ABI PRISM® SNaPshot™ Multiplex Kit

Protocol

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Contents

Product and Protocol Overview

About the Kit

The ABI PRISM® SNaPshot™ Multiplex Kit is designed to interrogate up to ten single nucleotide polymorphisms (SNPs) at known locations on one to ten DNA templates in a single tube.

Control Template DNA and Primer Mix included in the SNaPshot Multiplex Kit provides reagents for control reactions.

Product Overview

Single nucleotide polymorphisms detection using the ABI PRISM SNaPshot Multiplex Kit requires the following components:

- SNaPshot Multiplex Ready Reaction Mix
- Your template and primers

The ABI PRISM SNaPshot Multiplex Control Template DNA and Primer Mix provides Control Primers and Control Template to perform control reactions only.

Kit Chemistry Based on Single Base Extension

The chemistry is based on the dideoxy single-base extension of an unlabeled oligonucleotide primer (or primers).

- Each primer binds to a complementary template in the presence of fluorescently labeled ddNTPs and AmpliTaq® DNA Polymerase, FS.
- The polymerase extends the primer by one nucleotide, adding a single ddNTP to its 3´ end.

Dye Assignments

The fluorescent dyes are assigned to the individual ddNTPs as follows:

ddNTP	Dye Label	Color of Analyzed Data
A	dR6G	Green
C	dTAMRA™	Black
G	dR110	Blue
T (U)	dROX™	Red

Platforms and Software

Products generated using the ABI PRISM SNaPshot Multiplex Kit can be analyzed with GeneScan® Analysis Software version 3.1 or higher. The kits can be run on the following platforms:

- ABI PRISM® 310 Genetic Analyzer
- ABI PRISM® 3100 Genetic Analyzer
- ABI PRISM® 3700 DNA Analyzer

About This Protocol

This protocol describes how to:

- Prepare sample reactions using your own template(s) and primer(s) or the control template and control primers.
- Perform SNaPshot reactions by thermal cycling and conduct post-extension treatment of the products.
- Electrophorese the samples and analyze the data.

To view a flowchart of the procedure refer to “Overview of the Procedure” on page 9.

Kit Contents and Storage

SNaPshot Multiplex Kit

The ABI PRISM SNaPshot Multiplex Kit is available in three reaction sizes. Using this kit, you can perform your own reactions and also perform 30 control reactions with the control template and primers provided.

One Kit Available in Three Formats

Kit	Number of Reactions[a]	Part Number
ABI PRISM SNaPshot™ Multiplex Kit	100	4323151
	1000	4323154
	5000	4323155

a. Each kit contains Multiplex Control Template and Multiplex Control Primers for 30 control reactions

The kit contains the following items:

Kit Components	Contents
SNaPshot Multiplex Ready Reaction Mix	AmpliTaq® DNA Polymerase, FS
	Fluorescently labeled ddNTPs
	Reaction buffer
SNaPshot Multiplex Control Primer Mix (30 µL total)	20A primer (0.05 pmol/µL)
	28G/A primer (0.10 pmol/µL)
	36G primer (0.05 pmol/µL)
	44T primer (0.30 pmol/µL)
	52C/T primer (0.30 pmol/µL)
	60C primer (0.30 pmol/µL)
SNaPshot Multiplex Control Template (60 µL total)	Amplicon from CEPH DNA
Protocol	P/N 4323357
Quick Reference Card	P/N 4323975

Storing the Reagents

Upon receipt, store the ABI PRISM SNaPshot Multiplex Kit at –15 to –25 °C in a constant-temperature freezer.

Required Software and Materials

Overview

This section describes the software and materials necessary for using the ABI PRISM SNaPshot Multiplex Kit.

GeneScan-120 LIZ Size Standard Recommended

Primers used in a single reaction for multiloci interrogation need to differ significantly in length to avoid overlap between the final SNaPshot products. To analyze the final products successfully and robustly, a 5th dye-labeled internal size standard specifically designed for small fragments should be used. The GeneScan™-120 LIZ™ size standard has been designed specifically for use with the SNaPshot Muiltiplex Kit.

Data Collection Software and/or GeneScan Run Module Required

One of the following Data Collection Software and/or GeneScan Run Modules is required:

310 Genetic Analyzer:

- 310 Data Collection version 2.1
- GS STR POP4 (1 mL) E5

3100 Genetic Analyzer:

- 3100 Data Collection version 1.0
- SNP36_POP4 default module

3700 DNA Analyzer:

- 3700 Data Collection version 1.1 (enabled with 3700 Data Collection 5-Dye Update File P/N 4324208)
- SNP1_1POP5

Materials Required but Not Included

The following materials are required but not included:

Item	Source
One of the following instruments with 5-dye capability: ♦ ABI PRISM 310 Genetic Analyzer ♦ ABI PRISM 3100 Genetic Analyzer with POP-4 polymer and 36-cm array ♦ ABI PRISM 3700 DNA Analyzers with POP-5 polymer and 50-cm array.	Applied Biosystems
GeneAmp® PCR System 9600 thermal cycler with appropriate tubes or plate, and caps	
GeneScan® software v. 3.1 or higher	
Matrix Standard Set DS-02 [dR110, dRGG, dTAMRA™, dROX™, LIZ™] 310 3100 3700 GeneScan™-120 LIZ™ size standard	Applied Biosystems 4323050 4323014 4323785 4324211
Hi-Di™ formamide, 25-mL bottle	Applied Biosystems (P/N 4311320)
1X TE, pH 7.0	Major laboratory supplier (MLS)
Centrifuge with 96-well plate adapter	MLS
Deionized water	MLS
Disposable gloves	MLS
Pipette tips, aerosol resistant	MLS
Shrimp Alkaline Phosphatase (SAP) or Calf Intestinal Phosphatase (CIP)	USB Corporation (P/N 70092X, 5000 Units) (P/N 70092Z, 1000 Units) (P/N 70092Y, 500 Units) New England BioLabs (P/N 290L, 5000 Units) (P/N 290S, 1000 Units)

The following materials are required but not included: *(continued)*

Item	Source
Exo I or	USB Corporation (Exonuclease I, P/N 70073Z)
PCR Clean Up Kit or	Roche Molecular Biochemicals (P/N 1696513, 100 reactions)
High Pure™ PCR Product Purification Kit	Roche Molecular Biochemicals (P/N 1732668, 50 reactions) (P/N 1732676, 250 reactions)

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.

⚠ 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.

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 and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Do not leave chemical containers open. Use only with adequate ventilation.
- 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.

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.

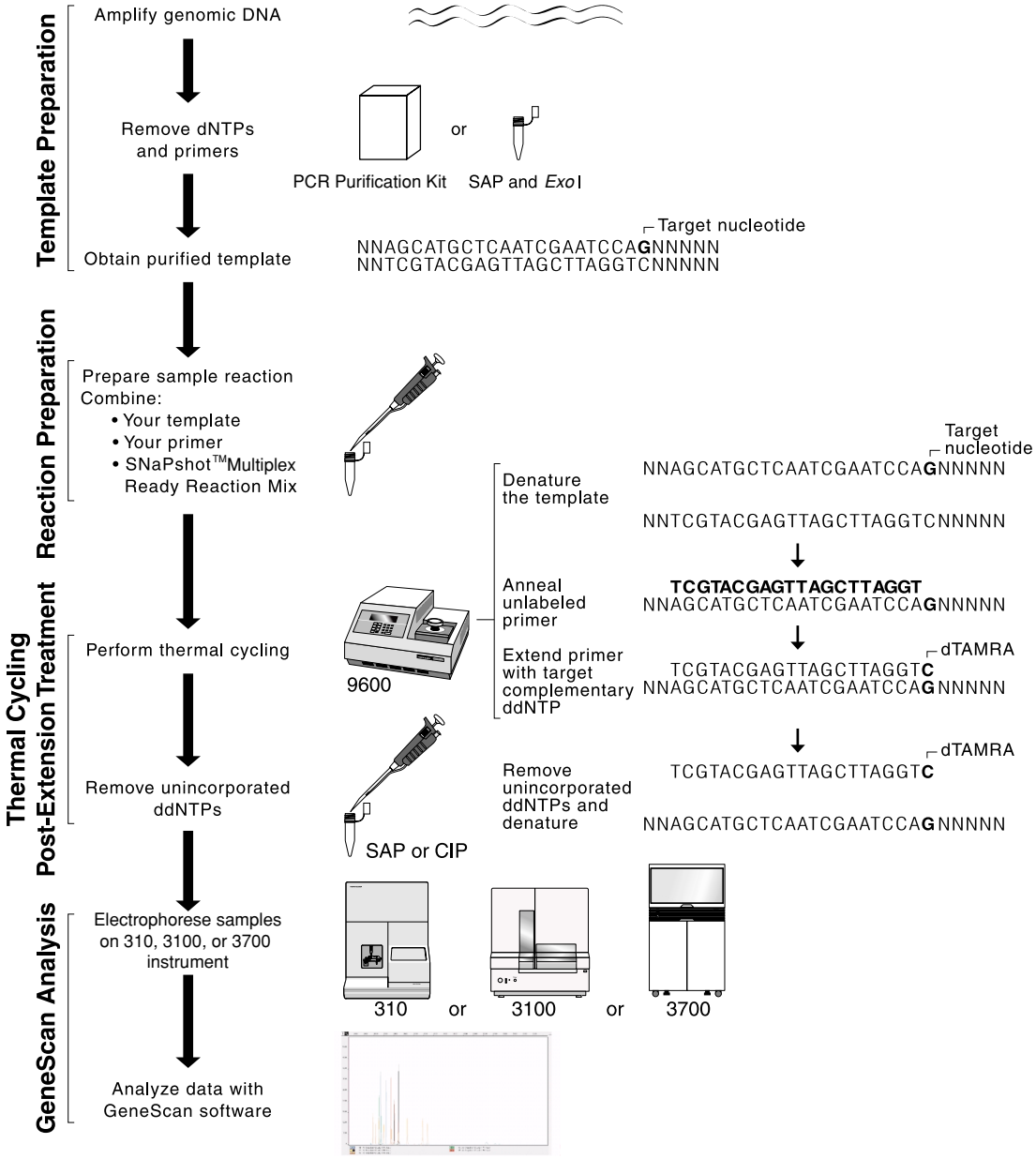
Ordering MSDSs

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

<table>
<tr><th>To order MSDSs...</th><th>Then...</th></tr>
<tr><td>Over the Internet</td><td>a. Go to our Web site at www.appliedbiosystems.com/techsupp.
b. Click MSDSs.

<table><tr><th>If you have...</th><th>Then...</th></tr><tr><td>The MSDS document number or the Document on Demand index number</td><td>Enter one of these numbers in the appropriate field on this page</td></tr><tr><td>The product part number</td><td rowspan="2">Select Click Here, then enter the part number or keyword(s) in the field on this page.</td></tr><tr><td>Keyword(s)</td></tr></table>
c. You can open and download a PDF (using Adobe® Acrobat Reader) of the document by selecting it, or you can choose to have the document sent to you by fax or email.</td></tr>
<tr><td>By automated telephone service</td><td>Use “Documents on Demand” under “Technical Support.”</td></tr>
<tr><td>By telephone in the United States</td><td>Dial 1-800-327-3002, then press 1.</td></tr>
<tr><td>By telephone from Canada</td><td><table><tr><th>To order in...</th><th>Dial 1-800-668-6913 and...</th></tr><tr><td>English</td><td>Press 1, then 2, then 1 again</td></tr><tr><td>French</td><td>Press 2, then 2, then 1</td></tr></table></td></tr>
<tr><td>By telephone from any other country</td><td>See “Regional Offices Sales and Service” under “Technical Support.”</td></tr>
</table>

Overview of the Procedure



Preparing Your PCR Template for Primer Extension

Purpose

This section describes how to prepare your PCR product template before primer extension.

About the Templates

There are two kinds of templates that you can use in primer extension reactions:

- Plasmid templates
- PCR products

While plasmid templates do not require cleanup before primer extension, PCR product templates must be purified.

Depending on the specific template, 0.01 to 0.40 pmol of the template should be used in the SNaPshot reactions.

Methods for Preparing PCR Templates

After PCR amplification, the resulting template is in solution, along with primers, dNTPs, and enzyme and buffer components. To avoid participation in the subsequent primer-extension reaction, primers and unincorporated dNTPs must be removed.

We recommend the following methods for purifying PCR products:

PCR Purification Kits

High Pure™ PCR Product Purification (P/N 1732668, 50 reactions, 1732676, 250 reactions) or PCR Clean Up Kits (P/N 1696513, 100 reactions) can be purchased from Roche Molecular Biochemicals. Refer to the manufacturer's instructions for the procedure.

SAP and *Exo* I Treatment

To treat PCR products using SAP and *Exo* I:

Step	Action
1	Add the following to 15 µL of PCR product: ♦ 5 units of SAP ♦ 2 unit of *Exo* I Use the following guidelines for enzyme treatment: ♦ Reaction volume can be adjusted up or down. PCR products can be from a single PCR reaction or multiple PCR reactions. We recommend that you purify individual PCR products and combine the purified products in the next step. ♦ **To ensure a low background**, we strongly recommend that the relative ratio of PCR product, SAP, and *Exo* I be kept constant, *i.e.*, 5 units of SAP and 2 units of *Exo* I for every 15 µL of PCR product. ♦ Because of the high glycerol concentration in undiluted SAP and *Exo* I, add each enzyme into the PCR mixture one at a time. ♦ *Exo* I can be freshly diluted in a buffer containing 80 mM Tris-HCl (pH = 9.05) and 2 mM $MgCl_2$. Do not store diluted *Exo* I.
2	Mix thoroughly and incubate at 37 °C for 1 hour. **Note** Because of the high glycerol concentration in undiluted SAP and *Exo* I, vortex briefly to mix.
3	Incubate at 75 °C for 15 minutes to inactivate the enzymes.
4	Keep on ice or at 4 °C. For longer storage, store at –20 °C.

Preparing the Control Reactions

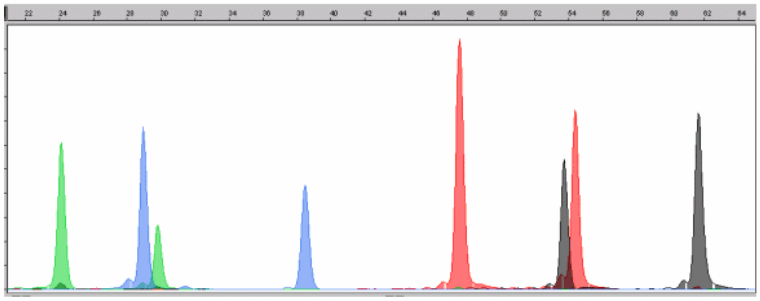
About the Control Reactions

Included in each kit are a Multiplex Control Primer Mix tube containing six distinct primers and a Multiplex Control Template tube containing an amplicon from CEPH DNA.

The control primers are listed in the table below.

Multiplex Control Primer Mix	Length of Final Products (nt)	Signal Color	Heterozygosity
20A primer	21	Green	Homozygote
28G/A primer	29	Blue/green	Heterozygote
36G primer	37	Blue	Homozygote
44T primer	45	Red	Homozygote
52C/T primer	53	Black/red	Heterozygote
60C primer	61	Black	Homozygote

Note Due to the influence of the dye on the mobility shift of the DNA fragments, the reported sizes will differ by a few bases from the actual sizes. This is particularly true with the shorter fragments as the relative contribution of the dye is greater.



Primer	20A	28G/A	36G	44T	52C/T	60C
Relative size of SNaPshot product*	24	29	39	46	53	62

*:As determined by sizing against GeneScan-120 Liz size standard

Negative Control Reaction

Run one negative control reaction without control template DNA.

Preparing the Control Reactions

To prepare the control reactions:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Label two 0.2-mL MicroAmp® tubes, one for the positive control reaction and one for the negative control reaction.</td></tr>
<tr><td>2</td><td>Thaw the SNaPshot Multiplex Ready Reaction Mix, Control Template, and Control Reaction Primer Mix on ice. Prepare the following reaction mix on ice:
<table>
<tr><th>Item</th><th>Positive Control (µL)</th><th>Negative Control (µL)</th></tr>
<tr><td>SNaPshot Multiplex Ready Reaction Mix</td><td>5</td><td>5</td></tr>
<tr><td>SNaPshot Multiplex Control Template</td><td>2</td><td>0</td></tr>
<tr><td>SNaPshot Multiplex Control Primer Mix</td><td>1</td><td>1</td></tr>
<tr><td>Deionized water</td><td>2</td><td>4</td></tr>
<tr><td>Total</td><td>10</td><td>10</td></tr>
</table></td></tr>
<tr><td>3</td><td>Mix and spin briefly.

Note Keep the SNaPshot mixture on ice before putting it into the thermal cycler. Leaving the mixture at ambient temperature for 20 minutes or longer may result in a higher background.</td></tr>
<tr><td>4</td><td>Proceed to “Thermal Cycling and Post-Extension Treatment” on page 16.</td></tr>
</table>

Preparing Your Sample Reactions

Overview

This section describes how you set up multiplex SNaPshot reactions using your templates and primers.

SNaPshot Primer Design

See “SNaPshot Primer Design and Evaluation Recommendations” on page 29 for recommendations on designing and evaluating primers.

Pooling PCR Amplified SNaPshot Templates

If you have multiple purified PCR amplified samples to run in a single SNaPshot reaction, mix equal volumes (*e.g.*, 2 µL each) of these products in a tube and place the tube on ice.

Note SNaPshot Multiplex Ready Mix gives satisfactory results over a range of 0.01 to 0.40 pmol of PCR products (depending on template) in a 10-µL reaction.

Note For a description of how to convert nanograms per microliter to picomoles per microliter, refer to Appendix B on page 31.

Pooling SNaPshot Primers

All the primers to be used in a single SNaPshot reaction should be premixed to give a final concentration of 0.2 µM for each primer. Place the primer mixture on ice.

Note SNaPshot Multiplex Ready Mix has been designed to exhaust all primers in the reaction. The recommended starting concentration for each primer is 0.2 µM. If a particular primer has a consistently low or high signal, increase or decrease the concentration of that primer. Successful results have been obtained using primers with concentrations that range between 0.05 µM and 1 µM in a six-primer mixture. Adjusting the template concentration is usually not required.

Setting Up Your Sample Reaction

To set up your sample reaction:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Thaw the SNaPshot Multiplex Ready Reaction Mix on ice.

Note Adjust the volume of deionized water to accommodate any changes in primer or template volumes.

Note Make a master mix if you are running several samples containing common components.

Combine the following:

<table>
<tr><th>Item</th><th>Volume (µL/ Sample)</th></tr>
<tr><td>SNaPshot Multiplex Ready Reaction Mix</td><td>5</td></tr>
<tr><td>Pooled PCR products</td><td>3</td></tr>
<tr><td>Pooled SNaPshot primers</td><td>1</td></tr>
<tr><td>Deionized water</td><td>1</td></tr>
<tr><td>Total</td><td>10</td></tr>
</table>
</td></tr>
<tr><td>2</td><td>Mix thoroughly and spin briefly.

Aliquot 10 µL into each MicroAmp tube/well.

Note It is important to keep the reaction mixture on ice before putting it into the thermal cycler. Leaving the mixture at ambient temperature for 20 minutes or longer may lead to higher background.</td></tr>
<tr><td>3</td><td>Proceed to “Thermal Cycling and Post-Extension Treatment” on page 16.</td></tr>
</table>

Thermal Cycling and Post-Extension Treatment

Overview

This section describes how to conduct thermal cycling and how to remove unincorporated ddNTPs after thermal cycling.

Thermal Cycling

To conduct thermal cycling:

Step	Action
1	Place the tubes in a GeneAmp 9600 thermal cycler, and set the volume to 10 µL.
2	Repeat the following for 25 cycles: ♦ Rapid thermal ramp to 96 °C ♦ 96 °C for 10 seconds ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 5 seconds ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 30 seconds **Note** Thermal cycling takes approximately 1 hour and 10 minutes to complete.
3	Rapid thermal ramp to 4 °C, and hold until ready for post-extension treatment.

Post-Extension Treatment

IMPORTANT Left untreated, the unincorporated [F]ddNTPs will co-migrate with the fragment(s) of interest. Removal of the 5´ phosphoryl groups by phosphatase treatment alters the migration of the unincorporated [F]ddNTPs and thus prohibits interference.

To conduct post-extension treatment:

Step	Action
1	Add one of the following to the reaction mixture, mix thoroughly, and incubate at 37 °C for 1 hour. **Note** Because of the high glycerol concentration in the undiluted SAP, vortex briefly to mix. ♦ 1.0 Unit of Shrimp Alkaline Phosphatase (SAP) or ♦ 1.0 Unit of Calf Intestinal Phosphatase (CIP)

To conduct post-extension treatment: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>2</td><td>Deactivate the enzyme by incubating at 75 °C for 15 minutes.</td></tr>
<tr><td>3</td><td>Samples may be placed at 4 °C for up to 24 hours prior to electrophoresis on the 310/3100/3700 systems.

For storage longer than 24 hours, store the samples at –20 °C.</td></tr>
<tr><td>4</td><td>
<table>
<tr><th>If you are running an...</th><th>Then proceed to...</th></tr>
<tr><td>ABI PRISM 310 Genetic Analyzer</td><td>“Electrophoresis on the ABI PRISM 310 Genetic Analyzer” on page 18.</td></tr>
<tr><td>ABI PRISM 3100 Genetic Analyzer</td><td>“Electrophoresis on the ABI PRISM 3100 Genetic Analyzer” on page 22.</td></tr>
<tr><td>ABI PRISM 3700 DNA Analyzer</td><td>“Electrophoresis on the ABI PRISM 3700 DNA Analyzer” on page 24.</td></tr>
</table>
</td></tr>
</table>

Electrophoresis on the ABI PRISM 310 Genetic Analyzer

Overview

This section describes electrophoresis of SNaPshot products on the ABI PRISM 310 Genetic Analyzer using the 310 Data Collection version 2.1.

Note For more information about using the ABI PRISM 310 Genetic Analyzer, refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* (P/N 903565).

The Polymer

The SNaPshot kits may be used with:

- POP-4™ polymer, in conjunction with GS POP-4 (1mL) E5 module

⚠ CAUTION **CHEMICAL HAZARD. POP-4 polymer** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Use for research and development purposes only.

GeneScan E5 Run Module Parameters

The GeneScan E5 Run Module encodes the following parameters on the 310 instrument:

Parameter	Control Module GS POP-4 (1mL) E5
Injection time	5 seconds
Electrophoresis voltage	15 kV
Collection time	24 minutes
EP voltage	15 kV
Heat plate temperature	60 °C
Syringe pump time	150 seconds
Preinjection EP	120 seconds

Adjusting the Run Time

Depending upon primer length, the peaks of interest may appear well before the run ends. For this reason, you may want to shorten the collection time.

Adjusting the Injection Time for Signal Variability

If increased or decreased signal is routinely observed, you may want to decrease or increase injection times, respectively. For a description of

how to adjust the injection time on the 310 Genetic Analyzer, refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* (P/N 903565).

Running Matrix Standards

If you are running the ABI PRISM SNaPshot Multiplex Kit reactions for the first time, you will need a Matrix Standard Set DS-02 [dR110, dRGG, dTAMRA, dROX, LIZ] for the 310 Genetic Analyzer system. Run the ABI PRISM DS-02 Matrix Standards Kit (P/N 4323050), along with the other control and sample reactions.

Refer to the DS-02 Matrix Standards Kit product insert for directions on how to prepare the DS-02 matrix standards.

Preparing Samples

Follow the instructions below if you are using an ABI PRISM 310 Genetic Analyzer to run your samples.

To prepare samples for the 310 Genetic Analyzer:

Step	Action
1	Thaw Hi-Di formamide, SNaPshot products, and the GeneScan-120 LIZ size standard. Vortex to mix and spin briefly. **⚠ 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 eyewear, clothing, and gloves.
2	Add 9 µL of Hi-Di formamide into each tube.
3	Add 0.5 µL of the SNaPshot product and 0.5 µL GeneScan-120 LIZ size standard into each tube. **Note** Total volume for injection is 10 µL **Note** Dilute the control reaction 1:2. Mix 0.5 µL of the diluted products with 0.5 µL of GeneScan-120 LIZ size standard. **Note** If you want to use volumes greater than 0.5 µL, the following mixing steps are suggested: a. Dilute 2 µL of SNaPshot product in 6 µL of Hi-Di formamide b. Dilute 2 µL of GeneScan-120 LIZ in 6 µL of Hi-Di formamide (enough for 4 samples) c. Mix: – 2 µL of diluted SNaPshot product – 2 µL of diluted GeneScan-120 LIZ size standard – 6 µL of Hi-Di formamide
4	Vortex briefly and quick spin.
5	Denature the samples by placing them at 95 °C for 5 minutes.
6	Place the samples on ice or at 4 °C until you are ready to load them on the 310 Genetic Analyzer.
7	Quick spin or tap the tubes or plates to bring liquid to the bottom of the tubes.

To prepare samples for the 310 Genetic Analyzer: *(continued)*

Step	Action
8	Refer to the *ABI PRISM 310 User's Manual* for specific directions on the following: a. Verify that you have chosen GeneScan Run Module E5. b. Confirm the injection time. c. Verify that you have selected the DS-02 GeneScan Matrix Set for the 310 Genetic Analyzer system. d. Verify that you have selected the GeneScan-120 LIZ size standard analysis parameter for automatic data analysis. **Note** To set up the GeneScan-120 LIZ size standard automatic analysis, refer to the instructions in the GeneScan-120 LIZ size standard product insert.

Electrophoresis on the ABI PRISM 3100 Genetic Analyzer

Setting Up the Analyzer

Before any run, make sure that the 3100 Genetic Analyzer is set up with a 36-cm capillary array and POP-4 polymer.

Running Matrix Standards

If you are running the ABI PRISM SNaPshot Multiplex Kit reactions for the first time, you will need a Matrix Standard Set DS-02 [dR110. dRGG, dTAMRA, dROX, LIZ] for the 3100 Genetic Analyzer (P/N 4323014).

Refer to the DS-02 Matrix Standards Kit product insert for directions on how to prepare the DS-02 matrix standards.

Preparing the Samples

To prepare samples for the 3100 Genetic Analyzer:

Step	Action
1	Thaw Hi-Di formamide, SNaPshot products, and the GeneScan-120 LIZ size standard. Vortex to mix and spin briefly. **⚠ 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 eyewear, clothing, and gloves.
2	Add 9 µL of Hi-Di formamide to each well.
3	Add 0.5 µL of the SNaPshot products and 0.5 µL of GeneScan-120 LIZ size standard into each well and seal the plates. **Note** Total volume for injection is 10 µL. **Note** If you want to use volumes greater than 0.5 µL, the following mixing steps are suggested: a. Dilute 2 µL of SNaPshot product in 6 µL of Hi-Di formamide. b. Dilute 2 µL of GS120 in 6 µL of Hi-Di formamide (enough for four samples). c. Mix: – 2 µL of diluted SNaPshot product – 2 µL of diluted GeneScan-120 LIZ size standard – 6 µL of Hi-Di formamide

To prepare samples for the 3100 Genetic Analyzer: *(continued)*

Step	Action
4	Vortex briefly and spin briefly.
5	Denature the samples by placing them at 95 °C for 5 minutes.
6	Place the samples on ice or at 4 °C until you are ready to load the analyzer.

GeneScan Run Parameters

To start the run:

Step	Action
1	In the **New Plate** setup, select **Dye Set E5** and SNP36_POP4 default module.
2	Start the run. **Note** To set up the GeneScan-120 LIZ size standard automatic analysis, refer to the instructions in the GeneScan-120 LIZ size standard product insert.

Electrophoresis on the ABI PRISM 3700 DNA Analyzer

Setting Up the Analyzer

Before any run, make sure that the 3700 DNA Analyzer is set up with a 50-cm capillary array and POP-5 polymer.

Running Matrix Standards

If you are running the ABI PRISM SNaPshot Multiplex Kit reactions for the first time, you will need a Matrix Standard Set DS-02 for the 3700 DNA Analyzer (P/N 4323785).

Refer to the DS-02 Matrix Standards Kit product insert for directions on how to prepare the DS-02 matrix standards.

Preparing the Samples

To prepare samples for the 3700 DNA Analyzer:

Step	Action
1	Thaw Hi-Di formamide, SNaPshot products, and the GeneScan-120 LIZ size standard. Vortex to mix and spin briefly. **⚠ 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 eyewear, clothing, and gloves.
2	Add 9 µL of Hi-Di formamide to each well.
3	Add 0.5 µL of SNaPshot products and 0.5 µL of GeneScan-120 LIZ size standard to each well, and seal the plates. **Note** Total volume for injection is 10 µL. **Note** If you want to use volumes greater than 0.5 µL, the following mixing steps are suggested: a. Dilute 2 µL of SNaPshot product in 6 µL of Hi-Di formamide. b. Dilute 2 µL of GeneScan-120 LIZ in 6 µL of Hi-Di formamide (enough for four samples). c. Mix: – 2 µL of diluted SNaPshot product – 2 µL of diluted GeneScan-120 LIZ size standard – 6 µL of Hi-Di formamide
4	Vortex briefly and spin briefly.

To prepare samples for the 3700 DNA Analyzer: *(continued)*

Step	Action
5	Denature the samples by placing them at 95 °C for 5 minutes. Place the samples on ice or at 4 °C until you are ready to load the analyzer.

Setting Up GeneScan Parameters

Setting up the GeneScan application:

Step	Action
1	In the **New Plate** setup, select **Dye Set E5** and **SNP1_1POP5 module**. **Note** Data collection time in the default SNP1_1POP5 module is 900 seconds. To ensure that all 9 peaks in GeneScan-120 LIZ size standard are collected, extend the data collection time to 1100 seconds. **Note** If the signal variation from the left to the right side of the array becomes a concern, try lowering the running voltage to 6 KV. You will also need to extend the data delay time from 900 seconds to 1200 seconds and the data collection time from 900 seconds to 1800 seconds (refer to step 3 for information on modifying the module). **Note** To set up the GeneScan-120 LIZ size standard automatic analysis, refer to the instructions in the GeneScan-120 LIZ size standard product insert.
2	Start the run.

Setting up the GeneScan application: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>3</td><td>Use the table below to adjust the signal intensity using the Module Editor.
<table>
<tr><th>Observation</th><th>Possible Cause</th><th>Recommended Action</th></tr>
<tr><td rowspan="2">Signal varies across the capillary array</td><td>The run temperature and the run voltage need adjusting</td><td>Adjust the run conditions (in the following order):
a. Lower the temperature to 50 °C
b. Lower the run voltage to 6 KV
c. Increase the data delay and run times to accommodate the slower run times caused by a. and b. above</td></tr>
<tr><td>The cuvette temperature is not optimized</td><td>Test in increments of 5 °C through the range 35–50 °C until you identify the temperature that produces the best signal uniformity across the array</td></tr>
</table></td></tr>
</table>

Data Analysis

Overview	This section describes how to perform GeneScan data analysis.
Analyzing Sample Files on the 310 Instrument	Analyze the files using GeneScan Analysis Software version 3.1 and GeneScan-120 LIZ size standard analysis parameter files. For a detailed explanation, refer to the *ABI PRISM GeneScan Analysis Software User's Manual* (P/N 4303242) and the GeneScan-120 LIZ size standard product insert.
Analyzing Sample Files on the 3100 and 3700 Instruments	Analyze the files using GeneScan Analysis Software version 3.5 and GeneScan-120 LIZ size standard analysis parameter files. For a detailed explanation, refer to the *ABI PRISM GeneScan Analysis Software User's Manual* (P/N 4303242) and the GeneScan-120 LIZ size standard product insert.

Example of Control Reaction

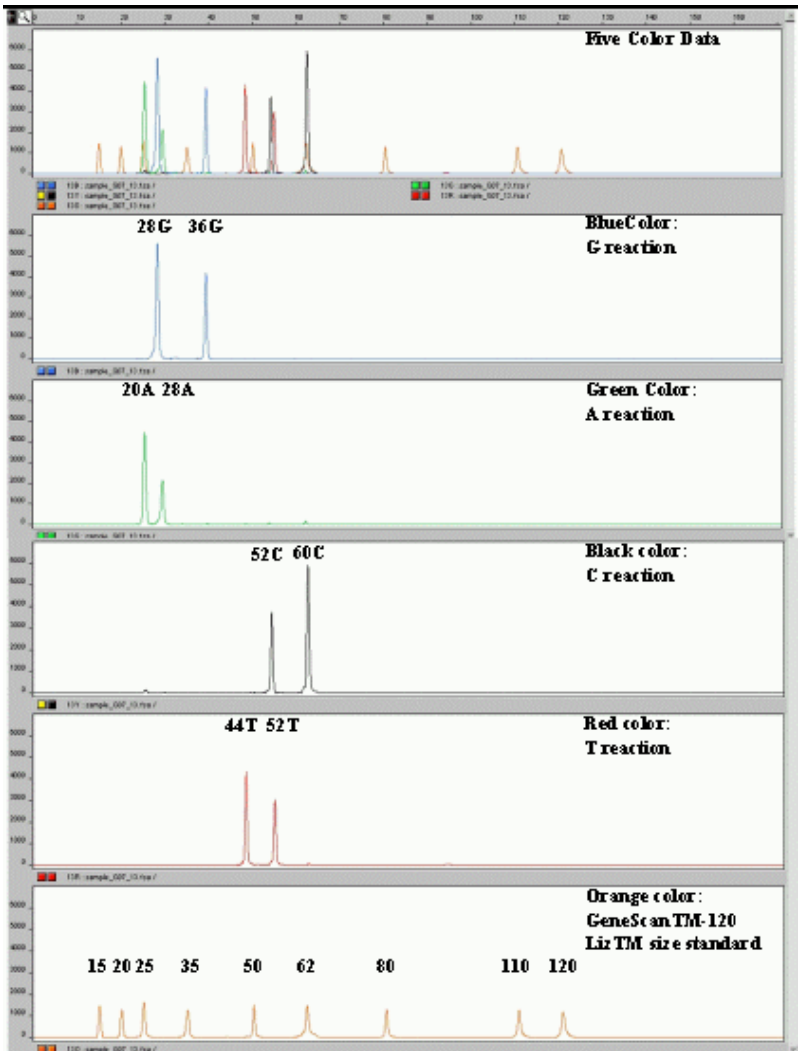


Figure 1 Electropherogram of the Multiplex Control Reaction products along with GeneScan™-120 LIZ™ size standard

Allele Calling

Genotyper 3.7 can be used to analyze this data. Please refer to the *Genotyper User's Manual* for more information. Applied Biosystems is constantly developing new software solutions particularly for SNP analysis. Please check our web site.

Appendix A. SNaPshot Primer Design and Evaluation Recommendations

Follow these recommendations for designing and evaluating primers:

- Primers included in a single reaction need to differ significantly in lengths in order to avoid overlap between the final SNaPshot products. A difference of 4–6 nucleotides between primer lengths is recommended as a starting point.
- The length of a primer can be modified by the addition of nonhomologous polynucleotides at the 5′ end. Since the recommended annealing temperature for a SNaPshot control primer is 50 °C, the melting temperature for the complementary region between any primer and its corresponding template should be at least 50 °C.
- Poly (dT), poly (dA), poly (dC), and poly (dGACT) are 5′ non-homologous tails which are predicted to have minimal secondary structures. They have all been used successfully. Generally the signal patterns are not affected by the kinds of tails that are used. The 5′ poly (dT) tails however may interfere with the addition of 3′ ddA.
- The mobility of an oligonucleotide in capillary electrophoresis is determined by its size, nucleotide composition, and dye. Thus the effect of nucleotide composition on mobility can be significant when the primer is short. We strongly recommend that primers shorter than 36 nucleotides be tested before being multiplexed to ensure that the final products are spatially resolved when analyzed on the instrument.
- Check primers for possible extendable hairpin structures within each primer and for extendable dimer formation between primers.
- HPLC purification of primers is recommended for oligonucleotides longer than 30 nucleotides. Heterogenous primer mixtures containing mixed molecular weight oligonucleotides may yield undesired products that will confuse analysis.
- Since SNP interrogation using primer extension does not permit any flexibility with respect to the location of the 3´ end of the primer, use primers that are complementary to the negative (–) DNA strand if the positive (+) DNA strand is difficult to assay.

- Run a negative control reaction (lacking template DNA) when evaluating a new primer.
- Certain primer/template combinations may require adjusting the annealing temperature or annealing time. Refer to Appendix C on page 32.
- For an illustration of the use of multiplexed primers in a SNP validation application see the following reference:

Lindblad-Toh, K., *et al*. Large -scale discovery and genotyping of single-nucleotide polymorphisms in the mouse. 2000, *Nature Genetics* 24: 381–386.

Appendix B. Converting Nanograms to Picomoles

Procedure To convert nanograms per microliter (A_{260}) to picomoles per microliter:

Step	Action
1	Measure the absorbance of your sample and multiply by a dilution factor. a. Using a spectrophotometer, measure the DNA sample absorbance at 260 nm (A_{260}). It may be necessary to dilute the sample for an accurate measurement. b. Multiply the A_{260} by any dilution factor used. For example, if the A_{260} sample reading is 0.060 and the dilution factor is 10, then: A_{260} = 0.060 x 10 = 0.600
2	Multiply the A_{260} value by 50 µg/mL (50 ng/µL) to obtain nanograms per microliter of double-stranded DNA. For example, if the A_{260}=0.600, then: 0.600 x 50 µg/mL= 30 ng/µL **Note** 1.0 OD =50 µg/mL of double-stranded DNA
3	Determine the molecular weight of the PCR product by multiplying the number of base pairs by 650 daltons/base pair. For example, if the oligo is 120 base pairs in length, then: 120 x 650 Da/bp = 78,000 Da
4	Convert nanograms per microliter to picomoles per microliter by a. dividing the molecular weight into 10^3 b. multiplying by the concentration determined in step 2 For example: (10^3 / 78,000 Da) x 30 ng/µL = 0.38 pmol/µL

Appendix C. Troubleshooting

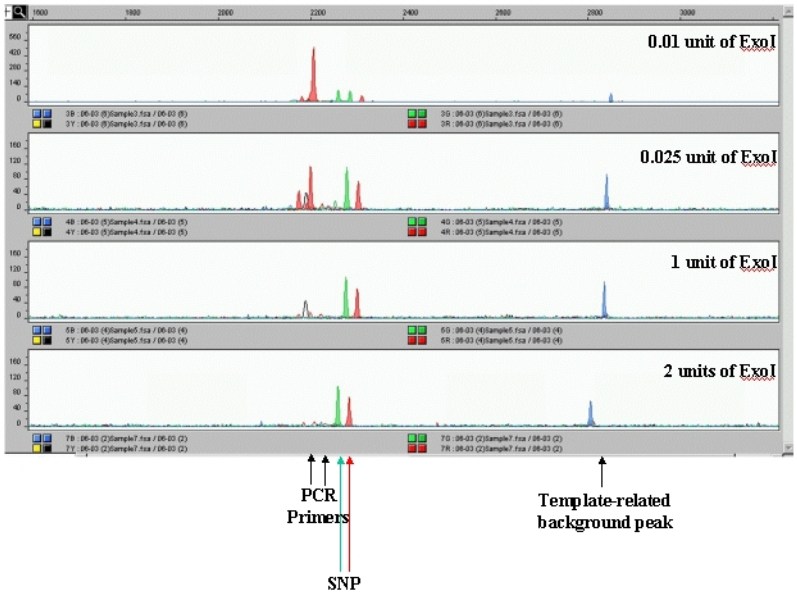
Troubleshooting Low Signal

Here are some possible causes of Low Signal

Observation	Possible Cause(s)	Recommended Action
Low signal	Insufficient concentration of annealed primer, possibly because of low annealing and extension efficiency.	Increase the primer concentration to 1 pmol per reaction. Combined primer concentrations greater then 4 pmol are not recommended as they may cause ddNTP mis-incorporation.
	Suboptimized thermal cycling conditions Primers annealing to templates occur at a much slower rate than that of ddNTP incorporation by Taq DNA Polymerase at the suggested temperature.	If you consistently observe low signals, try optimizing the annealing temperature and/or the annealing time. The annealing temperature may be the same as the extension temperature.
	Insufficient amplification of template DNA	Measure the absorbance of the DNA template at 260 nm to confirm the DNA concentration in the amplification products. Satisfactory results have been obtained using 0.01 pmol of DNA template per reaction. **Note** This is a less likely cause of low signal than insufficient concentration of primers.
	Inappropriate injection time.	Increase the injection time.

Troubleshooting Extraneous Peaks Use the following table to troubleshoot extraneous peaks:

Causes of Extraneous Peaks

Observation	Possible Cause(s)	Recommended Action
Extraneous peaks	Incomplete removal of PCR primers. **Note** PCR primers that have not been removed can participate in the SNaPshot primer extension reaction and resemble signal derived from the SNaPshot interrogation primer.	Since the primer size and sequence is known, look at the data to determine if the peak observed is the expected size (primer + 1 nt) and color of the expected peak.
		Use fresh SAP and *Exo* I, or employ an alternative method of primer removal. **Note** There have been some reports of PCR primers that are refractive to digestion by *Exo* I. See Figure 1.
	 **Figure 2** Electropherogram of SNaPshot products.Increasing the amount of *Exo* I resulted in less peaks from PCR primers.	
	Incomplete removal of [F]ddNTPs by SAP digestion results in comigration of [F]ddNTPs with the fragments of interest.	The undigested [F]ddNTPs normally appear as peaks larger than 70 bp. Excess [F]ddNTPs also result in peaks of smaller sizes. In this case, use fresh CIP or SAP.

Causes of extraneous peaks (*continued*):

Observation	Possible Cause	Recommended Action
Extraneous peaks	PCR-amplified templates. **Note** These products are usually longer than 60 base pairs.	To determine if the peaks are from templates, run a SNaPshot reaction using the templates without SNaPshot primers. Any peaks that appear will be from the PCR amplification of the templates. To decrease the amount of these extraneous peaks, try decreasing the amount of *Exo* I used. If you are using column purification, try a more stringent elution condition to minimize short fragment recovery. Alternatively you can decrease the concentration of templates in the SNaPshot reaction. See Figure 3.

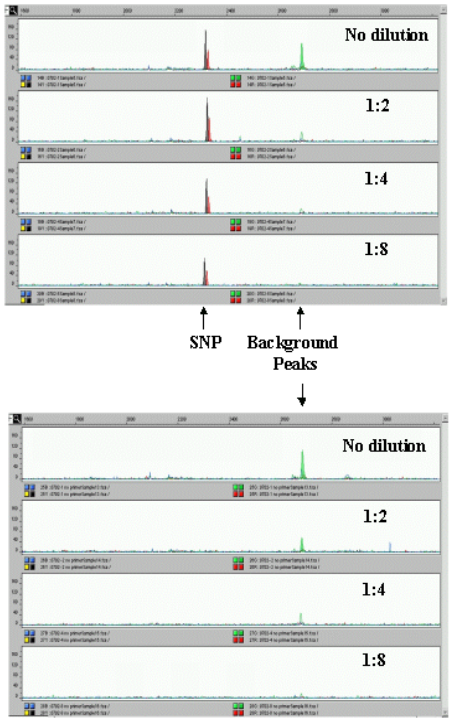
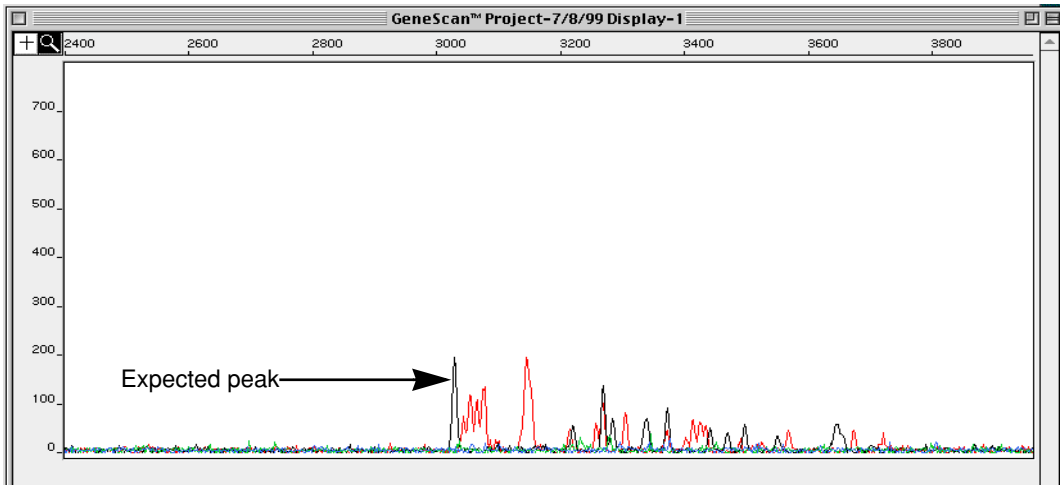


Figure 3 Electropherogram of SNaPshot products. Two microliters of PCR-amplified templates (undiluted, 1:2 diluted, 1:4 diluted, 1:8 diluted) was used in the SNaPshot reaction with a SNaPshot primer (top panel) or without a SNaPshot primer (bottom panel). Background peaks (green peaks) are the same in samples with or without SNaPshot primers and decrease as the templates become more diluted.

Causes of extraneous peaks (*continued*);

Observation	Possible Cause	Recommended Action
Extraneous peaks that resemble a conventional Sanger sequencing reaction. The peak of interest has significantly reduced amplitude.	Incomplete removal of dNTPs from PCR reactions. This enables dNTPs to participate in the ddNTP extension reaction. Refer to Figure 4.	Use fresh SAP. Use an alternate means of PCR reaction purification such as those listed on page 9 under PCR Purification Kits.
 **Figure 4** Electropherogram resulting from the presence of residual dNTPs.		
Extraneous peaks	Primer hairpin extension. Primer dimer extension.	Carefully analyze the primer sequence. ♦ Avoid using primers that are capable of annealing to themselves and leaving a recessed 3´ end. ♦ Use primer analysis software to help identify problems associated with primer design. ♦ Try designing primers using the complementary DNA strand.

Troubleshooting Sizing Problems

Use the following table to troubleshoot sizing problems.

Observation	Possible Cause	Recommended Action
The fragment sizes observed are different from the expected sizes	Incorporation of dye greatly effects the mobility of the extension products. Often shorter fragments will appear to be nearly five bases longer than their actual size.	No action required.
The sizes of identical fragments vary between runs or capillaries.	Size standard improperly called due to low-intensity peaks being called instead of the real peaks.	Reanalyze the samples after increasing the minimum peak height value. Make sure that you change the analysis parameter settings used for your analysis (refer to the *GeneScan User's Manual* for more information).
	Off-scale peaks in the region of a size standard peak is causing that size standard peak to fail to be recognized. Older versions of GeneScan (earlier than GS 3.52) have a size matching algorithm that can cause this problem.	Get the new patch from the Applied Biosystems web site.

Appendix D. Technical Support

Contacting Technical Support

You can contact Applied Biosystems for technical support by telephone or fax, by e-mail, or through the Internet. You can order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents 24 hours a day. In addition, you can download documents in PDF format from the Applied Biosystems Web site (please see the section “To Obtain Documents on Demand” following the telephone information below).

To Contact Technical Support by E-Mail

Contact technical support by e-mail for help in the following product areas:

Product Area	E-mail address
Genetic Analysis (DNA Sequencing)	galab@appliedbiosystems.com
Sequence Detection Systems and PCR	pcrlab@appliedbiosystems.com
Protein Sequencing, Peptide and DNA Synthesis	corelab@appliedbiosystems.com
Biochromatography, PerSeptive DNA, PNA and Peptide Synthesis systems, CytoFluor®, FMAT™, Voyager™, and Mariner™ Mass Spectrometers	tsupport@appliedbiosystems.com
Applied Biosystems/MDS Sciex	api3-support@sciex.com
Chemiluminescence (Tropix)	tropix@appliedbiosystems.com

Hours for Telephone Technical Support

In the United States and Canada, technical support is available at the following times:

Product	Hours
Chemiluminescence	8:30 a.m. to 5:30 p.m. Eastern Time
Framingham support	8:00 a.m. to 6:00 p.m. Eastern Time
All Other Products	5:30 a.m. to 5:00 p.m. Pacific Time

To Contact Technical Support by Telephone or Fax

In North America

To contact Applied Biosystems Technical Support, use the telephone or fax numbers given below. (To open a service call for other support needs, or in case of an emergency, dial **1-800-831-6844** and press **1**.)

Product or Product Area	Telephone Dial...	Fax Dial...
ABI PRISM® 3700 DNA Analyzer	**1-800-831-6844,** then press **8**	**1-650-638-5981**
DNA Synthesis	**1-800-831-6844**, then press **21**	**1-650-638-5981**
Fluorescent DNA Sequencing	**1-800-831-6844**, then press **22**	**1-650-638-5981**
Fluorescent Fragment Analysis (includes GeneScan® applications)	**1-800-831-6844**, then press **23**	**1-650-638-5981**
Integrated Thermal Cyclers (ABI PRISM®877 and Catalyst 800 instruments)	**1-800-831-6844**, then press **24**	**1-650-638-5981**
ABI PRISM® 3100 Genetic Analyzer	**1-800-831-6844**, then press **26**	**1-650-638-5981**
BioInformatics (includes BioLIMS®, BioMerge®, and SQL GT™ applications)	**1-800-831-6844**, then press **25**	**1-505-982-7690**
Peptide Synthesis (433 and 43X Systems)	**1-800-831-6844**, then press **31**	**1-650-638-5981**
Protein Sequencing (Procise® Protein Sequencing Systems)	**1-800-831-6844**, then press **32**	**1-650-638-5981**
PCR and Sequence Detection	**1-800-762-4001**, then press **1** for PCR, **2** for the 7700 or 5700, **6** for the 6700 or dial **1-800-831-6844**, then press **5**	**1-240-453-4613**

Product or Product Area	Telephone Dial...	Fax Dial...
Voyager™ MALDI-TOF Biospectrometry and Mariner™ ESI-TOF Mass Spectrometry Workstations	**1-800-899-5858**, then press **13**	**1-508-383-7855**
Biochromatography (BioCAD® Workstations and Poros® Perfusion Chromatography Products)	**1-800-899-5858**, then press **14**	**1-508-383-7855**
Expedite™ Nucleic acid Synthesis Systems	**1-800-899-5858**, then press **15**	**1-508-383-7855**
Peptide Synthesis (Pioneer™ and 9050 Plus Peptide Synthesizers)	**1-800-899-5858**, then press **15**	**1-508-383-7855**
PNA Custom and Synthesis	**1-800-899-5858,** then press **15**	**1-508-383-7855**
FMAT™ 8100 HTS System and CytoFluor® 4000 Fluorescence Plate Reader	**1-800-899-5858**, then press **16**	**1-508-383-7855**
Chemiluminescence (Tropix)	**1-800-542-2369** (U.S. only), or **1-781-271-0045**	**1-781-275-8581**
Applied Biosystems/MDS Sciex	**1-800-952-4716**	**1-650-638-6223**

Outside North America

Region	Telephone Dial...	Fax Dial...
Africa and the Middle East		
Africa (English Speaking) and West Asia (Fairlands, South Africa)	**27 11 478 0411**	**27 11 478 0349**
South Africa (Johannesburg)	**27 11 478 0411**	**27 11 478 0349**
Middle Eastern Countries and North Africa (Monza, Italia)	**39 (0)39 8389 481**	**39 (0)39 8389 493**

Region	Telephone Dial...	Fax Dial...
Eastern Asia, China, Oceania		
Australia (Scoresby, Victoria)	**61 3 9730 8600**	**61 3 9730 8799**
China (Beijing)	**86 10 64106608**	**86 10 64106617**
Hong Kong	**852 2756 6928**	**852 2756 6968**
Korea (Seoul)	**82 2 593 6470/6471**	**82 2 593 6472**
Malaysia (Petaling Jaya)	**60 3 758 8268**	**60 3 754 9043**
Singapore	**65 896 2168**	**65 896 2147**
Taiwan (Taipei Hsien)	**886 2 2358 2838**	**886 2 2358 2839**
Thailand (Bangkok)	**66 2 719 6405**	**66 2 319 9788**
Europe		
Austria (Wien)	**43 (0)1 867 35 75 0**	**43 (0)1 867 35 75 11**
Belgium	**32 (0)2 712 5555**	**32 (0)2 712 5516**
Czech Republic and Slovakia (Praha)	**420 2 61 222 164**	**420 2 61 222 168**
Denmark (Naerum)	**45 45 58 60 00**	**45 45 58 60 01**
Finland (Espoo)	**358 (0)9 251 24 250**	**358 (0)9 251 24 243**
France (Paris)	**33 (0)1 69 59 85 85**	**33 (0)1 69 59 85 00**
Germany (Weiterstadt)	**49 (0) 6150 101 0**	**49 (0) 6150 101 101**
Hungary (Budapest)	**36 (0)1 270 8398**	**36 (0)1 270 8288**
Italy (Milano)	**39 (0)39 83891**	**39 (0)39 838 9492**
Norway (Oslo)	**47 23 12 06 05**	**47 23 12 05 75**
Poland, Lithuania, Latvia, and Estonia (Warszawa)	**48 (22) 866 40 10**	**48 (22) 866 40 20**
Portugal (Lisboa)	**351 (0)22 605 33 14**	**351 (0)22 605 33 15**
Russia (Moskva)	**7 095 935 8888**	**7 095 564 8787**
South East Europe (Zagreb, Croatia)	**385 1 34 91 927**	**385 1 34 91 840**
Spain (Tres Cantos)	**34 (0)91 806 1210**	**34 (0)91 806 1206**
Sweden (Stockholm)	**46 (0)8 619 4400**	**46 (0)8 619 4401**
Switzerland (Rotkreuz)	**41 (0)41 799 7777**	**41 (0)41 790 0676**
The Netherlands (Nieuwerkerk a/d IJssel)	**31 (0)180 331400**	**31 (0)180 331409**

Region	Telephone Dial...	Fax Dial...
United Kingdom (Warrington, Cheshire)	**44 (0)1925 825650**	**44 (0)1925 282502**
All other countries not listed (Warrington, UK)	**44 (0)1925 282481**	**44 (0)1925 282509**
Japan		
Japan (Hacchobori, Chuo-Ku, Tokyo)	**81 3 5566 6006**	**81 3 5566 6505**
Latin America		
Del.A. Obregon, Mexico	**305-670-4350**	**305-670-4349**

To Reach Technical Support Through the Internet

We strongly encourage you to visit our Web site for answers to frequently asked questions and for more information about our products. You can also order technical documents or an index of available documents and have them faxed or e-mailed to you through our site. The Applied Biosystems Web site address is

http://www.appliedbiosystems.com/techsupp

To submit technical questions from North America or Europe:

Step	Action
1	Access the Applied Biosystems Technical Support Web site.
2	Under the **Troubleshooting** heading, click **Support Request Forms**, then select the relevant support region for the product area of interest.
3	Enter the requested information and your question in the displayed form, then click **Ask Us RIGHT NOW** (blue button with yellow text).
4	Enter the required information in the next form (if you have not already done so), then click **Ask Us RIGHT NOW**. You will receive an e-mail reply to your question from one of our technical experts within 24 to 48 hours.

To Obtain Documents on Demand

Free, 24-hour access to Applied Biosystems technical documents, including MSDSs, is available by fax or e-mail or by download from our Web site.

To order documents...	Then...
by index number	a. Access the Applied Biosystems Technical Support Web site at **http://www.appliedbiosystems.com/techsupp** b. Click the **Index** link for the document type you want, then find the document you want and record the index number. c. Use the index number when requesting documents following the procedures below.
by phone for fax delivery	a. From the U.S. or Canada, call **1-800-487-6809,** or from outside the U.S. and Canada, call **1-858-712-0317**. b. Follow the voice instructions to order the documents you want. **Note** There is a limit of five documents per request.
through the Internet for fax or e-mail delivery	a. Access the Applied Biosystems Technical Support Web site at **http://www.appliedbiosystems.com/techsupp** b. Under **Resource Libraries**, click the type of document you want. c. Enter or select the requested information in the displayed form, then click **Search**. d. In the displayed search results, select a check box for the method of delivery for each document that matches your criteria, then click **Deliver Selected Documents Now** (or click the PDF icon for the document to download it immediately). e. Fill in the information form (if you have not previously done so), then click **Deliver Selected Documents Now** to submit your order. **Note** There is a limit of five documents per request for fax deliver but no limit on the number of documents you can order for e-mail delivery.

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