in the size standard definition are reflected in the analysis range.

2. Make sure the primer peak is not interfering with smaller fragments. If it is, exclude the primer peak from the analysis.

3. Make sure the higher fragments are resolved. If they are not, reduce the analysis range and change the size standard definition to reflect the missing peaks.

4. Attempt to get a better ratio match by changing the size standard definition and analysis range to analyze a smaller range containing only peaks of interest, if plausible.

5. Attempt to guess values for any split peaks so that every peak displayed has a value.

Pass ↓

Size standard curve is constructed using the method selected by the user (*e.g.,* local southern).

↓

Size standard curve used to size all peaks in sample.

Figure E-29   Peak sizing flowcharts

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F

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