GeneScan® Reference Guide

Chemistry Reference for the ABI PRISM® 310 Genetic Analyzer

Applied Biosystems

For Research Use Only. Not for use in diagnostic procedures.

P/N 4303189B

Contents

1 Introduction 1-1

Using This Manual 1-1
Technical Support 1-3

2 Experimental Design Considerations 2-1

Using GeneScan Analysis Software to Analyze DNA Fragments 2-1
Working with Multiple Colors 2-2
Choosing Fluorescent Labeling Methods 2-5
Determining Loading Concentrations for Samples 2-8
Optimizing Electrokinetic Injection Parameters 2-9
Optimizing Electrophoresis Conditions 2-13
Using Control DNA 2-16

3 General Analysis and Evaluation Techniques 3-1

Overview 3-1
Analyzing the Data 3-2
Evaluating Data Quality 3-8
Quantitating Nucleic Acids 3-10
Evaluating Matrix Quality 3-11

4 ABI PRISM Dyes 4-1

Overview 4-1
Available Dyes 4-2
Understanding Dye Spectra 4-3
Choosing Dye/Filter Set Combinations 4-5
Emission Spectra for Representative Dye/Virtual Filter Set Combinations 4-8

5 Sizing and Size Standards 5-1

Overview 5-1
Introduction to Sizing 5-2
Preventing/Troubleshooting Sizing Problems 5-5
GeneScan Internal Lane Size Standards 5-7
GeneScan-350 Size Standard 5-8
GeneScan-400HD Size Standard 5-10
GeneScan-500 Size Standard 5-12

GeneScan-1000 Size Standard. 5-14
GeneScan-2500 Size Standard. 5-16

6 Optimizing PCR 6-1

Overview 6-1
Choosing Reaction Volumes and Tube Types 6-2
Designing Custom Primers 6-3
Determining Reagent Concentrations 6-6
Choosing the Right Enzyme 6-8
Multiplexing PCR 6-10
Using RNA Templates 6-12
Preventing Competing Side Reactions—Hot Start PCR 6-13
Modifying Thermal Cycling Parameters 6-15
Avoiding Contamination 6-16
3´ A Nucleotide Addition. 6-18
Stutter Products 6-21
Preparing PCR Products for Analysis 6-23

7 SSCP Analysis 7-1

Overview 7-1
Introduction to SSCP Analysis 7-2
Before You Begin. 7-3
PCR Amplification, Labeling, and Controls 7-4
To Save Time—Prerun Checklist. 7-7
Preparing for a Run 7-8
Analyzing the Data 7-12
Optimizing SSCP Run Conditions. 7-14
Troubleshooting 7-17

8 Microsatellite Analysis 8-1

Overview 8-1
Introduction to Microsatellite Analysis 8-2
Before You Begin. 8-3
PCR Amplification, Labeling, and Controls for Microsatellite Analysis 8-4
To Save Time—Prerun Checklist. 8-6
Preparing for a Run 8-7
Analyzing the Data, Part I—Using GeneScan. 8-10
Analyzing the Data, Part II—Allele Binning Using Genotyper 2.0 8-12
Troubleshooting Microsatellite Analysis 8-25

9 Microsatellite Analysis Applications 9-1

Overview 9-1
Microsatellite Analysis Using the LMS V2 9-2
Troubleshooting the LMS V2 9-4
LOH Screening 9-5
Performing LOH Screening 9-7
RER Screening 9-13
Troubleshooting LOH and RER Screening 9-16
Animal Paternity 9-17
Human Identification 9-19

10 AFLP Mapping 10-1

Introduction 10-1
The AFLP Technique 10-5

11 Troubleshooting 11-1

Troubleshooting PCR Amplification 11-1
Troubleshooting PCR Product Detection 11-7

A Reagent Preparation A-1

B Creating Matrix Files B-1

Creating the GeneScan Matrix File B-1

C Preparing 5´-End Labeled Primers C-1

Overview C-1
Introduction to 5´-end Labeling C-2
Instrument Setup C-4
Synthesis and Purification of 5´-end Labeled Primers C-6
Calculating Absorbance for DNA Samples C-10

D References D-1

E Part Numbers E-1

ABI PRISM DNA Fragment Analysis Kits and Reagents E-1

ABI PRISM 310 Genetic Analyzer E-5

Reference Materials E-7

Updates E-8

Index

1 Introduction

Using This Manual

How This Manual Is Organized

This manual contains four major topic divisions:

- General knowledge (Chapter 2 through Chapter 6):
  - Designing experiments
  - Analyzing and evaluating data
  - Fluorescent dyes and recommended dye sets
  - Choosing size standards and size-calling methods and troubleshooting sizing problems
  - Optimizing PCR
- Applications (Chapter 7 through Chapter 10):
  - SSCP analysis protocols and optimization suggestions
  - Generic microsatellite analysis protocols and optimization suggestions
  - Specialized applications of microsatellite analysis
  - Information about AFLP™ microbial fingerprinting and plant mapping applications
- Troubleshooting (Chapter 11):
  - PCR amplification
  - PCR product detection
- Appendices (A through E):
  - Reagent preparation
  - Preparing 5´-end labeled primers
  - Creating matrix files
  - Literature references
  - Part numbers

continued on next page

Conventions Used in This Manual

The following words and styles draw your attention to specific details of the information presented in this manual:

Note A note calls attention to useful and/or interesting information.

IMPORTANT This information is emphasized because it is critical to the success of your experiments.

! WARNING ! Indicates that physical injury to yourself or others could result if you do not follow the recommended precautions.

Safety Information

For information on the safe operation of the ABI PRISM® 310 Genetic Analyzer, refer to the ABI PRISM 310 *Genetic Analyzer Site Preparation and Safety Guide* (P/N 903558).

Updates

Visit our World Wide Web site (www.appliedbiosystems.com/techsupport) for updates to this manual.

Other Manuals

This manual is designed to be used in conjunction with the following manuals:

- ABI PRISM 310 *Genetic Analyzer User's Manual* (P/N 903565)
- *GeneScan® Analysis Software User's Manual* (P/N 904435)

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Experimental Design Considerations 2

Using GeneScan Analysis Software to Analyze DNA Fragments

Introduction

The GeneScan® Analysis Software analyzes the data collected by the ABI PRISM 310 Genetic Analyzer to size and quantitate DNA fragments. GeneScan analysis of sample files includes establishing a baseline, adjusting for spectral overlap of the dyes, peak detection, and size calling.

The GeneScan Analysis Software sizes and quantitates DNA fragments automatically, allowing faster and more accurate analysis than traditional methods such as radiolabeling. Depending on your run conditions, you can achieve resolution sufficient to differentiate between fragments that have apparent sizes up to 5000 base pairs.

When you use the GeneScan system, you can label different DNA fragments with up to three different color fluorescent dyes. A fourth color is reserved for the GeneScan Internal Lane Size Standard. The size standard is used for precise size calling without the problems often encountered using other techniques, such as band-shift artifacts and run-to-run variation.

You can display the results of an experiment as electropherograms, as tabular data, or as both. Electropherograms show fluorescence intensity as a function of fragment size or migration time. Each electropherogram represents a single injection. The tabular data provides precise sizing and quantitative information. The data can be exported to downstream applications, such as Genotyper® software.

In This Chapter

This chapter summarizes the basic design factors that you should consider before beginning any GeneScan fragment analysis experiment.

This chapter contains the following topics:

Topic	See Page
Working with Multiple Colors	2-2
Choosing Fluorescent Labeling Methods	2-5
Determining Loading Concentrations for Samples	2-8
Optimizing Electrokinetic Injection Parameters	2-9
Optimizing Electrophoresis Conditions	2-13
Using Control DNA	2-16

Working with Multiple Colors

Introduction

Fluorescent labeling enables you to analyze many independent loci in the same capillary injection, using color in addition to size to distinguish between fragments. To take advantage of this, you need to consider more factors than you would with traditional techniques.

Guidelines

For Performing Any Experiment

You should always:

- Use the same GeneScan Internal Lane Size Standard labeled with the same dye for all samples in a single study.
- Compare peak areas, heights, and sizes in nucleotide bases only for fragments that are labeled with the same dye.

  For example, compare FAM-labeled fragments only to other FAM-labeled fragments.

Note It is possible to compare samples labeled with different dyes when comparing relative sizes and peak height and area ratios.

For Working with Similarly Sized DNA Fragments

If the sizes of different fragments overlap, then you can do one of the following to differentiate between them:

- Label overlapping products with different dye colors.
- Choose new primer sites to alter the PCR-product fragment lengths.
- Load overlapping products during different capillary injections.

Multiplexing to Increase Throughput

To exploit the potential for increased throughput using ABI PRISM™ multicolor fluorescent dye technology, you will want to multiplex electrophoresis by co-loading the products of multiple PCR reactions in the same capillary injection. Depending upon your application you may also want to multiplex the PCR.

Co-electrophoresis

Before performing PCR for co-electrophoresis, be sure that you follow these guidelines:

- Use different dye labels for PCR reactions with overlapping product sizes.
- Use a combination of dyes that display in different colors and can be detected by the same virtual filter set.

  Table 4-3 on page 4-6 lists recommended filter/dye set combinations.
- Use greater dye concentrations in PCR reactions with dyes of low emission intensity than for dyes of high emission intensity. (See “Accounting for Differences in Dye Signal Strengths” later in this section.)

After pooling PCR products, you may need to perform a desalting step prior to loading samples. Pooling products from multiple PCR reactions often increases the salt concentration in the loaded samples. (See “Decreasing the Salt Concentration” on page 2-3.)

Multiplexing PCR

You can multiplex the PCR by combining more than one pair of primers in the same PCR reaction tube. Do not multiplex primers with similar product lengths labeled with similar dyes.

Note For microsatellite applications, do not multiplex primers labeled with the same fluorescent dye for loci with overlapping allele size ranges. If you do not know the full extent of the allele size ranges leave at least 15–20 base pairs between the known size ranges.

Before performing the PCR, perform a preliminary check for primer compatibility. Avoid excessive regions of complementarity among the primers. Also, choose primer pairs with similar melting temperatures. For more information on primer-pair compatibility, see "Designing Custom Primers" on page 6-3.

After identifying compatible primer pairs, test the pairs for successful co-amplification. You will often need to optimize reaction conditions and, occasionally, you will need to redesign the primers. For more information on optimizing reaction conditions, see "Multiplexing PCR" on page 6-10.

Ensuring Adequate Signal Intensity

Accounting for Differences in Dye Signal Strengths

The intensity of emitted fluorescence is different for each dye. For example, to generate signals of equal intensity you need to load approximately eight times as much ROX as you do 6-FAM or TET.

The following lists ABI PRISM dyes in order of increasing emission intensity when excited by the dual-mode argon laser:

- ROX (lowest signal strength)
- TAMRA
- HEX
- JOE, NED
- 5-FAM, 6-FAM, TET (highest signal strength)

See Chapter 4 for more information about ABI PRISM dyes, dye spectra, and the argon ion laser.

Decreasing the Salt Concentration

Salt anions compete with negatively charged DNA for entry into the capillary during electrokinetic injection. As the salt concentration increases, less DNA enters the capillary, weakening the signal. Excess salt can also precipitate the DNA in the sample tube in the presence of formamide.

You might be able to compensate for the decreased signal intensity by increasing the sample injection time and/or injection voltage. If this does not work you will need to desalt and concentrate the samples.

To desalt:

- Use an Amicon Centricon-100 (or Centricon-30 for fragments smaller than 130 base pairs in length) Microconcentrator.
- Precipitate the pooled PCR product and resuspend in distilled, deionized water.

♦ Dialyze the sample on a filter membrane to remove salt from the solution:

Step	Action
1	Float a Millipore VS filter (Millipore P/N VSWP 02500), shiny side up, on top of 50 mL of deionized, autoclaved water in a 50-mL conical plastic tube.
2	Carefully spot approximately 15 µL of sample on top of the filter, using an appropriate pipette. Dialyze the sample for 20 minutes.
3	Using a pipette, very carefully remove the sample and dilute.

Note Do not increase sample concentration by evaporating the samples without performing a desalting step. This increases the salt concentration, making it more difficult to denature the DNA and decreasing the signal strength.

Adjusting the Signal Intensity

For more information on adjusting the signal intensity see “Determining Loading Concentrations for Samples” on page 2-8 and “Optimizing Electrokinetic Injection Parameters” on page 2-9.

Choosing Fluorescent Labeling Methods

Recommended Methods

Most GeneScan applications analyze PCR products. You can use either of two fluorescent labeling methods during PCR amplification:

- Incorporating a 5´-end labeled primer during the primer-annealing step
- Incorporating fluorescent dye-labeled dUTPs or dCTPs ([F]dNTPs) during the primer-extension step

IMPORTANT With any labeling technique you should use only ABI PRISM dyes. Other dyes, or mixed isomers of dyes, have variable spectral shifts that will interfere with making a multicomponent matrix. (The matrix compensates for the spectral overlap between the dyes.)

Comparing Labeling Methods

Advantages of Using 5´-end Labeled Primers

- High precision

  All fluorophores affect DNA mobility to some degree. Different fluorophores have different mobilities. Because fragments labeled with 5´-end labeled primers have a single fluorophore at the 5´ end, the mobility of all detectable fragments is comparably affected. Therefore, fragment peaks tend to be narrow.

  By contrast, fragments labeled with [F]dNTPs have a variable number of fluorophores attached in variable positions on both strands. Thus, peaks tend to be wider with [F]dNTPs, and can appear to be split.

- Consistent quantitation

  Every fragment in a peak contributes a single fluorophore to the total signal. Thus peak area is directly proportional to the number of molecules. A population of fragments labeled with [F]dNTPs has a variable number of attached fluorophores. (The average number of attached fluorophores depends upon the fragment's base composition and length and upon the ratio of [F]dNTPs to dNTPs added to the reaction mixture.) Thus it is inadvisable to compare peak areas between fragments labeled with [F]dNTPs.

- Distinguishing between the forward and reverse strands

  By attaching a different fluorophore to the forward and reverse primers, you can distinguish the peaks corresponding to the forward strand, the reverse strand, and residual double-stranded product.

Advantages of Using [F]dNTPs

- High sensitivity

  Because most fragments contain multiple fluorophores, a given number of [F]dNTP-labeled fragments will produce a greater signal than the same number of 5´-end labeled fragments. The increased signal strength allows you to use smaller reaction volumes and fewer amplification cycles during PCR.

- Low cost

  You can add [F]dNTPs to any PCR reaction. You do not need to order or synthesize fluorescently-labeled primers before each PCR and you can use [F]dNTPs with your existing primer sets.

- Post-PCR end-labeling

  Post-PCR end-labeling with [F]dNTPs using Klenow is an alternative to labeling during PCR. See Iwahana *et al.* (1995) and Inazuka *et al.* (1996) for details.

  You can also label with [F]dNTPs using traditional techniques such as random priming or nick translation.

Figure 2-1 and Figure 2-2 compare the results obtained using 5´-end labeled primers and [F]dNTPs. 5´-end labeled primers give better resolution, but [F]dNTPs give better sensitivity. Note also the unincorporated fluorescently-labeled nucleotides in the [F]dNTP-labeled sample (bottom panel of Figure 2-1).

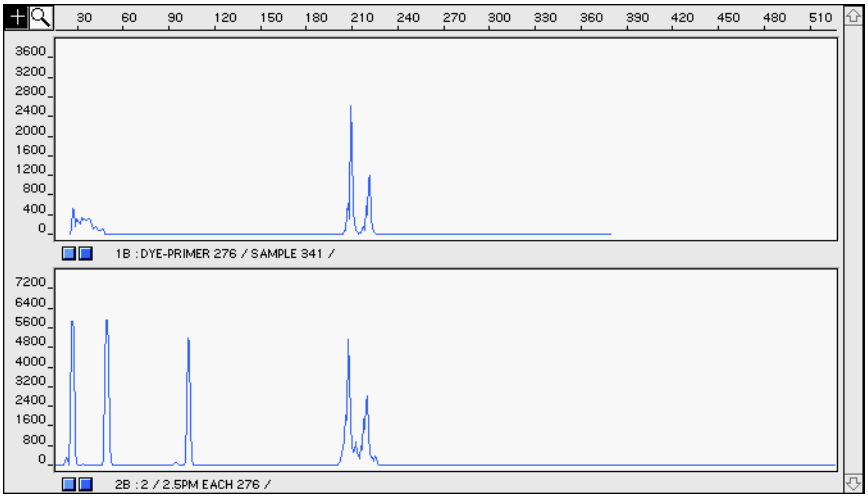


Figure 2-1 Results from 5´-end labeling (top) and [F]dNTPs (bottom)

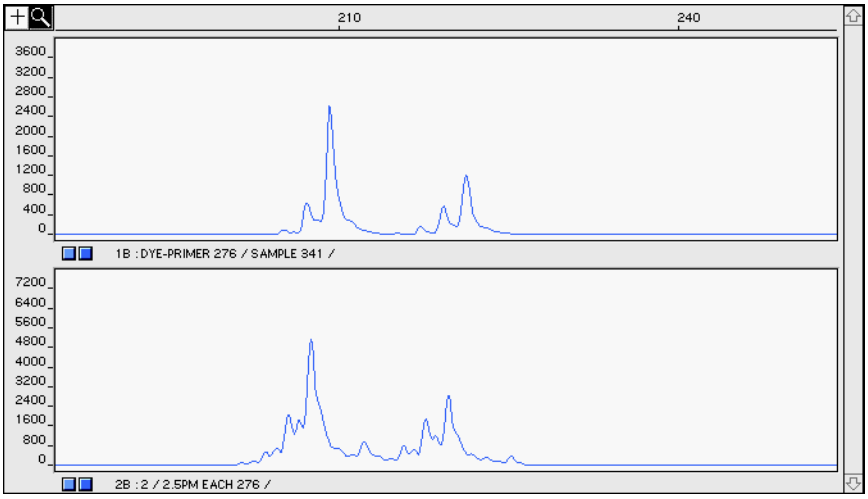


Figure 2-2 Expanded view of the electropherogram from Figure 2-1, showing the differences in resolution and peak height between the two labeling methods

continued on next page

For More Information

You can obtain custom 5´-end labeled primers from the Applied Biosystems Custom Oligonucleotide Synthesis Service either by phone (800-345-5224), by e-mail (support@appliedbiosystems.com), or online (www.appliedbiosystems.com/techsupport).

For information on synthesizing 5´-end labeled primers, see Appendix C.

For information on labeling with [F]dNTPs, consult the *[F]dNTP Reagents Protocol* (P/N 402774).

Determining Loading Concentrations for Samples

Why Problems Arise

Using too little or too much sample can cause problems. Your ABI PRISM® instrument can convert a limited range of fluorescent signal into digital values. For optimal results, you should keep the fluorescent signal between approximately 150 and 4000 relative fluorescent units (RFU).

Too Little Signal

Below this range, the signal-to-noise ratio is too low to discriminate between sample peaks and background fluctuations.

Too Much Signal—The Most Common Problem

Above this range, the instrument cannot measure the true value of the signal and consequently cannot compensate for the spectral overlap among the dyes. As a result, artifact peaks (called “bleedthrough” or “pull-up” peaks, see page 3-11) can appear in other colors. Artifact peaks can corrupt both automated size-calling (extra peaks in the size standard color) and the analysis of co-loaded samples.

How to Proceed

- Typically, dilute 1 µL of each PCR product and 0.5 µL of the GeneScan Internal Lane Size Standard (see Chapter 5), in 12 µL of distilled, deionized water (for non-denaturing applications) or deionized formamide (for denaturing applications).

! WARNING ! CHEMICAL HAZARD. Formamide is a known teratogen. It can cause birth defects. Wash thoroughly after handling formamide. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves. Wash thoroughly after handling formamide.

- If you anticipate an extremely high sample concentration, run dilutions of the sample as a precautionary measure. If the signal is too strong, you can further dilute the sample or you can decrease the sample injection time and/or injection voltage.
- If the signal is too weak, first try increasing the signal by increasing the sample injection time or voltage.

Note To optimize the signal intensity for a given sample, inject the same sample multiple times using a range of injection parameters.

- If the signal intensity is still too weak or the resolution is poor, concentrate the sample (see “Decreasing the Salt Concentration” on page 2-3).
- If the signal intensity is too low after concentration, see “Problems with Signal Strength and Quality” on page 11-10 or “Problems with Poor Amplification” on page 11-1.

Optimizing Electrokinetic Injection Parameters

Introduction

Optimizing electrokinetic injection parameters can greatly improve data quality, run-to-run precision in sizing, and reproducibility in the amount of sample loaded. The goal is to inject sufficient DNA to yield peaks of adequate height (that is, data with a good signal-to-noise ratio) while maintaining the resolution and precision required by the application.

The ABI PRISM® 310 run modules have preset values for injection times and voltages. These values are adequate for many applications. However, you should consider modifying the injection parameters if the signal is too strong or too weak or if the resolution is poor. (The maximum recommended injection time is 30 seconds and the maximum possible injection voltage is 15 kV.)

When selecting values for injection parameters, consider the following:

- The range of fragment lengths
- The resolution required

Definition of Resolution

The resolution, R_s, of two peaks in an electropherogram is defined as follows:

$$R_s = \frac{|P_1 - P_2|}{0.5 \times (W_1 + W_2)}$$

where the P_i are the peak positions measured below the peak apex and the W_i are the peak widths measured at half peak maximum.

Modifying Injection Time

When you modify the injection time, you will encounter a tradeoff between increasing signal strength and increasing resolution.

Effects on Signal Intensity

For the range of parameter values and sample concentrations used in most experiments, the signal strength (as measured both by peak height and by peak area) increases linearly with increasing injection time.

However, as shown in Figure 2-3 and Figure 2-4 on page 2-10, it is not true that an n-fold increase in injection time results in an n-fold increase in peak height. No improvement is seen after 10 seconds for the larger fragment. The signal decreases dramatically after 40 seconds for the smaller fragment. As the injection time increases, the resolution decreases (Figure 2-5 on page 2-11), leading to increasing peak widths and decreasing peak heights.

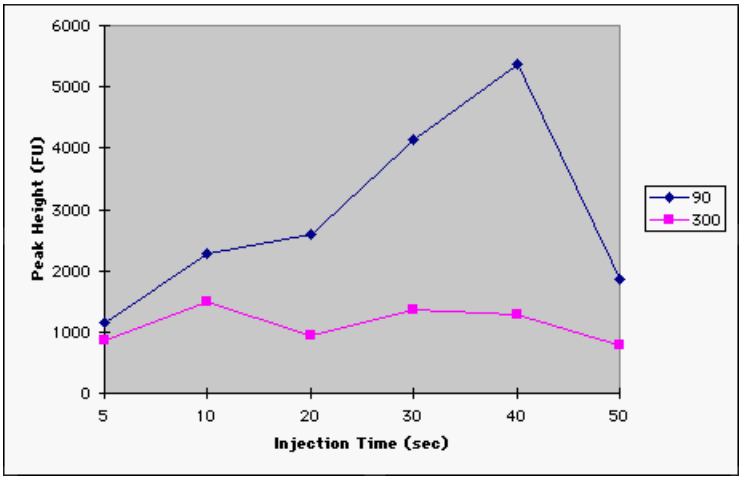


Figure 2-3 Peak height vs. injection time for two different-sized fragments (90 bp and 300 bp)

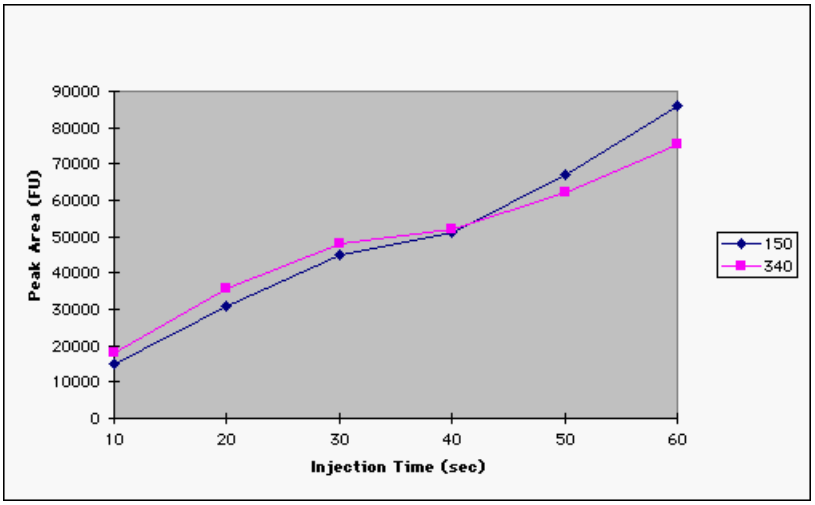


Figure 2-4 Peak area vs. injection time for two different-sized fragments (150 bp and 340 bp)

Effects on Resolution

Increasing the injection time decreases the resolution. As shown in Figure 2-5 on page 2-11, the deleterious effect on resolution is more pronounced for larger fragments.

The decrease in resolution results from an increase in peak width (as opposed to a decrease in peak separation).

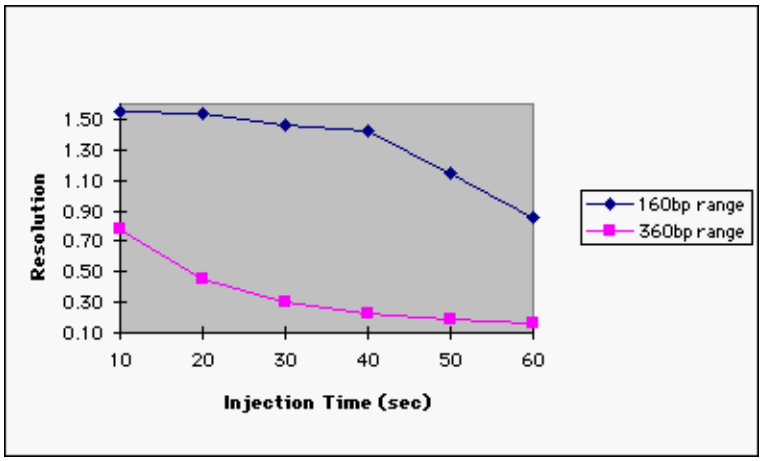


Figure 2-5 Resolution vs. injection time for different-sized fragments

Modifying Injection Voltage

No trade-off between increasing signal strength and increasing resolution exists when modifying injection voltage. Resolution with injection voltages of 319 V/cm (the highest possible setting) is often indistinguishable from resolution with injection voltages of 53 V/cm. However, lower voltages, which produce lower currents, are often preferable because injection timing is more accurate. Accurate timing ensures reproducibility in sample loading.

Effects on Signal Intensity

Peak height and peak area increase linearly with increasing injection voltage. Figure 2-6 below and Figure 2-7 on page 2-12 show the effect of increasing the injection voltage from 53 V/cm to 319 V/cm on peak height and peak area, respectively, for two different-sized fragments.

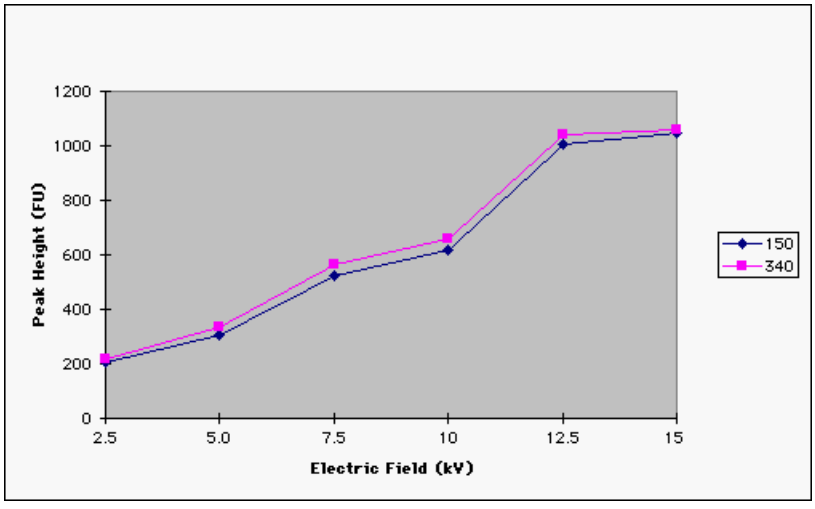


Figure 2-6 Peak height vs. injection voltage for two different-sized fragments (150 bp and 340 bp

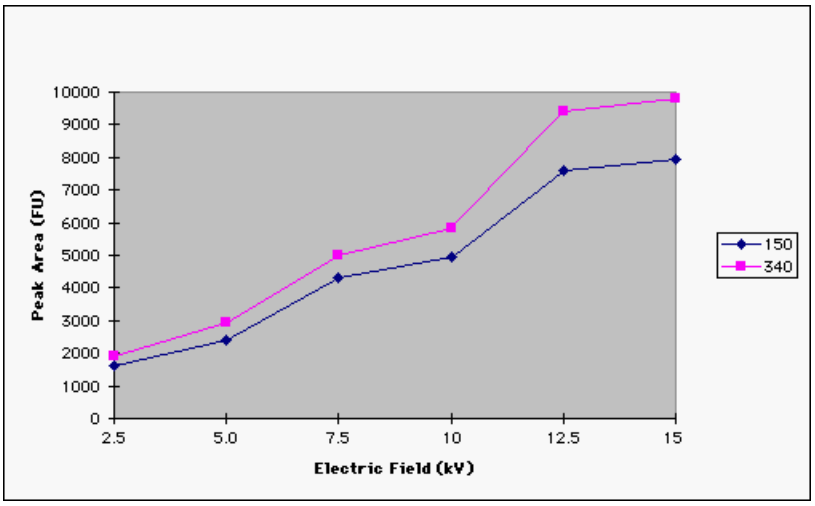


Figure 2-7 Peak area vs. injection voltage for two different-sized fragments (150 bp and 340 bp

Effects on Resolution

Injection voltage has little effect on peak resolution (Figure 2-8).

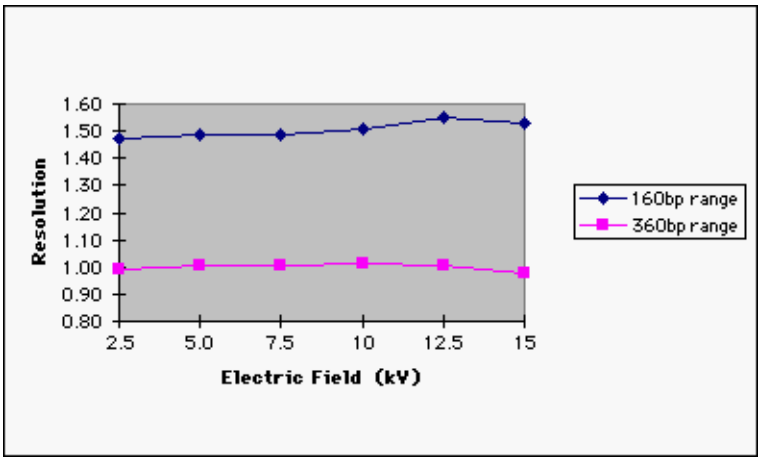


Figure 2-8 Resolution vs. injection voltage for different-sized fragments

Figure 2-5 on page 2-11 and Figure 2-8 above show calculated R_s values over increasing injection time and electric field. The plots measure single nucleotide intervals in the 160-bp and 360-bp ranges. An R_s value of 1 corresponds to fragments that can be discriminated by one nucleotide.

If Results Are Poor

If after adjusting the electrokinetic time and voltage, the signal is still too weak or the resolution is poor, you may need to concentrate or desalt the sample (see page 2-3).

Setting Electrokinetic Injection Values

For information on setting electrokinetic injection values, see the ABI PRISM 310 *Genetic Analyzer User's Manual*.

Optimizing Electrophoresis Conditions

Introduction

Optimizing electrophoresis conditions (run time, run voltage, and run temperature) can greatly improve data quality, run-to-run precision, and/or throughput. When selecting values for these parameters, consider the following factors:

- Range of fragment lengths
- Required degree of resolution
- Type of genetic analysis you will be performing

  For example, does the application require native or denaturing conditions?

The preset electrophoresis parameters in the application modules are set to ensure the following:

- Detection of all fragments in the typical size range permitted by the application

  For example, microsatellite loci are rarely over 400 base pairs in length.
- Acceptable run times
- Acceptable resolution

Modifying Run Time

Determining Required Run Time

To determine the minimal acceptable run time for a given run voltage, you will need to perform a trial run. To ensure that you collect sufficient data to perform analysis, set the electrophoresis run time approximately 10% higher than the migration time of the largest fragment of interest.

Note The “largest fragment of interest” will most probably be a size-standard peak that is needed for sizing the largest sample fragments of interest. The set of size-standard peaks that GeneScan uses to generate the sizing curve can vary with the size-calling method. In general, be sure to include the two size-standard peaks immediately larger than the largest sample fragment of interest.

Decreasing Run Time

For faster run times, you can increase the electrophoresis voltage, but this can decrease the resolution.

continued on next page

Modifying Run Voltage

Effects on Fragment Migration Rates

Figure 2-9 shows the effect of electrophoresis voltage on migration time. Higher voltages give faster run times but can affect the resolution (Figure 2-10).

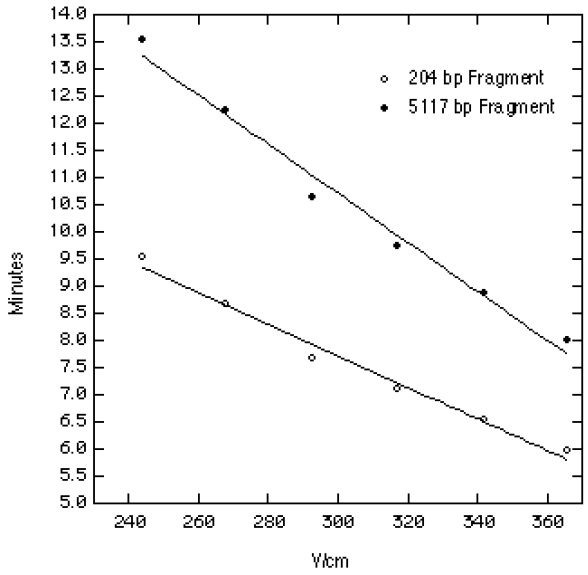


Figure 2-9 Migration time to detector vs. electrophoresis voltage for 204-bp and 5117-bp GeneScan-2500 fragments, 2.5% GeneScan Polymer in 41-cm capillary at 30 °C

Effects on Resolution

Figure 2-10 shows the effect of increasing electrophoresis voltage on resolution. In general, resolution is better at lower field strengths.

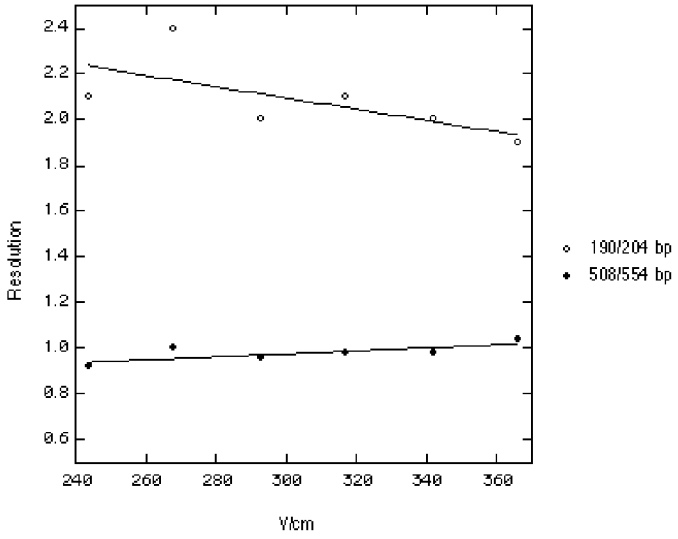


Figure 2-10 Resolution vs. electrophoresis voltage for different-sized GeneScan-2500 fragments, 2.5% GeneScan Polymer in 41-cm capillary at 30 °C

When to Increase Voltage

If the fragments of interest are well separated or if you need a quick answer, you can increase the voltage.

Modifying Electrophoresis Temperature

Perform native applications and non-denaturing applications, such as SSCP, at lower temperatures (27–42 °C).

Protocols for most denaturing applications using the POP-4™ polymer specify a 60 °C run temperature.

Laboratory Temperature and Humidity

The laboratory temperature should be maintained between 15 and 30 °C. Once the ABI PRISM 310 Genetic Analyzer is set up and in operation, the laboratory temperature should not fluctuate more than ±2 °C. The instrument can tolerate up to 80% non-condensing relative humidity. Avoid placing it near heaters or cooling ducts.

For More Information

For information on setting electrophoresis parameters, see the ABI PRISM 310 *Genetic Analyzer User's Manual*.

Using Control DNA

Purpose of Control DNA

Control DNA:

- Serves as a positive control for troubleshooting PCR amplification

  Knowing whether the control DNA amplifies will allow you to distinguish between problems with the sample DNA (the control DNA amplifies but samples do not) and problems with reagents, thermal cyclers, or protocols (the control DNA does not amplify).

- Allows you to monitor sizing precision

  Because the control DNA is not used to calculate the sizing curve, you can use the sizes obtained during different capillary injections to verify that sizing precision (reproducibility) is within acceptable limits.

- Allows you to correlate the fragment sizes that you obtain with the fragment sizes obtained by others, *e.g.*, for the CEPH families in the Linkage Mapping Set

Guidelines for Use

- Amplify at least one control DNA sample in every PCR run.
- Include at least one injection of amplified control DNA during every capillary run. Use more than one injection if you vary the electrophoresis parameters during the run.

CEPH 1347-02 Control DNA

In addition to the GeneScan Internal Lane Size Standards, Applied Biosystems sells the CEPH 1347-02 standard used to generate the Généthon map of the human genome (P/N 403062).

General Analysis and Evaluation Techniques 3

Overview

In This Chapter

This chapter will help you evaluate and analyze data obtained on the ABI PRISM® 310 Genetic Analyzer. In particular, it should help you avoid many common causes of poor quality data.

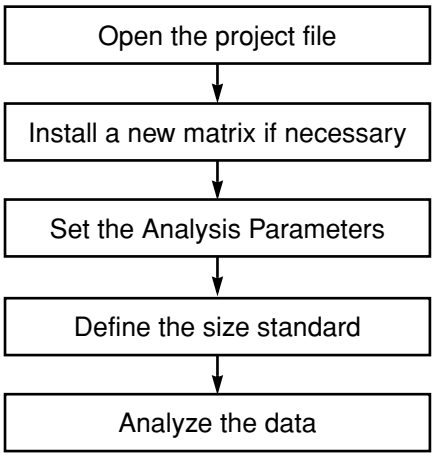
This chapter contains the following topics:

Topic	See Page
Analyzing the Data	3-2
Evaluating Data Quality	3-8
Quantitating Nucleic Acids	3-10
Evaluating Matrix Quality	3-11

Analyzing the Data

Process Overview

The following diagram summarizes the data analysis process using the GeneScan Analysis software.

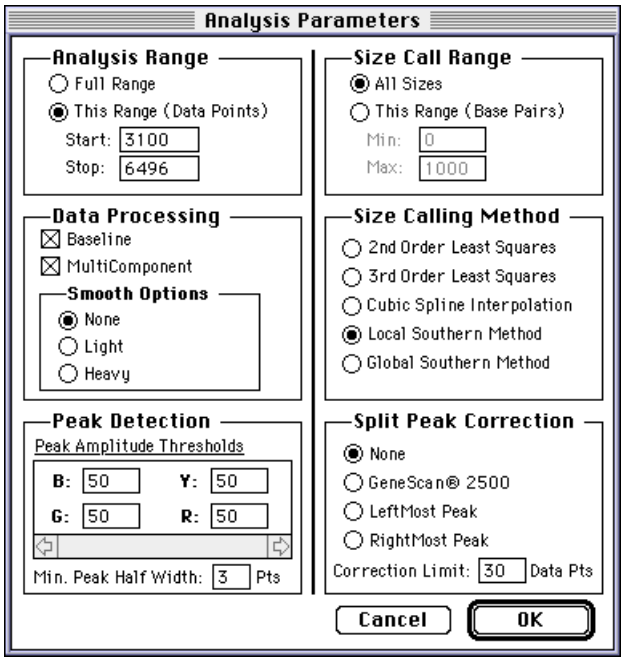


Analyze Sample Files

To analyze sample files:

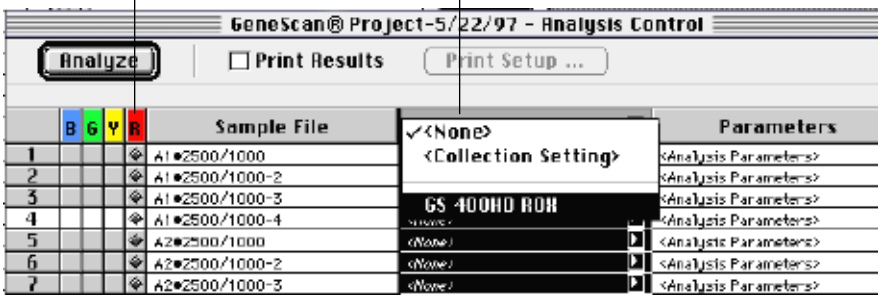
Step	Action
1	Launch the GeneScan Analysis Software.
2	Assign the matrix file to the sample files if you have not already done so in the ABI PRISM 310 Collection Software. **Note** For detailed instructions see "Assigning a New Matrix to a Sample File" in Chapter 4 of the *GeneScan Analysis Software User's Manual.*
3	If the Analysis Control window is not visible, choose Analysis Control from the Windows pull-down menu.
4	Assign a size standard to all sample files as described in "Define and Select the Size Standard" on page 3-4.
5	Highlight the appropriate dye colors for each sample.

To analyze sample files: *(continued)*

Step	Action
6	Define analysis parameters: a. From the File menu, select New. b. Click Analysis Parameters. The Analysis Parameters dialog box appears.  c. Set the appropriate analysis parameters for your application as described in Chapter 4 of the *GeneScan Analysis Software User's Manual*. Be sure to exclude the primer peak from the analysis range.
7	Click Analyze.
8	After the analysis is complete, verify the peak assignments for the size standard in all sample files using one of the methods described below.

continued on next page

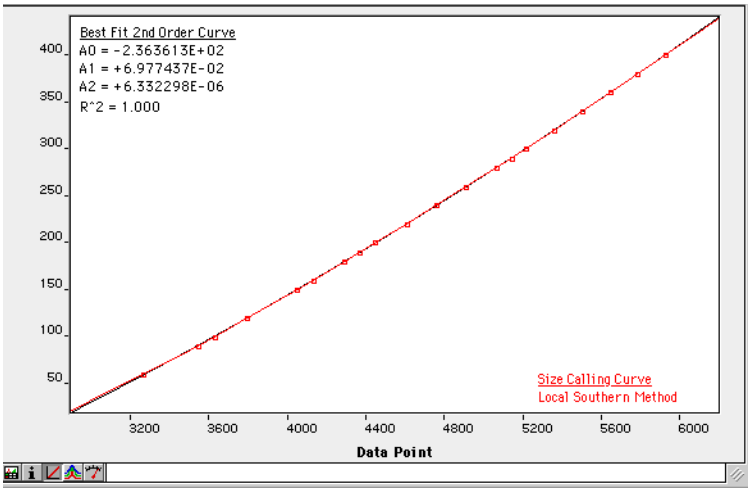
Define and Select the Size Standard

Step	Action
1	Select one of the sample files in the Analysis Control window.
2	If a size standard file already exists, proceed to step 8. Otherwise, continue to step 3 and create a size standard file now.
3	Open the Size Standard pop-up menu for the highlighted sample, and select Define New.
4	Define the size standard. See Chapter 5 for the sizes of the fragments in the size standards. For detailed information on defining size standards, refer to Chapter 4 of the *GeneScan Analysis Software User's Manual*.
5	Close the window.
6	Click Save.
7	Name the size standard file, and click Save.
8	Open the Size Standard pop-up menu at the top of the Size Standard column, and select the appropriate size standard. A diamond in this column indicates the size standard will be applied to the corresponding sample file Size standard pop-up menu 
9	The size standard must be selected for all the samples except matrix standard samples. The size standard is selected if a diamond appears in the R (red) column for a particular sample. To select or deselect the size standard, hold down the command key and click in the appropriate square

continued on next page

Verify Size-Standard Peak Assignments

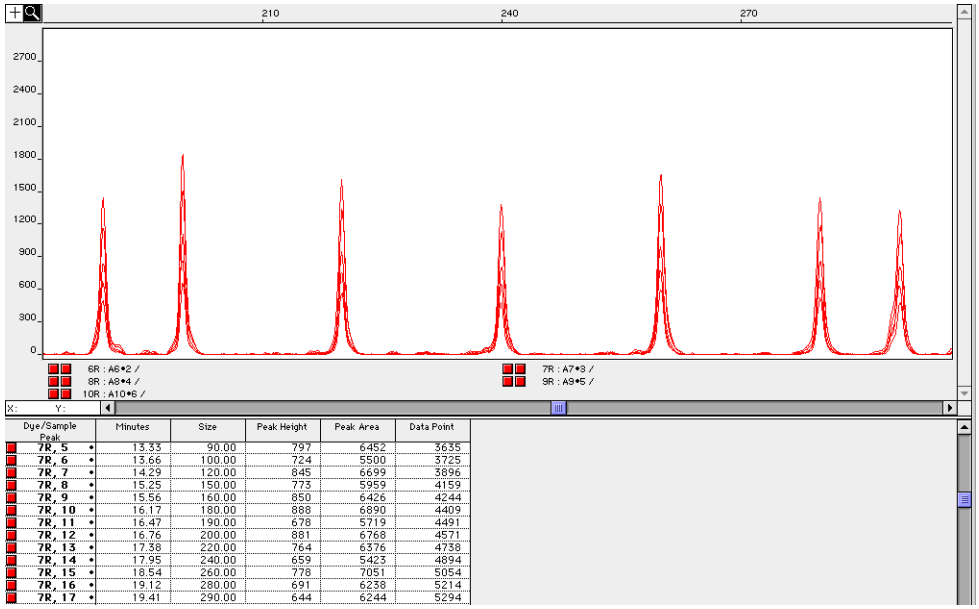
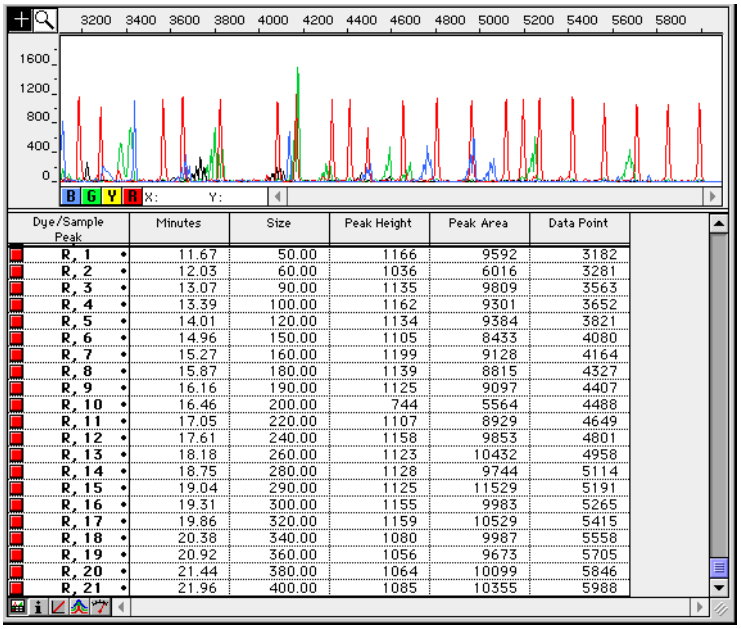
First Method

Step	Action
1	Highlight the sample files of interest and display the sizing curve by selecting Size Curve from the Sample menu.  The R^2 value and the coefficients of the curve are provided. The R^2 value is a measure of the accuracy of fit of the best-fit second order curve. **Note** You can only display the sizing curve for a sample if a valid sizing curve exists for that sample.
2	Check whether all the defined size standard peaks fall on the sizing curve and note peaks that lie off the curve. **Note** The 250-bp fragment in the GeneScan-350 and GeneScan-500 Internal Lane Size Standards does not fall on the sizing curve.
3	If all of the size standard peaks did not lie on the sizing curve for any samples, define a new size standard for those samples as described in the *GeneScan Analysis Software User's Manual* and reanalyze.

Second Method

Step	Action
1	Select Results Control from the Window menu (⌘ R).
2	From the View menu, select Align By Size. **Note** If fragments are aligned by size, this option will not be available.
3	Examine the GeneScan size standard peaks in overlapping groups of 16 samples (Quick Tile Off). Verify that the size standard peaks are superimposed, as shown below.

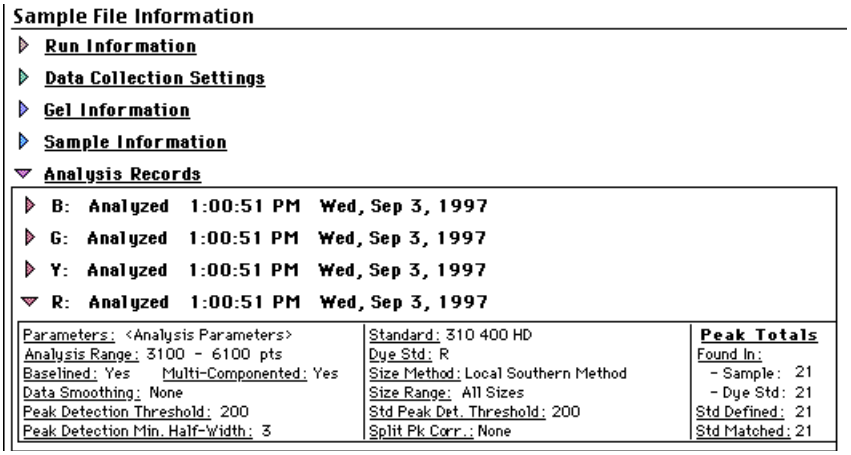
Second Method *(continued)*

Step	Action
	
4	In the Results display table, peaks in the size standard definition are marked with a bullet as shown below.  Scroll through the tables to verify the size standard peak assignments and note which samples (if any) have incorrect assignments.
5	Define a new size standard for those samples with incorrect peak assignments, as described in the *GeneScan Analysis Software User's Manual*.

Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
7R, 5 •	13.33	90.00	797	6452	3635
7R, 6 •	13.66	100.00	724	5500	3725
7R, 7 •	14.29	120.00	845	6699	3896
7R, 8 •	15.25	150.00	773	5959	4159
7R, 9 •	15.56	160.00	850	6426	4244
7R, 10 •	16.17	180.00	888	6890	4409
7R, 11 •	16.47	190.00	678	5719	4491
7R, 12 •	16.76	200.00	881	6768	4571
7R, 13 •	17.38	220.00	764	6376	4738
7R, 14 •	17.95	240.00	659	5423	4894
7R, 15 •	18.54	260.00	778	7051	5054
7R, 16 •	19.12	280.00	691	6238	5214
7R, 17 •	19.41	290.00	644	6244	5294

Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
R, 1 •	11.67	50.00	1166	9592	3182
R, 2 •	12.03	60.00	1036	6016	3281
R, 3 •	13.07	90.00	1135	9809	3563
R, 4 •	13.39	100.00	1162	9301	3652
R, 5 •	14.01	120.00	1134	9384	3821
R, 6 •	14.96	150.00	1105	8433	4080
R, 7 •	15.27	160.00	1199	9128	4164
R, 8 •	15.87	180.00	1139	8815	4327
R, 9 •	16.16	190.00	1125	9097	4407
R, 10 •	16.46	200.00	744	5564	4488
R, 11 •	17.05	220.00	1107	8929	4649
R, 12 •	17.61	240.00	1158	9853	4801
R, 13 •	18.18	260.00	1123	10432	4958
R, 14 •	18.75	280.00	1128	9744	5114
R, 15 •	19.04	290.00	1125	11529	5191
R, 16 •	19.31	300.00	1155	9983	5265
R, 17 •	19.86	320.00	1159	10529	5415
R, 18 •	20.38	340.00	1080	9987	5558
R, 19 •	20.92	360.00	1056	9673	5705
R, 20 •	21.44	380.00	1064	10099	5846
R, 21 •	21.96	400.00	1085	10355	5988

Third Method

Step	Action
1	Highlight the sample files of interest. From the Sample window, select Sample Info (⌘ I). The Sample File Information window appears.
2	Each dye is listed in the Analysis records. Select the Red dye as shown below.  **Note** The sample must already be analyzed.
3	In the column marked Peak Totals, confirm that the Dye Std and the Sample contain the same numbers of peaks.

Evaluating Data Quality

Good Data Looks Like This

Electropherogram peaks should be sharp, well-defined, and scaled above 150 relative fluorescent units (RFU) as shown in Figure 3-1. The peaks corresponding to different color dyes need not be of equal intensity but the data for the less intense colors should be clearly resolvable at higher magnification (Figure 3-2).

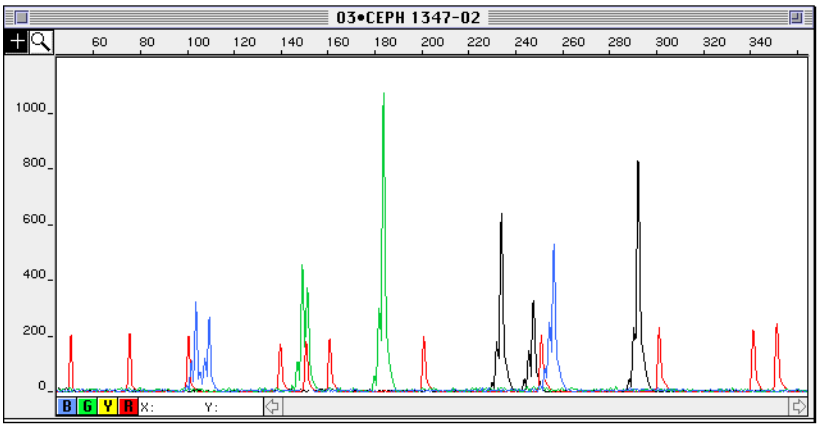


Figure 3-1 Example of data from the Fluorescent Genotyping Demonstration Kit B. Two dinucleotide repeat loci are labeled with each dye, except for red, which is used for the size standard.

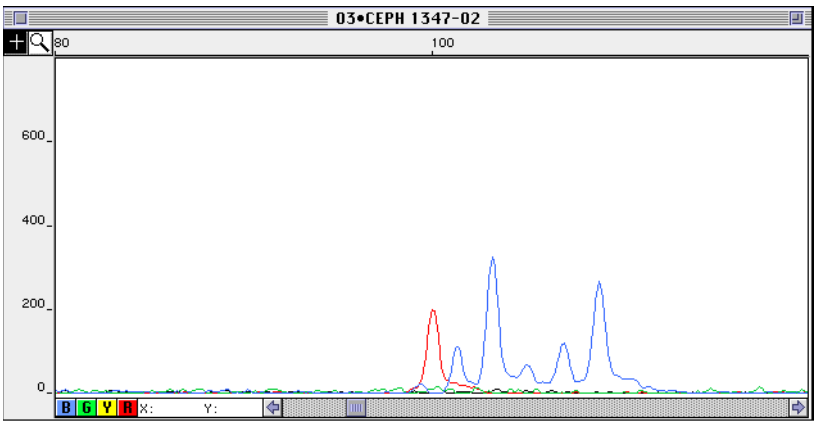


Figure 3-2 Expanded view of the D12S83 locus from the same sample as Figure 3-1

continued on next page

Bad Data Looks Like This

An example of data where the sample was greatly overloaded is shown in Figure 3-3. Note that even the “pull-up” peaks (see page 3-11) are truncated.

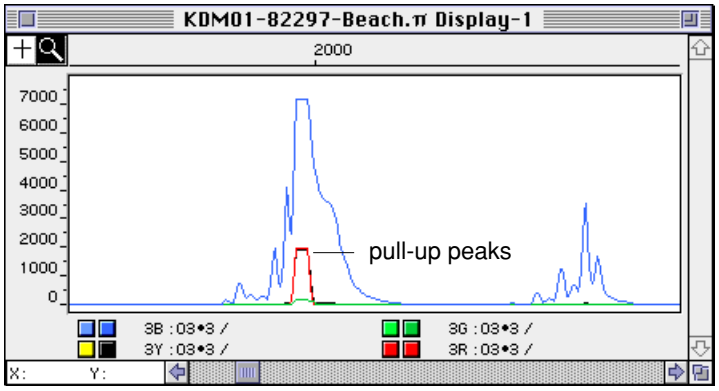


Figure 3-3 Ridiculously overloaded sample

A more subtle example of overloading is shown in Figure 3-4 and Figure 3-5. The off-scale peaks in the raw data in Figure 3-4 (note that the peaks are truncated at 8100 RFU) appear smaller in the analyzed data in Figure 3-5. The peaks are broadened and blue “pull-up” peaks (see page 3-11) appear under the green peaks.

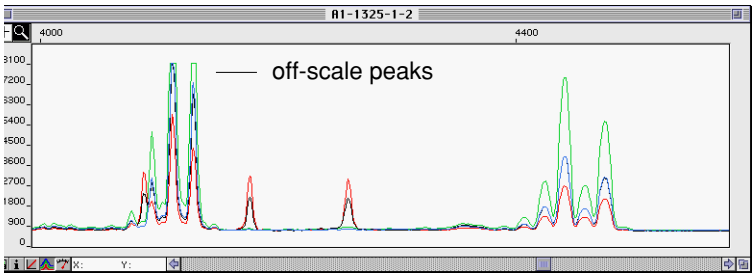


Figure 3-4 Raw data from an overloaded sample

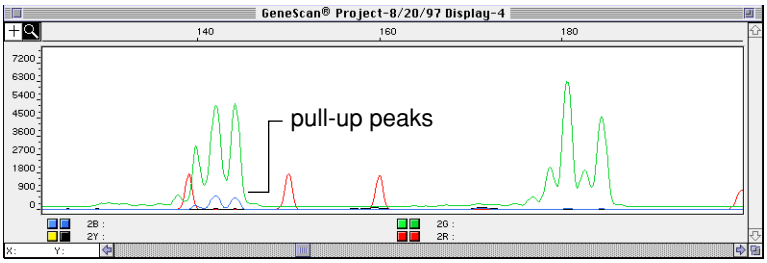


Figure 3-5 Analyzed data from sample in Figure 3-4

Quantitating Nucleic Acids

Introduction

You can determine the relative quantities of two 5´-end labeled fragments run on your ABI PRISM 310 Genetic Analyzer by comparing the corresponding peak areas or peak heights on a GeneScan electropherogram.

IMPORTANT Do not use internally-labeled ([F]dNTP-labeled) fragments in your quantitative experiments. Variations in the per-fragment number of labeled nucleotides and the increased peak spreading with this method make relative quantitation unreliable.

How to Proceed

To determine the relative number of molecules of two different-sized fragments, you calculate the ratio of respective peak areas or heights. Always compare peak area to peak area and peak height to peak height.

- If two fragments are similar in size, it is often better to compare peak heights, especially if the peaks overlap slightly.

  If the peaks are well defined, peak area and peak height will give similar results. If the peaks are irregularly shaped, *e.g.*, have shoulders, peak heights will often give better results than peak areas.

- If two fragments are far apart in size, it is often better to compare peak areas because large peaks tend to spread considerably more than small peaks.

Ensuring Precise Relative Quantitation

Depending upon the value you set for the Minimum Peak Half Width and the smoothing option that you choose, the GeneScan software may interpret a noisy peak as multiple peaks. Thus, these parameters can influence the measurement of peak area and peak height. Overestimating the number of peaks can be a particular problem with the jagged peaks characteristic of noisy data. Either increase the Minimum Peak Half Width or use a stronger smoothing option when analyzing noisy data.

Evaluating Matrix Quality

Purpose of a Matrix

While the most intense fluorescence emitted by an ABI PRISM™ dye will fall within a small wavelength detection range, some fluorescence emission in the detection ranges of the other dyes will always occur. The multicomponent matrix compensates for this overlap by subtracting out, in each dye's detection range, the portion of the signal due to fluorescence from other dyes.

For directions on how to create a matrix file, see Appendix B.

Why the Matrix Must Be Remade

When you create a matrix, you must run each relevant dye matrix standard separately to determine the proportional amount of fluorescence that is emitted in all four detection regions. Because the emission spectra of the dyes vary with the physical environment (such as the pH or polymer type and concentration), the matrix must be remade if run conditions change.

Factors Affecting Matrix Quality

- Aging reagents
- Buffer type and concentration
- Polymer type
- Denaturing vs. non-denaturing conditions
- Run temperature

Virtual Filter Set C

The emission maximum of 6-FAM, the recommended blue-displaying dye for this filter set, is very close to the laser wavelength of 514.5 nm. Thus, the window for collected blue light-intensity data is offset to longer wavelengths and does not contain the emission maximum of 6-FAM. It is also very close to the detection region for the green-displaying dye TET (see "Virtual Filter Set C" on page 4-9).

Matrix files made for Virtual Filter Set C are especially susceptible to minor changes in run conditions. If you are using Virtual Filter Set C for GeneScan applications, watch for evidence of matrix problems and remake the matrix as soon as problems appear.

How to Recognize Matrix Problems

A poor or incorrect matrix results in too much or too little subtraction of dye spectral overlap during data analysis. Each causes a recognizable electropherogram anomaly:

- Bleedthrough peaks, also called "pull-ups" (caused by too little subtraction)
- Elevated interpeak baseline (caused by too much subtraction)

Bleedthrough Peaks (or Pull-ups)

Bleedthrough peaks are small peaks of one color lying directly under a large peak of another color even though there is no PCR product corresponding to the smaller peak. (The large-peak signal is "pulling-up" peaks in other colors.) An example is shown in Figure 3-6 on page 3-12.

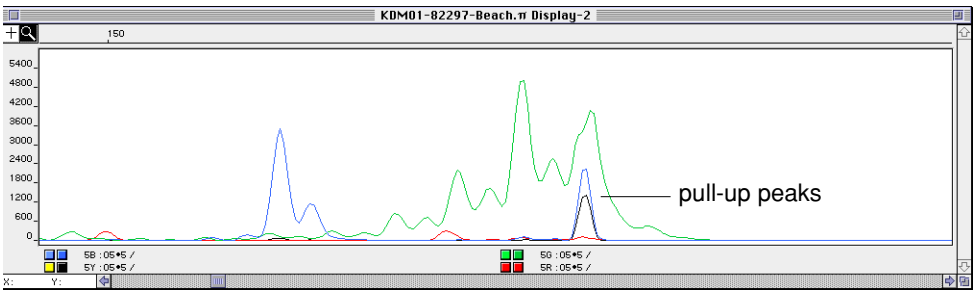


Figure 3-6 Characteristic appearance of bleedthrough peaks

Bleedthrough can occur for two reasons:

- The matrix was made with the wrong dyes or filter set.

  For a list of recommended dye/filter set combinations, see “Recommended Dye/Virtual Filter Set Combinations” on page 4-6.

- The signal from the large peak is off-scale because of sample overloading. In the example shown in Figure 3-6, the peak showing bleedthrough is actually off-scale (Figure 3-7).

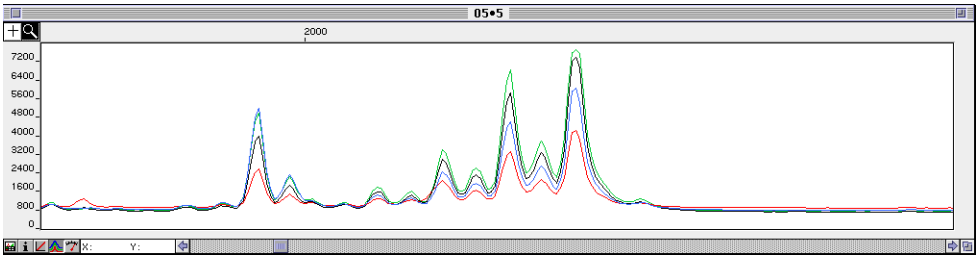


Figure 3-7 Raw data from the bleedthrough example shown in Figure 3-6

Keep peak heights between approximately 150 and 4000 RFU. If sample data is off-scale, do one of the following:

- Rerun the samples using a shorter injection time.
- Dilute and rerun the samples.

Elevated Interpeak Baseline

Figure 3-8 shows a typical example of an elevated interpeak baseline:

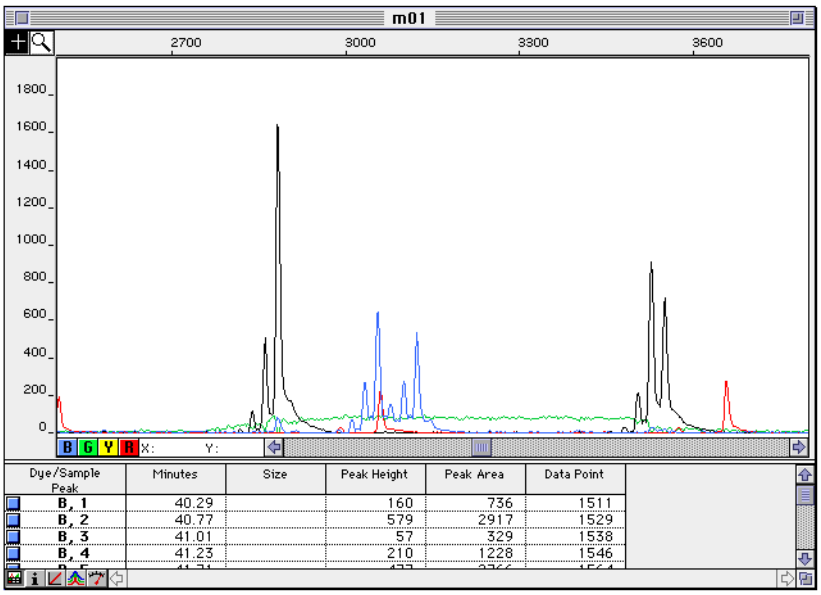


Figure 3-8 Characteristic appearance of an elevated baseline caused by a bad or incorrect matrix

In this example, the green baseline is elevated in the region between two large black peaks (representing the yellow dye signal) because too much green signal is subtracted from the yellow signal (see Figure 3-9 on page 3-14). The GeneScan software uses these low data points to calculate the baseline for the green signal. Therefore the original green baseline is elevated.

Note If the baseline is sufficiently elevated, random fluctuations in the baseline can lie above the Peak Amplitude Threshold and might be falsely interpreted as product peaks.

If you suspect that an elevated interpeak baseline is caused by matrix problems, inspect the data before baselining. This can be done by reanalyzing the data with baselining deselected in the Analysis Parameters dialog box. As shown in Figure 3-9, low data points are apparent as troughs in one color beneath peaks in another.

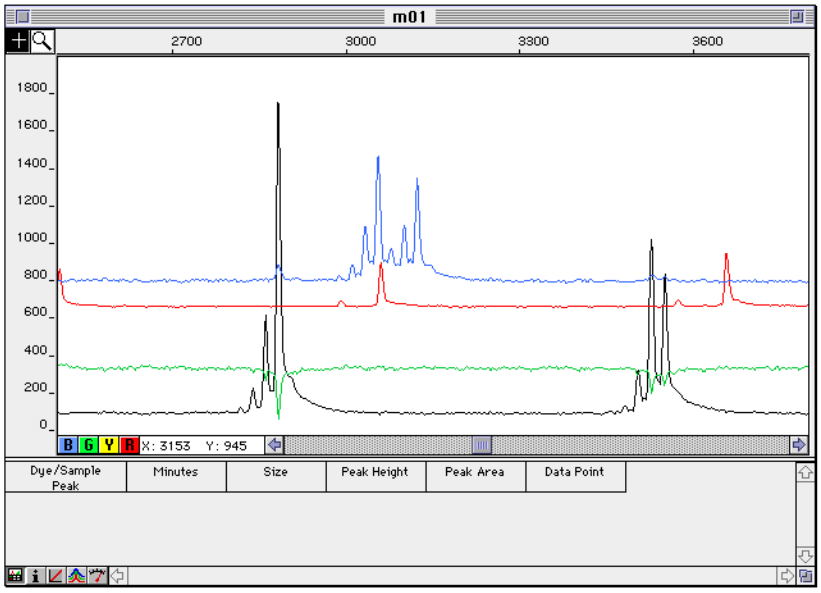


Figure 3-9 The data of Figure 3-8 before baselining

What To Do If You Have Matrix Problems

If problems related to bleedthrough peaks or to an elevated interpeak baseline appear consistently, you should remake the matrix. Apply the new matrix to the old sample data and reanalyze the data.

For directions on how to create a matrix file, see Appendix B.

4 ABI PRISM Dyes

Overview

In This Chapter

This chapter describes the dyes used in GeneScan® fragment analysis applications. It also provides background information on the following issues:

- How to choose dye/virtual filter set combinations
- What factors determine the relative signal intensities of ABI PRISM™ dyes
- Why matrix files depend on the run conditions

This chapter contains the following topics:

Topic	See Page
Available Dyes	4-2
Understanding Dye Spectra	4-3
Choosing Dye/Filter Set Combinations	4-5
Emission Spectra for Representative Dye/Virtual Filter Set Combinations	4-8

Available Dyes

Introduction

Applied Biosystems supplies ten fluorescent dyes for use on ABI PRISM instruments: 5-FAM, 6-FAM, R110, TET, JOE, R6G, HEX, NED, TAMRA, and ROX. Each emits a continuous spectrum of light upon laser excitation.

During an electrophoresis run, the ABI PRISM® 310 Genetic Analyzer records the fluorescence intensity as a function of time and wavelength from regions on a CCD camera that correspond to different detection wavelength ranges. A multicomponent matrix is applied to the fluorescence intensity data to correct for spectral overlap between the dyes. After correction, the fluorescence intensities are color-coded and displayed as peaks in the electropherogram.

Dye Chemical Forms

ABI PRISM dyes are available in multiple chemical forms. Some forms are supplied coupled to primers and others you can use to label your own custom primers. Each form has distinct advantages and disadvantages depending upon the intended application and your laboratory setup.

The following table summarizes the uses of and dyes available in each chemical form:

Chemical Form	Used For...	Available Dyes
NHS-esters	Post-synthesis 5´-end labeling of oligonucleotides containing a 5´ Aminolink 2	NED[a], TAMRA, ROX
Phosphoramidite reagents	Preparing custom, 5´-end labeled primers directly on any Applied Biosystems DNA synthesizer[b]	6-FAM, HEX, TET, NED[a]
[F]dNTPs	Simple internal fluorescent labeling of multiple nucleotides during PCR amplification	R6G, R110, TAMRA
Labeled primers in reagent kits	Microsatellite and human identification applications	5-FAM[c], JOE[c], 6-FAM, HEX, TET, NED[a]
Labeled size standard	Generating the sizing curve to size unknown sample fragments	TAMRA, ROX

a. NED-labeled primers are available only in kits or through the Applied Biosystems Custom Oligo Service. Call Applied Biosystemsor visit the Applied Biosystems WorldWideWeb page at www.appliedbiosystems.com/techsupport for information on how to order custom-labeled oligos.

b. For directions on synthesizing labeled oligonucleotides, see Appendix C.

c. 5-FAM and JOE are only available as labeled primers in select reagent kits.

See “Choosing Fluorescent Labeling Methods” on page 2-5 for a detailed comparison of fluorescent labeling with 5´-end labeled primers and [F]dNTPs.

Understanding Dye Spectra

Background

An electron, in a molecule or atom, that has been transferred to an excited (high energy) state, *e.g.*, after absorption of a photon, will eventually return to the more stable (lower energy) ground state. The return to the ground state can occur either through the evolution of heat (radiationless decay) or through the emission of a photon (fluorescence or phosphorescence).

Definitions

The emission spectrum of a fluorescent dye is the intensity of emitted light (fluorescence) as a function of the wavelength of the emitted light.

The excitation spectrum of a dye is the intensity of emitted light as a function of the wavelength of the exciting light.

The excitation efficiency of a dye is a measure of the probability that it will absorb light of a certain wavelength, as a percentage of the probability of absorption at the wavelength of maximum absorption.

The quantum yield of a dye is the probability that its excited state will emit a photon as it decays back to the ground state.

Factors That Affect Spectra

The emission and excitation spectra of a dye attached to DNA depend upon the dye's:

- Chemical structure
- Physical environment

  Relevant parameters include the buffer pH and concentration, the polymer composition, and whether the DNA is single- or double-stranded.

The effect of the physical environment on dye spectra explains why you need to remake the matrix every time you change the run conditions.

Although altered by the physical environment, the wavelengths of maximum emission and excitation for each ABI PRISM dye always lie within a small wavelength range. This relative invariance of the emission spectra is crucial to experimental success.

Experimental Considerations

Detecting a Dye Signal

The ability of the instrument to detect a dye signal depends upon the dye's:

- Excitation efficiency at the wavelengths of light emitted by the laser

  The argon ion laser in the ABI PRISM 310 Genetic Analyzer emits the greatest intensity of light at 488 nm and 514.5 nm.
- Quantum yield
- Concentration

Distinguishing Between Two Dye Signals

The instrument identifies a dye by determining the ratio of the dye's emission intensities in a series of filters. Each dye's maximum emission intensity lies in a unique filter.

The ability of any instrument to distinguish between two dye signals is determined by the relative differences in the measurements of the two dyes in each of two filters. The

larger the difference in the intensity of the signal in the two filters, the easier it is to identify each dye. The closeness of the ratios of the measurements in each of the filters will be determined by the amount of overlap in the two emission spectra.

In general, the ability to discern between two dye signals is enhanced by:

- A larger separation between the emission maxima of the two dyes
- A narrower emission spectrum of one or both dyes

ABI PRISM Dye Maximum Emission and Excitation Wavelengths

The following table lists the maximum fluorescence emission and excitation wavelengths for oligonucleotides labeled with ABI PRISM dye NHS-esters, dye phosphoramidites, and [F]dNTPs. (The actual maximum emission and excitation wavelengths may differ from the listed values due to the influence of the physical environment upon the dye.)

Table 4-1 Maximum Emission and Excitation Wavelengths

Dye[a]	Emission λ_{max} (nm)	Excitation λ_{max} (nm)
6-FAM	517	494
5-FAM	522	493
R110	525	501
TET	538	522
R6G	549	529
HEX	553	535
JOE	554	528
TAMRA ([F]dNTP)	572	555
NED	575	553
TAMRA	583	560
ROX	607	587

a. All dye-labeled oligonucleotides were run at 10^{-7} M in 1X TE buffer, pH 8.0, room temperature, on a TaqMan® LS-50B PCR Detection System.

Choosing Dye/Filter Set Combinations

How Data Collection Works

The ABI PRISM 310 Genetic Analyzer determines the light intensity in four non-overlapping regions on a CCD camera. Each region corresponds to a wavelength range that contains or is close to the emission maximum of an ABI PRISM dye. The exact positions of the regions and the dye combinations appropriate to these positions depend upon the virtual filter set used.[1] For example, with Virtual Filter Set A the instrument records the light intensity in four regions, or "windows," centered at 540 nm, 560 nm, 580 nm, and 610 nm. The window positions in each virtual filter set have been optimized to provide the maximum possible separation among the centers of detection for the different dyes while maintaining an excellent signal-to-noise ratio.

The GeneScan Analysis Software color-codes the intensity displays from the four light-collection regions. These appear as the blue, green, black, and red peaks in the Raw Data window. The blue display represents the total light intensity from the shortest wavelength range monitored and the red display represents the total light intensity from the longest wavelength range monitored.

It is important to realize that the same four colors are used to color-code fluorescence data from all dye/virtual filter set combinations. Thus, the display colors represent the relative, not the actual, detection wavelengths.

The process is similar to using physical filters to separate light of different wavelengths. However, the ABI PRISM 310 Genetic Analyzer filter sets are called "virtual filters" because the instrument uses no physical filtering hardware to perform the separation.[2]

Available Virtual Filter Sets

The ABI Prism 310 Genetic Analyzer uses six virtual filter sets, A–F.

Virtual filter sets A, C, D, and F are used for GeneScan applications, while A, B, and E are used for DNA sequencing applications.

Note You can change the virtual filter set used for one or more injections in a series of capillary injections by selecting a different run module in the GeneScan Injection List.

Rules for Dye Choice

Each filter set should be used with a combination of four or fewer dyes (including the dye reserved for the internal lane size standard) that:

- Display in four different colors
- Are available in compatible chemical forms

  You can combine phosphoramidite labels with NHS-ester labels. You should not combine [F]dNTP-labeling with any other labeling method.

Table 4-2 through Table 4-4 on pages 4-6 and 4-7 will help you chose the appropriate dye and filter set combination for your particular application.

1. Each GeneScan run module contains instructions for a specific virtual filter set. Once you choose a module, the choice of virtual filter set is automatic.
2. The ABI PRISM 310 Genetic Analyzer has a long-pass filter to prevent light from the instrument's argon ion laser from interfering with the detection of the dye signals.

Table 4-2 Chemical Forms and Color Displays of ABI PRISM Dyes

Dye	Available As...	Color Display (in Virtual Filter Set)
5-FAM[a]	Labeled primer in reagent kits	Blue (A, F)
6-FAM	Phosphoramidite	Blue (A, C, D)
R110	[F]dNTP	Blue (A)
TET	Phosphoramidite	Green (C)
JOE[a]	Labeled primer in reagent kits	Green (A, F)
R6G	[F]dNTP	Green (A)
HEX	Phosphoramidite	Green (A, D), Yellow (C)
NED[b]	NHS-ester, phosphoramidite	Yellow (D, F)
TAMRA	NHS-ester, [F]dNTP, or GeneScan Internal Lane Size Standard	Yellow (A), Red (C)
ROX	NHS-ester or GeneScan Internal Lane Size Standard	Red (A, D, F)

a. 5-FAM and JOE are only available as labeled primers in certain reagent kits (see Table 4-4 on page 4-7).

b. NED-labeled primers are available only in kits or through the Applied Biosystems Custom Oligo Service. Call Applied Biosystems or visit the Applied Biosystems WorldWideWeb site at www.appliedbiosystems.com/techsupport for information on how to order custom-labeled oligos.

Table 4-3 Recommended Dye/Virtual Filter Set Combinations

Dye Combination	Chemical Forms Combined	Use with Virtual Filter Set...
6-FAM, HEX, TAMRA, ROX (std)	Phosphoramidites, NHS-esters	A
R110, R6G, TAMRA, ROX (std)	[F]dNTPs	
5-FAM, JOE, TAMRA, ROX (std)	Reagent kit primers, NHS-esters	
6-FAM, TET, HEX, TAMRA (std)[a]	Phosphoramidites	C
6-FAM, HEX, NED, ROX (std)	Phosphoramidites	D
5-FAM, JOE, NED, ROX (std)	Reagent kit primers, NHS-esters	F

a. This combination, although recommended, can sometimes give poor quality data due to spectral overlap among the dye signals. If you experience matrix problems with this combination, you may see an improvement in data quality by switching to 6-FAM, HEX, NED, and ROX and using Virtual Filter Set D.

Table 4-4 Reagent Kit and Custom-labeled Primer Dye Sets

Application	Dye Set	Virtual Filter Set
AmpFℓSTR Blue™ PCR Amplification Kit	5-FAM (Blue), ROX (Red, std)	A, F
AmpFℓSTR Green™ I PCR Amplification Kit	JOE (Green), ROX (Red, std)	A, F
StockMarks® for Horses Equine Paternity PCR Typing Kit	5-FAM (Blue), JOE (Green), TAMRA (Yellow), ROX (Red, std)	A
ABI PRISM™ Linkage Mapping Set	6-FAM (Blue), TET (Green), HEX (Yellow), TAMRA (Red, std)	C
StockMarks for Cattle® Bovine Paternity PCR Typing Kit		
Custom-labeled primers for fragment analysis	6-FAM (Blue), HEX (Green), NED (Yellow), ROX (Red, std)	D
ABI PRISM Linkage Mapping Set Version 2		
AFLP™ Microbial Fingerprinting Kit	5-FAM (Blue), JOE (Green), NED (Yellow), ROX (Red, std)	F
AFLP Plant Mapping Kits		
AmpFℓSTR™ Profiler™ PCR Amplification Kit		
AmpFℓSTR Profiler Plus™ PCR Amplification Kit		

Emission Spectra for Representative Dye/Virtual Filter Set Combinations

About the Spectra

In the following four figures, the collection windows of the four GeneScan virtual filter sets are shown overlaid with the normalized emission spectra of a recommended dye set.

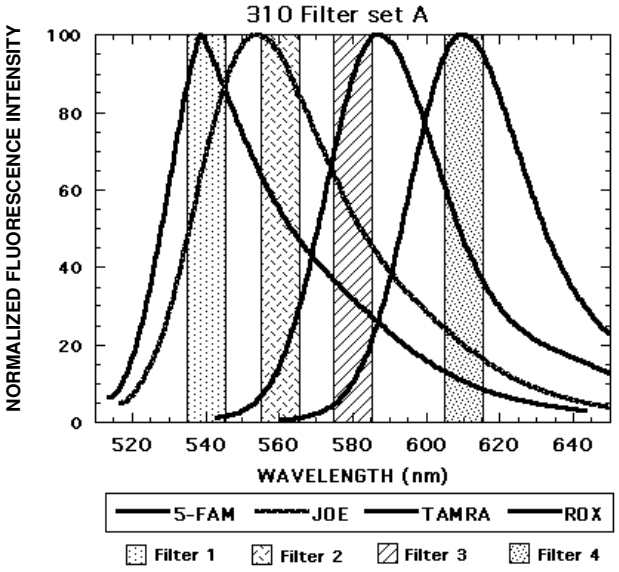
The wavelength of maximum emission of the dyes shown in these figures differs from the value given in Table 4-1 on page 4-4 for the following reasons:

- The long-pass blocking filter (see footnote on page 4-5) blocks much of the light emitted by 6-FAM and 5-FAM, the dyes with emission maxima closest to the laser wavelength.
- The environment of the capillary polymer shifts the emission spectra of ABI PRISM dyes.

  See “Factors That Affect Spectra” on page 4-3 for an explanation of the observed shift.

Virtual Filter Set A

Virtual Filter Set A is used with the dyes in the Dye Primer Matrix Standards Kit (5-FAM, JOE, TAMRA, and ROX).



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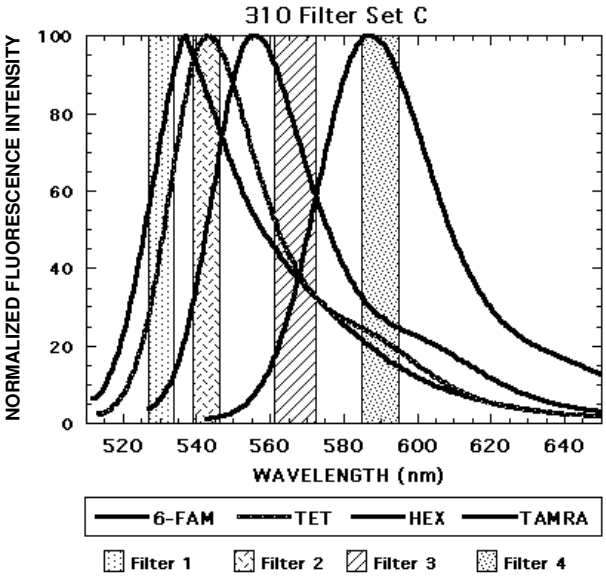
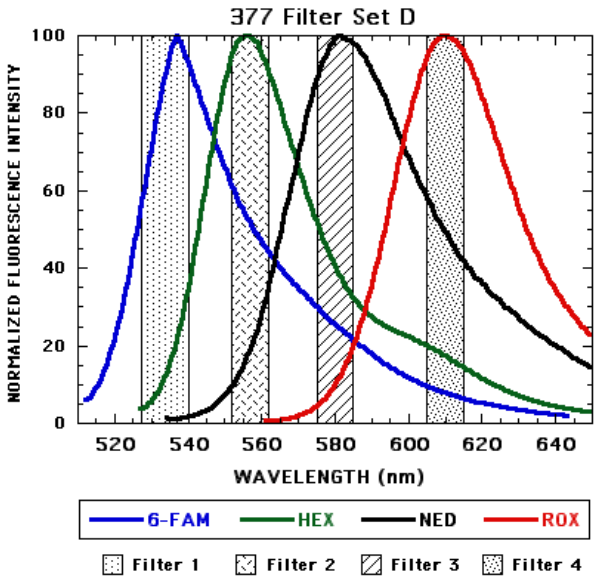
Virtual Filter Set C

The following figure demonstrates why matrix problems can occur using this virtual filter set. The light collection windows for 6-FAM and TET are close to one another and the spectral overlap between the two dyes is significant.



Virtual Filter Set D

The spectral resolution of this dye/virtual filter set combination is much greater than the spectral resolution of 6-FAM, TET, HEX, and TAMRA with Virtual Filter Set C. Switching to this combination reduces the potential for matrix problems and may yield cleaner data.



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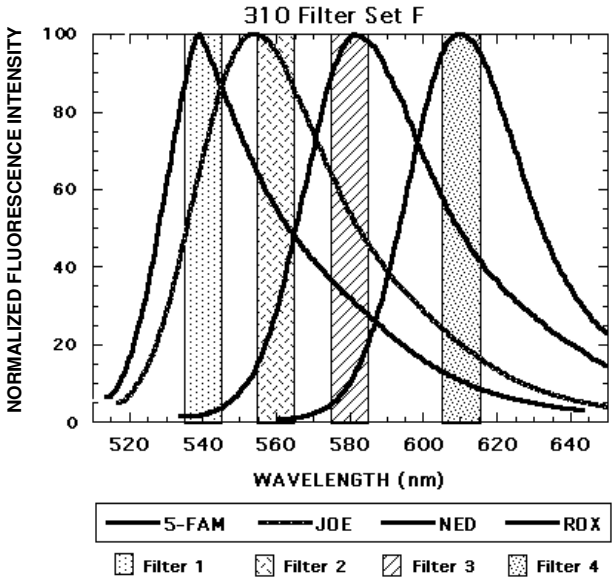
Virtual Filter Set F

The spectral resolution of this dye/virtual filter set combination is similar to the spectral resolution of 5-FAM, JOE, TAMRA, and ROX with Virtual Filter Set A. However, if you are experiencing problems with signal strength using TAMRA or you want to load less sample, you should use this combination. (Relative signal strength is not indicated in these normalized spectra.)

Virtual Filter Set F is used in the AmpFℓSTR Profiler and AmpFℓSTR Profiler Plus PCR Amplification Kits and in the AFLP Plant Mapping and AFLP Microbial Fingerprinting Kits.



Sizing and Size Standards

5

Overview

In This Chapter

This chapter describes the size-calling process in detail, including the following:

- The distinction between absolute accuracy and precision in size calling
- How cross-platform sizing differences arise
- How to avoid or recognize the most common sizing problems

This chapter also describes the available GeneScan Internal Lane Size Standards, including each standard's useful range.

This chapter contains the following topics:

Topic	See Page
Introduction to Sizing	5-2
Preventing/Troubleshooting Sizing Problems	5-5
GeneScan Internal Lane Size Standards	5-7
GeneScan-350 Size Standard	5-8
GeneScan-400HD Size Standard	5-10
GeneScan-500 Size Standard	5-12
GeneScan-1000 Size Standard	5-14
GeneScan-2500 Size Standard	5-16

Introduction to Sizing

Introduction

Size standards and sample fragments loaded in the same capillary run undergo the same electrophoretic forces. Therefore, the relative electrophoretic mobility of any sample fragment is a good indicator of its molecular weight because of injection-to-injection variation in electrophoretic forces no longer contributes to measurement error.

Process Overview

The three steps in the size-calling process are the following:

Fitting the Internal Lane Size Standard to the Size Standard Definition

During this first step, the GeneScan® Analysis Software tries to match the peaks of the internal lane standard with the peaks of the size standard definition, so that the overall fit:

- Maximizes the number of matched peaks

  To be considered a match, a size standard peak must lie within ±400 scans of its expected position (as defined by the corresponding size standard definition peak).

- Minimizes the total squared error of the matched peaks

  Once a size standard peak is matched, the “error” is defined as the distance between its expected position and its actual position in the internal lane standard.

To complete Step 1 successfully, the GeneScan Analysis Software must match at least three peaks.

Generating a Sizing Curve

From the fragment migration times of the internal lane standard, the GeneScan Analysis Software generates a sizing curve giving size in base pairs or nucleotides as a function of scan number (*i.e.*, migration time) using one of the following operator-chosen sizing methods:

- Global sizing methods
  - 2nd-Order Least Squares
  - 3rd-Order Least Squares
  - Global Southern
- Local sizing methods
  - Local Southern
  - Cubic Spline

Global methods, which generate the best-fit curve from all matched fragments in the size standard, are less affected than local methods by anomalies in the run times of single size standard fragments.

Local methods, which generate the best-fit curve from nearby internal lane standard data points, are less affected by changes in the electrophoresis conditions or in the analysis range. (A change in the analysis range will change the subset of size standard fragments that is available for generating the sizing curve.)

IMPORTANT For the Local Southern Method to work, you must have at least two size standard fragments larger than your largest unknown fragment.

For detailed information on the different sizing methods, refer to the *GeneScan Analysis Software User's Manual*.

Converting Fragment Migration Times to Sizes

This step is a straightforward mapping of any given fragment's migration time onto the sizing curve.

Accuracy Versus Precision

Accuracy in size calling is a measure of the instrument's ability to generate fragment sizes that are close to the actual size of the fragment as determined by sequencing.

Precision, or reproducibility, in size calling is a measure of the instrument's ability to generate the same size consistently for a given fragment independent of whether the called size is close to the actual size for a given set of run conditions.

Comparing Sizes Obtained Within and Across Platforms

If care is taken to control for variations in run conditions, ABI PRISM® instruments are highly precise within a single set of injections or a single gel. However, the called size for the same fragment can differ between run conditions on a single instrument. In other words, the generated sizes are not necessarily accurate.

Between-run sizing differences arise from a number of factors including:

- Differences in the type and concentration of capillary or gel polymer
- Well-to-read or time-to-read differences
- Differences in run temperature
- Differences in electrophoresis conditions (*e.g.*, the denaturing ability of the separation matrix)
- Changes in the sizing method or specific GeneScan size standard used to generate the sizing curve

  When comparing across injections, it is important to use the same sizing method and the same size standard definition.

Table 5-1 on page 5-4 compares precision within and between the three instrument platforms for a typical data set from the AmpFℓSTR Blue™ PCR Amplification Kit. The three instrument platforms represented are the following:

- ABI PRISM® 377 DNA Sequencer, 36-cm wtr plates
- ABI™ 373 DNA Sequencer, 24-cm wtr plates
- ABI PRISM 310 Genetic Analyzer, POP-4™ polymer

All results were obtained within a gel or within a set of injections from a single capillary.

Table 5-1 Cross-platform precision results obtained from the AmpFℓSTR Blue PCR Amplification Kit

			ABI PRISM 377		ABI 373		ABI PRISM 310	
Allele	**n**	**Actual Size**	**Mean**	**S. D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
D3S1358								
12	3	114	114.23	0.05	114.53	0.15	111.89	0.07
19	3	142	143.36	0.08	143.06	0.04	140.55	0.01
vWA								
11	3	157	157.25	0.06	157.62	0.03	155.20	0.01
21	3	197	196.98	0.03	197.16	0.05	195.50	0.06
FGA								
18	4	219	220.25	0.05	217.73	0.11	217.15	0.05
30	18	267	268.87	0.05	265.20	0.13	265.68	0.10

For example, consider D3S1358 allele 12. On all three platforms, three times the standard deviation is less than 0.5 bp. By contrast, the mean called size for this allele differs by more than 2 bp between the ABI PRISM 310 and ABI PRISM 377 instruments.

IMPORTANT Because the called size for a fragment can differ from its actual size, you should convert fragment sizes to alleles before comparing microsatellite data generated on different instruments.

Preventing/Troubleshooting Sizing Problems

Preventing Sizing Problems

The following guidelines will help you avoid some of the more common sources of inconsistent sizing within a single experiment:

Guidelines	Comments
Use the same sizing method for all injections.	To verify, check the Analysis Record in the Sample Information window.
Define the same size standard peaks in the size standard definition for all injections (if using more than one size standard definition).	To verify, overlay the size standard peaks from each injection or display the sizing curve for each sample file.
Verify that all defined size standard peaks are called within all sample files.	In the Analysis Record in the Sample Information window, make sure that Standard Defined and Standard Matched are the same.
Use the same Analysis Range in the size standard definition and in the sample files to be analyzed.	To verify, check defined data points in the Analysis Parameters window.

What Can Go Wrong

If the position of a size standard peak differs by more than ±400 scans from the definition peak, the GeneScan Analysis Software will not recognize the peak.

The most common causes of failure to recognize a size standard peak are the following:

- Bad capillaries
- Bleedthrough peaks caused by:
  - Matrix problems
  - Off-scale data (*e.g.*, too much sample is loaded or the primer peak is not removed from the analysis range)
- Using anomalous size standard peaks in the size standard definition
- Changes in electrophoresis conditions

Bad Capillaries

A bad capillary (*e.g.*, one that is broken or has a dirty detection window) is the most common cause of inconsistency in the scan position of size standard peaks. If you are having sizing problems always double-check the condition of the capillary.

Signs of a bad capillary include:

- Loss of current
- Loss of resolution
- Low or no signal

See “Troubleshooting PCR Product Detection” on page 11-7 for more information.

Matrix Problems

If the multicomponent matrix is not correct, sample peaks in other colors will often bleed through to the size standard color, creating false peaks and disrupting sizing.

Off-Scale Data

Off-scale data can also be the source of peaks that bleed through to the size standard color, even when the matrix is good. See “Determining Loading Concentrations for Samples” on page 2-8 and “Optimizing Electrokinetic Injection Parameters” on page 2-9 for suggestions on evaluating and modifying signal strength.

Even if you load the correct amount of sample, sometimes the 35-bp fragment of the GeneScan-350 and GeneScan-500 size standards co-migrates with one of the primer peaks. In most cases the unused primer runs as a number of clustered, off-scale peaks. Because matrix files cannot correct for off-scale data, bleedthrough peaks inevitably appear in other colors. If the GeneScan Analysis Software mistakes one of the bleedthrough peaks for the 35-bp size standard peak, the size-calling curve will be inaccurate over part or all of its range.

Using Anomalous Size Standard Peaks

Using the following fragment peaks in the size standard definition can also cause sizing problems:

- The 250-bp fragment peak of the GeneScan-350 and the GeneScan-500 size standards under denaturing conditions
- The 262- and 692-bp fragment peaks of the GeneScan-1000 size standard under non-denaturing conditions
- The 508-bp fragment peak of the GeneScan-2500 size standard under non-denaturing conditions

The apparent size of these fragments is always smaller than their actual size. (For example, the 250-bp fragment frequently runs at 246 bp.) The reason that they should not be used is that their apparent size varies greatly with small changes in experimental conditions. Using any of these fragment peaks in the size standard definition will affect sizing precision.

Changes in Electrophoresis Conditions

All changes in electrophoresis parameters, buffers, and polymer composition affect the fragment migration rate and can therefore affect sizing.

For More Information

For more information on techniques for improving sizing accuracy on the ABI PRISM 310 Genetic Analyzer, refer to Rosenblum *et al.* (1997) and Wenz *et al.*, 1998.

GeneScan Internal Lane Size Standards

Definition

Internal lane size standards are fluorescently-labeled DNA ladders that you load in the same capillary injection as your experimental samples. The size standard fragments are subject to the same electrophoretic forces as the experimental samples and compensate for injection-to-injection variation in these forces. The uniform spacing of size standard fragments ensures precise size calling throughout the size-calling range.

Available Standards

Applied Biosystems provides five different size standards labeled with either TAMRA or ROX:

- GeneScan-350
- GeneScan-400HD
- GeneScan-500
- GeneScan-1000 (only available labeled with ROX)
- GeneScan-2500

IMPORTANT Choose a size standard such that there at least two size standard fragments larger than your largest unknown fragment.

GeneScan-350 Size Standard

Useful Range You can use the GeneScan-350 size standard to determine fragment lengths between 35 and 350 base pairs.

Fragment Lengths The following table lists the lengths of the 12 fragments comprising the GeneScan-350 size standard:

Table 5-2 GeneScan-350: Fragment Lengths (nt)

35	139	250[a]
50	150	300
75	160	340
100	200	350

a. Do not use this fragment for sizing. See IMPORTANT notice on page 5-9 for an explanation.

Preparation The GeneScan-350 size standard is prepared by digesting a proprietary DNA plasmid with Pst I, followed by ligating a TAMRA- or ROX-labeled, 22-mer oligodeoxynucleotide to the cut ends. A subsequent enzymatic digestion with BstU I yields DNA fragments containing a single TAMRA or ROX dye.

continued on next page

Denaturing Electropherogram

Although the GeneScan-350 size standard is made of double-stranded DNA fragments, only one of the strands is labeled. Consequently, even if the two strands migrate at different rates under denaturing conditions you will not need to worry about peak splitting. Figure 5-1 shows the peak patterns of GeneScan-350 fragments run under denaturing conditions.

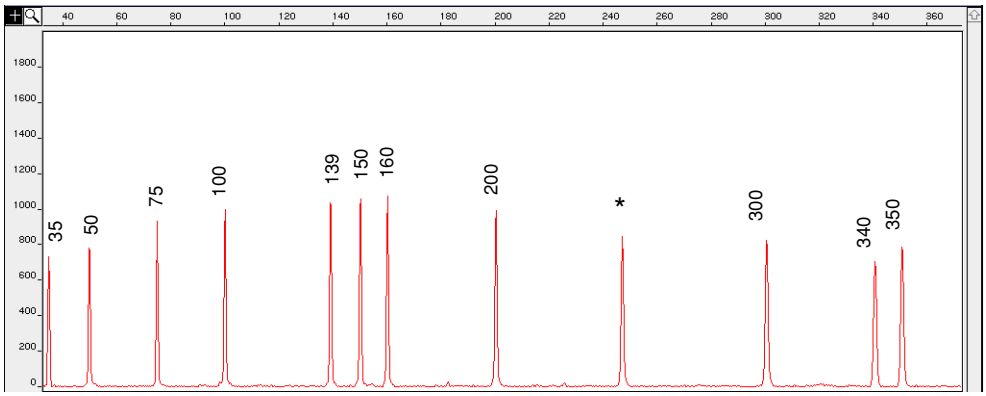


Figure 5-1 Electropherogram of the GeneScan-350 size standard run under denaturing conditions on the ABI PRISM 310 Genetic Analyzer. Fragments were run using the POP-4 polymer at 60 °C.

IMPORTANT An * for the 250-bp peak denotes a peak resulting from abnormal migration of double strands that did not completely separate under denaturing conditions. Do not use this peak to size samples. This peak shows variably smaller values than the actual size of the fragments.

Non-denaturing Electropherogram

Figure 5-2 shows the peak patterns of GeneScan-350 fragments run under non-denaturing conditions.

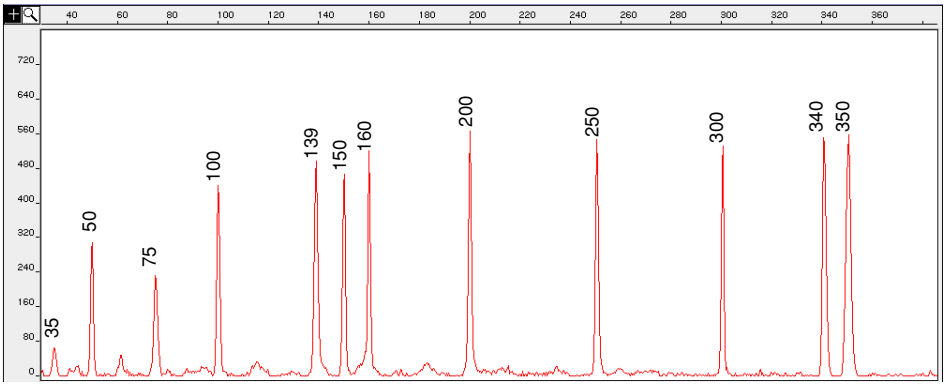


Figure 5-2 Electropherogram of the GeneScan-350 size standard run under non-denaturing conditions on the ABI PRISM 310 Genetic Analyzer. Fragments were run using 3% GeneScan Polymer at 30 °C.

GeneScan-400HD Size Standard

Useful Range You can use the GeneScan-400HD (High Density) size standard to determine fragment lengths between 50 and 400 base pairs.

Special Uses The high density of marker bands in this standard makes it particularly useful for microsatellite analysis. All fragments have been checked for migration that is true to size under a wide variety of run conditions on all ABI PRISM instruments. There are no anomalous fragments (*e.g.*, the 250-bp fragment in GeneScan-350 on the ABI PRISM 310 Genetic Analyzer).

Note GeneScan-400HD is the recommended size standard for use with the ABI PRISM Linkage Mapping Set Version 2.

Fragment Lengths The following table lists the lengths of the 21 fragments comprising the GeneScan-400HD size standard:

Table 5-3 GeneScan-400HD: Fragment Lengths (nt)

50	160	260	360
60	180	280	380
90	190	290	400
100	200	300	
120	220	320	
150	240	340	

Preparation All aspects of the preparation of the GeneScan-400HD size standard are proprietary. Each fragment contains a single TAMRA or ROX fluorophore.

continued on next page

Denaturing Electropherogram

Although the GeneScan-400HD size standard is made of double-stranded DNA fragments, only one of the strands is labeled. Consequently, even if the two strands migrate at different rates under denaturing conditions you will not need to worry about peak splitting. Figure 5-3 shows the peak patterns of GeneScan-400HD fragments run under denaturing conditions.

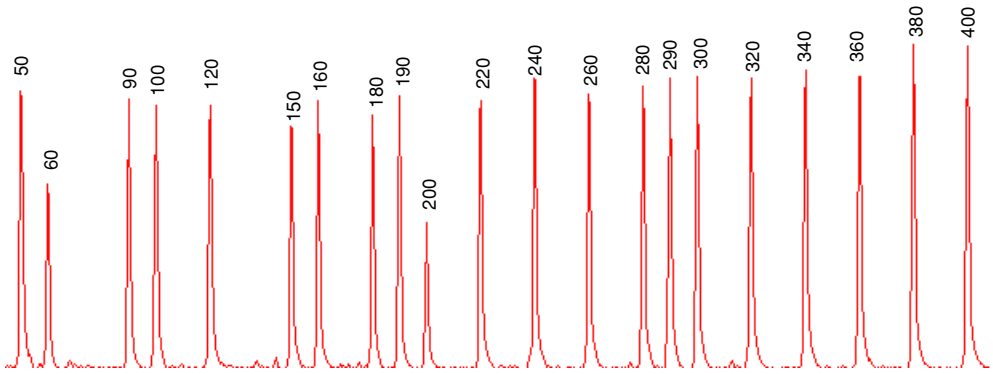


Figure 5-3 Electropherogram of the GeneScan-400HD size standard run under denaturing conditions on the ABI PRISM 310 Genetic Analyzer. Fragments were run using the POP-4 polymer at 60 °C.

Non-denaturing Electropherogram

Figure 5-4 shows the peak patterns of GeneScan-400HD fragments run under non-denaturing conditions.

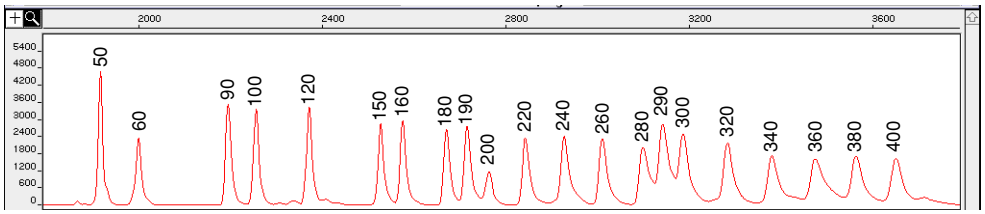


Figure 5-4 Electropherogram of the GeneScan-400HD size standard run under non-denaturing conditions on the ABI PRISM 310 Genetic Analyzer. Fragments were run using 3% GeneScan Polymer (GSP) at 30 °C.

GeneScan-500 Size Standard

Useful Range You can use the GeneScan-500 size standard to determine fragment lengths between 35 and 500 base pairs.

Special Uses These size standards are recommended for analysis of tri- and tetranucleotide microsatellite loci, which can often exceed 400 base pairs in length.

Fragment Lengths The following table lists the lengths of the 16 fragments comprising the GeneScan-500 size standard:

Table 5-4 GeneScan-500: Fragment Lengths (nt)

35	139	250[a]	400
50	150	300	450
75	160	340	490
100	200	350	500

a. Do not use this fragment for sizing. See IMPORTANT notice on page 5-13 for an explanation.

Preparation The GeneScan-500 size standard is prepared by digesting a proprietary DNA plasmid with Pst I, followed by ligating a TAMRA- or ROX-labeled, 22-mer oligodeoxynucleotide to the cut ends. A subsequent enzymatic digestion with BstU I yields DNA fragments containing a single TAMRA or ROX dye.

continued on next page

Denaturing Electropherogram

Although the GeneScan-500 size standard is made of double-stranded DNA fragments, only one of the strands is labeled. Consequently, even if the two strands migrate at different rates under denaturing conditions you will not need to worry about peak splitting.

Figure 5-5 shows the peak patterns of GeneScan-500 fragments run under denaturing conditions.

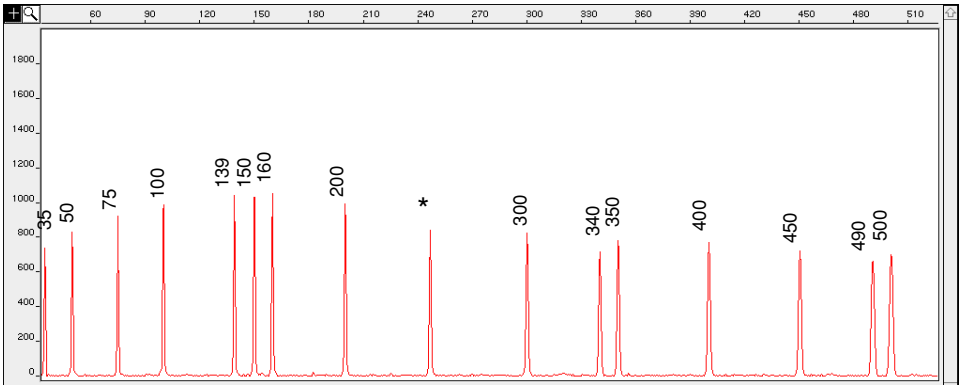


Figure 5-5 Electropherogram of the GeneScan-500 size standard run under denaturing conditions on the ABI PRISM 310 Genetic Analyzer. Fragments were run using the POP-4 polymer at 60 °C.

IMPORTANT An * for the 250-bp peak denotes a peak resulting from abnormal migration of double strands that did not completely separate under denaturing conditions. Do not use this peak to size samples. This peak shows variably smaller values than the actual size of the fragments.

Non-denaturing Electropherogram

Figure 5-6 shows the peak patterns of GeneScan-500 fragments run under non-denaturing conditions.

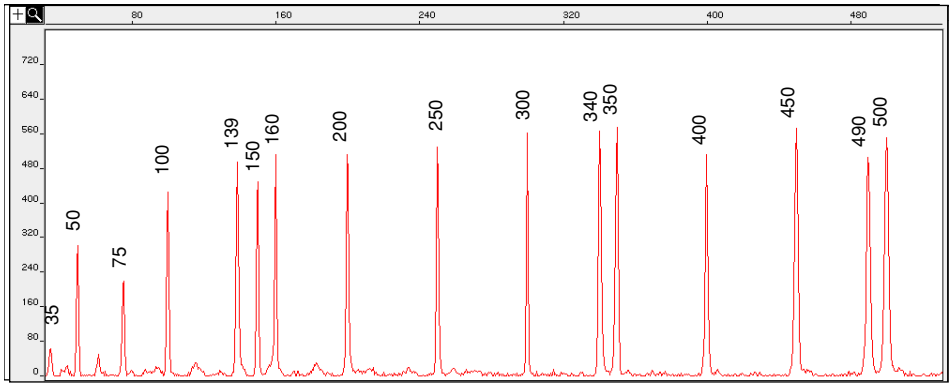


Figure 5-6 Electropherogram of the GeneScan-500 run under non-denaturing conditions on the ABI PRISM 310 Genetic Analyzer. Fragments were run using 3% GeneScan Polymer (GSP) at 30 °C.

GeneScan-1000 Size Standard

Useful Range

Under non-denaturing conditions, you can use the GeneScan-1000 size standard to determine fragment lengths between 100 and 900 base pairs. Under denaturing conditions using the POP-4 polymer, you can determine fragment lengths between 100 and 539 base pairs, the linear range (with respect to length in bp versus scan number) of the separation.

Special Uses

The GeneScan-1000 size standard fragments are labeled on both strands and thus are most suited for non-denaturing applications. If run under denaturing conditions, some or all of the peaks will appear split, making interpretation difficult. (Under denaturing conditions, all fragments will run 18 nucleotides smaller than the sizes shown in Table 5-5.)

Fragment Lengths

The following table lists the lengths of the 17 fragments comprising the GeneScan-1000 size standard.

Table 5-5 GeneScan-1000: Non-denatured Fragment Lengths (bp)

47	93	292	695
51	99	317	946
55	126	439	
82	136	557	
85	262[a]	692[a]	

a. Do not use these fragments for sizing. See IMPORTANT notice on page 5-15 for an explanation.

Note Non-denatured fragments are 18 nucleotides longer than denatured fragments.

Preparation

The GeneScan-1000 size standard is prepared by digesting pBR322 with the restriction enzyme Alu I, followed by ligating a ROX-labeled, 22-mer oligodeoxynucleotide to the cut ends.

continued on next page

Denaturing Electropherogram

Figure 5-7 shows the peak patterns of GeneScan-1000 fragments run under denaturing conditions.

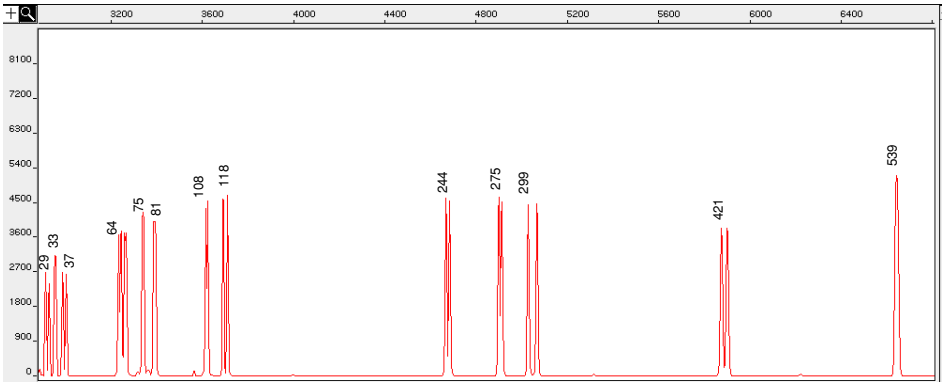


Figure 5-7 Electropherogram of the GeneScan-1000 size standard run under denaturing conditions on the ABI PRISM 310 Genetic Analyzer. Fragments were run using the POP-4 polymer at 60 °C.

Note Under denaturing conditions the two strands of the doubly-labeled GeneScan-1000 size standard fragments migrate at different rates, appearing as split peaks. To ensure size-calling precision and a reliable size standard definition, you must explicitly define one peak from each split peak pair in the size standard definition. To improve matching of size standard peaks, choose either LeftMost Peak or RightMost Peak in the Split Peak Correction section of the Analysis Parameters window.

Non-denaturing Electropherogram

Figure 5-8 shows the peak patterns of GeneScan-1000 fragments run under non-denaturing conditions.

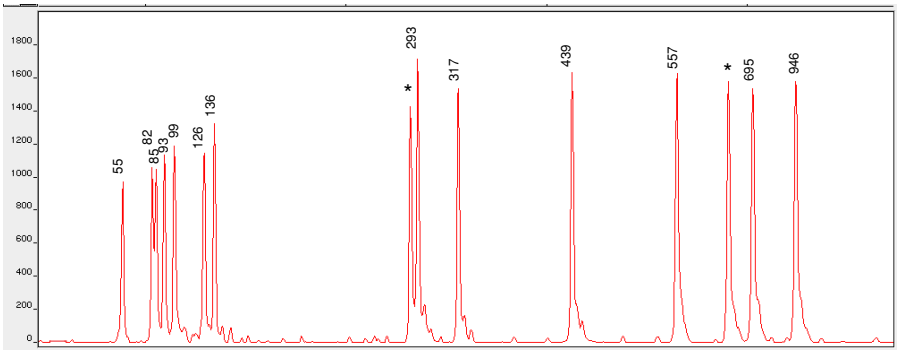


Figure 5-8 Electropherogram of the GeneScan-1000 size standard run under non-denaturing conditions on the ABI PRISM 310 Genetic Analyzer. Fragments were run using 3% GeneScan Polymer (GSP) at 30 °C.

IMPORTANT The * for the 262- and 692-bp peaks denote peaks resulting from abnormal migration. Do not use these peaks to size samples. These peaks show variably smaller values than the actual size of the fragments.

GeneScan-2500 Size Standard

Useful Range Under non-denaturing conditions, you can use the GeneScan-2500 size standard to determine fragment lengths between 100 and 5000 base pairs. Under denaturing conditions using the POP-4 polymer, you can determine fragment lengths between 100 and 536 base pairs, the linear range (with respect to length in bp versus scan number) of the separation.

Special Uses The GeneScan-2500 size standard fragments are labeled on both strands and thus are most suited for non-denaturing applications. If run under denaturing conditions, some or all of the peaks will appear split, making interpretation difficult. (Under denaturing conditions, all fragments will run 18 nucleotides smaller that the sizes shown in Table 5-6.)

Fragment Lengths The following table lists the lengths of the 27 fragments comprising the GeneScan-2500 size standard.

Table 5-6 GeneScan-2500: Non-denatured Fragment Lengths (bp)

55	240	488	1740	4547
112	251	508[a]	2026	4789
127	256	554	2180	5117
134	287	845	2483	14097
190	304	1133	2499	
204	379	1199	2878	

a. Do not use this fragment for sizing. See IMPORTANT notice on page 5-17 for an explanation.

Note Non-denatured fragments are 18 nucleotides longer than denatured fragments.

Preparation The GeneScan-2500 size standard is prepared by digesting phage λ DNA with Pst I, followed by ligating a TAMRA- or ROX-labeled, 22-mer oligodeoxynucleotide.

continued on next page

Denaturing Electropherogram

Figure 5-9 shows the peak patterns of GeneScan-2500 fragments run under denaturing conditions.

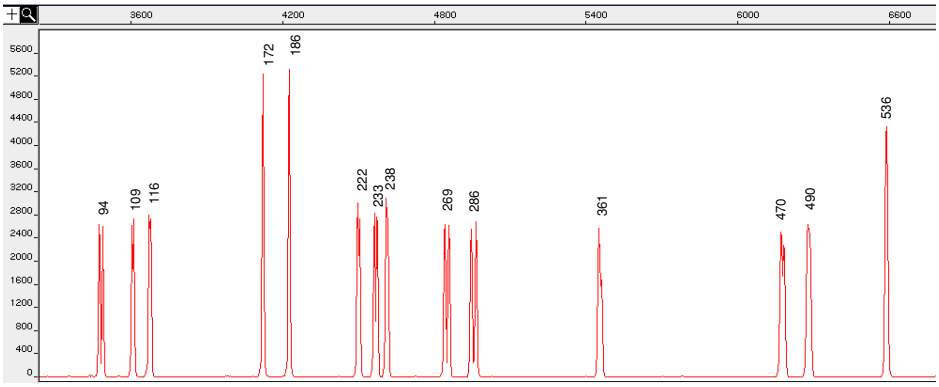


Figure 5-9 Electropherogram of the GeneScan-2500 size standard run under denaturing conditions on the ABI PRISM 310 Genetic Analyzer. Fragments were run using the POP-4 polymer at 60 °C.

Note Under denaturing conditions the two strands of the doubly-labeled GeneScan-2500 size standard fragments migrate at different rates, appearing as split peaks. To ensure size-calling precision and a reliable size standard definition, you must explicitly define one peak from each split peak pair in the size standard definition. To improve matching of size standard peaks, choose either LeftMost Peak or RightMost Peak in the Split Peak Correction section of the Analysis Parameters window.

Non-denaturing Electropherogram

Figure 5-10 shows the peak patterns of GeneScan-2500 fragments run under non-denaturing conditions.

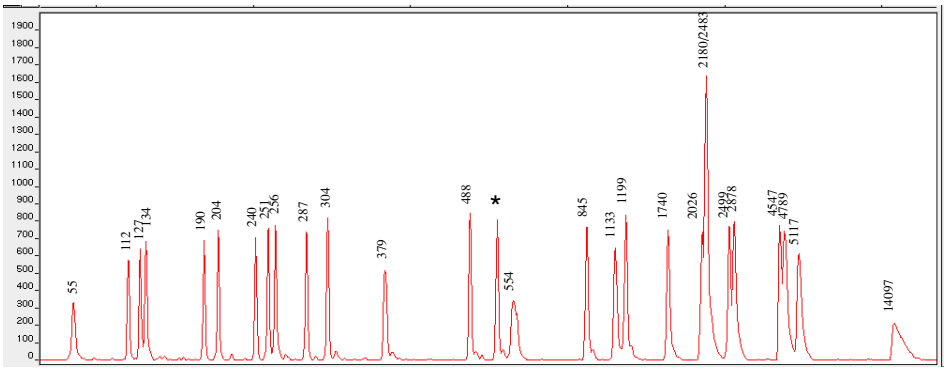


Figure 5-10 Electropherogram of the GeneScan-2500 size standard run under non-denaturing conditions on the ABI PRISM 310 Genetic Analyzer. Fragments were run using 3% GeneScan Polymer (GSP) at 30 °C.

IMPORTANT An * for the 508-bp peak denotes a peak resulting from abnormal migration. Do not use this peak to size samples. The peak has a smaller value than actual size of the fragment.

6 Optimizing PCR

Overview

In This Chapter

The success or failure of most GeneScan® fragment analysis experiments depends upon the success or failure of the PCR amplification step.

This chapter presents strategies that you can employ to optimize polymerase chain reaction (PCR) product yield in your experiments. Because no single set of PCR conditions is optimal for all applications, consider the optimization strategies discussed in this section as hints or tips for improving PCR product yield.

This chapter contains the following topics:

Topic	See Page
Choosing Reaction Volumes and Tube Types	6-2
Designing Custom Primers	6-3
Determining Reagent Concentrations	6-6
Choosing the Right Enzyme	6-8
Multiplexing PCR	6-10
Using RNA Templates	6-12
Preventing Competing Side Reactions—Hot Start PCR	6-13
Modifying Thermal Cycling Parameters	6-15
Avoiding Contamination	6-16
3´ A Nucleotide Addition	6-18
Stutter Products	6-21
Preparing PCR Products for Analysis	6-23

Choosing Reaction Volumes and Tube Types

Reaction Volumes

With Applied Biosystems PCR Instrument Systems, reaction volumes in the range of 25–100 µL are generally used. However, reactions with less than 25 µL have been successful.

Tube Types

The following table lists the type of reaction tube to use with each thermal cycler.

Thermal Cycler	Reaction Tube Type	Uses
DNA Thermal Cycler (TC1)	GeneAmp® PCR Reaction Tubes	Optimized for PCR amplification of reaction volumes between 25 µL and 100 µL. Mineral oil required.
DNA Thermal Cycler 480	0.5-mL GeneAmp Thin-walled Reaction Tubes with domed or flat caps[a]	Allows you to program shorter hold times (45 seconds or more) at each temperature in the PCR cycle. Mineral oil required.
GeneAmp PCR System 9600	0.2-mL MicroAmp® Reaction Tubes	Optimized for fast PCR amplification of reaction volumes between 25 µL and 100 µL. Use without mineral oil.
	0.5-mL GeneAmp Thin-walled Reaction Tubes with domed caps	Optimized for fast PCR amplification of 100-µL reaction volumes. 48-well adapter required. Mineral oil overlay or AmpliWax® PCR Gems required.
GeneAmp PCR System 9700	0.2-mL MicroAmp Reaction Tubes	Optimized for fast PCR amplification of reaction volumes between 25 µL and 100 µL. Use without mineral oil.
GeneAmp PCR System 2400	0.2-mL MicroAmp Reaction Tubes	Optimized for fast PCR amplification of reaction volumes between 25 µL and 100 µL. Use without mineral oil.

a. Can also be used with the DNA Thermal Cycler (TC1) using hold times of at least 60 seconds.

Using Small Amounts of Template

Although reaction tubes usually do not need to be sterilized or siliconized, autoclave tubes when dealing with small quantities (approximately 150–500 pg) of starting DNA template.

Autoclaved PCR tubes can also be ordered from Applied Biosystems:

- MicroAmp Autoclaved Reaction Tubes with Caps, P/N N801-0612 (for the GeneAmp PCR Systems 9600, 9700, and 2400)
- GeneAmp Autoclaved Thin-walled Reaction Tubes (with domed caps), P/N N801-0611 (for the DNA Thermal Cycler 480)

Designing Custom Primers

Definition

A PCR primer pair consists of two oligonucleotides, typically 15–30 nucleotides long, that hybridize to complementary strands of the DNA template and flank the region of interest.

To Ensure Successful Amplification

Choose primers with similar melting temperatures (T_m).

Choose primers that maximize the stability and specificity of binding to the desired template.

Note For best results, evaluate potential primers with the aid of primer-design software, *e.g.*, Primer Express™.

Melting Temperature

Choosing primers with similar T_ms makes it possible to find thermal cycling parameters that are optimal for all members of a primer pair or pairs. Be aware that the calculated T_m is only a guideline (based on base composition). The actual T_m is also influenced by the concentration of Mg^{2+}, K^+, and cosolvents.

Maximizing Stability

Binding stability is influenced by:

- Primer/template base composition
- Primer/template base order
- Primer or template secondary structure

Effects of Base Composition

G-C bonds contribute more to the stability (increased melting temperature) of primer/template binding than do A-T bonds.

To ensure stable binding of primer and template while avoiding problems with the internal secondary structure of primers or long stretches of any one base, choose primers with a 40% to 60% G+C content. However, do not let this rule interfere with primer choice based on T_m and primer-length considerations. Avoiding primer-dimers and gapped-duplex structures is more important than actual percent G+C.

Effects of Base Order

Two primer/template complexes with identical G+C content will have different melting temperatures because base order influences the overall stability. You can determine the exact effect of base order on complex stability using Table 6-1 (adapted from Salser, 1978).

Table 6-1 Base-Pairing Energies (kcal/dinucleotide pair)

	3´ Nucleotide			
5´ Nucleotide	**A**	**C**	**G**	**T**
A	–1.2	–2.1	–2.1	–1.8
C	–2.1	–4.8	–3.0	–2.1
G	–2.1	–4.3	–4.8	–2.1
T	–1.8	–2.1	–2.1	–1.2

Note In the table, larger negative values represent more stable interactions.

To illustrate the use of the table, consider the two sequences 3´–GAC–5´ and 3´–CGA–5´. The sequence 3´–GAC–5´ contained within a primer would contribute –4.2 kcal to the binding energy (–2.1 kcal [3´–GA–5´] + –2.1 kcal [3´–AC–5´] = –4.2 kcal). However, if the G and C are next to each other, as in 3´–CGA–5´, the contribution increases to –6.4 kcal (–4.3 kcal [3´–CG–5´] + –2.1 kcal [3´–GA–5´] = –6.4 kcal).

Note Although a G-C dinucleotide at the 3´ end of the primer can stabilize the binding complex when using thermostable enzymes such as AmpliTaq® DNA Polymerase, a 3´ G-C can also lead to false priming if you do not optimize PCR conditions. (For justification of the claim that a 3´ G-C can lead to false priming, see "Minimizing Binding to Secondary Sites" on page 6-4.)

Effects of Primer 2° Structure

Strings of Gs and Cs can form internal, non-Watson-Crick basepairs (Sarocchi *et al.*, 1970) that disrupt stable primer binding. Although this anomalous behavior is difficult to predict, a good general rule is to avoid runs of more than three consecutive Gs in primers. (See the following Note for exceptions to this rule.)

Note A short run of G's at or near the 5´ end of a primer will *not* disrupt stable primer binding because 5´ positioning does not lead to involvement in disruptive secondary structures (for example, primer-dimer or duplex loops).

Similarly, self-complementarity can lead to the formation of hairpin structures that disrupt stable primer binding. A stable hairpin can form with just four G-C basepairs in the stem and three bases in the loop (Summer *et al.*, 1985).

Effects of Template 2° Structure

Primers do not bind effectively to target sequences with known secondary structure. For example, RNA sequences often have regions of looped secondary structure.

Maximizing Specificity

Maximizing binding specificity requires minimizing primer binding to secondary sites in the DNA and to other primers.

Minimizing Binding to Secondary Sites

Note This section is most applicable if your starting template is genomic DNA. The probability of binding to secondary sites is greatly diminished for low-complexity templates such as plasmid DNA.

Ideally, the binding of the primer to the desired template is:

- Strongest at the 5´ end.
- More positive than -9.8 kcal/mole at the 3´ end.

  This is equivalent to saying that the binding at the 3´ end is weaker than -9.8 kcal/mole.

Polymerases only require the binding of the nucleotides at the 3´ end to begin elongation. If the 3´ nucleotides bind strongly (perhaps because of a 3´ G-C) any template sequences that are complementary to the 3´ end are amplified. In this case, because the entire primer is not used to discriminate among target sequences, specificity is lost.

Conversely, if binding is strongest at the 5´ end, the typical binding event on the template DNA begins at the 5´ end. Polymerases, however, cannot begin elongation

until the 3´ end binds. Therefore the entire primer is used to distinguish among target sequences.

Self-complementarity can lead to the formation of hairpin structures that decrease binding specificity (as well as disrupt binding stability as discussed earlier). Nucleotides in the hairpin structure are not available for recognition of the target sequence. The available nucleotides can be thought of as forming a “smaller,” and therefore less specific, primer.

When performing a computer-assisted search to evaluate binding to secondary sites in the target DNA, consider the potential for “gapped duplex” formation.[1]

Note Binding to secondary sites can also involve the formation of stable non-Watson-Crick base pairs (Topal and Fresco, 1976). Stable base-pairing is most likely to occur between G and T, but A-C and G-A pairs can also be stable (Hunter, 1986). All software programs have difficulty modeling these sorts of interactions.

Minimizing Binding to Other Primers

Complementarity between two primers, especially at the 3´ ends, can lead to the formation of product artifacts arising from amplified primer-dimers and primer-oligomers. Avoid primers with regions of complementarity between members of a primer pair or pairs.

Post-Amplification Manipulation

Adding 5´ primer-extensions (that are not complementary to the template) can facilitate a variety of useful post-amplification manipulations of the PCR product without adversely affecting yield. Examples include 5´ extensions that contain restriction sites, universal primer binding sites, or promoter sequences.

1. A gapped duplex can form when the primer and target are completely complementary except for a single base (Miller, Kirchoff *et al.*, 1987; Miller, Wlodawer *et al.*, 1987).

Determining Reagent Concentrations

Factors to Consider

When preparing reaction mixtures, consider the following factors that can affect overall yield of specific DNA target sequences:

- dNTP concentration
- Magnesium ion concentration
- Primer concentration
- Template concentration
- Enzyme concentration

dNTP Concentration

In the standard GeneAmp PCR protocol, the concentration of each deoxynucleoside triphosphate (dNTP) is 200 µM.

In most cases, lower dNTP concentrations do not significantly affect the yield of PCR amplification product and will increase the fidelity. However, for efficient base incorporation, keep the four dNTP concentrations balanced and above the estimated K_m of each dNTP (10–15 µM).

Some applications might require higher dNTP concentration (especially when dNTP analogues are used). However, excess dNTPs decrease enzyme fidelity.

Magnesium Ion Concentration

DNA polymerases require free magnesium ion in solution for activity. For most PCR amplifications, you can relate product yield and specificity and enzyme fidelity to the free magnesium ion (Mg^{2+}) concentration:

$$[\text{free } Mg^{2+}] = [\text{total } Mg^{2+}] - [\text{total dNTP}] - 2[\text{EDTA}]$$

In general, increasing the free magnesium concentration increases yield and decreases specificity and fidelity.

To identify the magnesium concentration that gives the best compromise between yield and specificity or fidelity for your particular application, perform the following experiment. In the presence of 800 µM total dNTP concentration, run a $MgCl_2$ reaction series in 50-µM increments over the range from 100–400 µM $MgCl_2$ and identify the optimal concentration.

continued on next page

Template Concentration

The concentration of template in your sample can affect the success of PCR amplification in a variety of ways. Too much template promotes nonspecific binding of primers to secondary sites or changes the pH of the reaction mix. Too little template can result in poor yields, especially if the template is degraded.

Even very low template concentrations (10 copies) are often sufficient for successful PCR amplification.

If your starting sample is DNA, you can use up to 20,000 copies of the target to start optimization trials. In general, this translates to:

- 1–5 ng of cloned template
- 200 ng to 1 µg of genomic DNA

  Start optimization trials with less genomic DNA if starting material is limited. With clean, good quality genomic DNA, 500–1000 pg of starting material almost always works well.

Enzyme Concentration

For most PCR applications, 2.0–2.5 units of AmpliTaq Gold™ DNA Polymerase is recommended for each 100-µL reaction volume.

Note You can avoid the tedium and inaccuracies involved in pipetting 0.5-µL amounts of enzyme by adding the enzyme to a fresh Master Mix prepared for a number of reactions.

Choosing the Right Enzyme

Introduction

For most applications AmpliTaq Gold DNA Polymerase is the enzyme of choice. However, Applied Biosystems supplies a number of PCR enzymes that have been optimized for specific needs. For quick reference, an enzyme-choice table is included at the end of this section.

PCR Enzyme Overview

A brief summary of the seven PCR enzymes supplied by Applied Biosystems follows.

Derivatives of Native Taq DNA Polymerase

- AmpliTaq DNA Polymerase

  AmpliTaq DNA Polymerase is a recombinant form of Taq DNA Polymerase obtained by expressing a modified Taq DNA Polymerase gene in an *E. coli* host. Like native Taq DNA Polymerase, it lacks endonuclease and 3´-5´ exonuclease (proofreading) activities, but has a 5´-3´ exonuclease activity.

- AmpliTaq Gold DNA Polymerase

  AmpliTaq Gold DNA Polymerase is a chemically modified form of AmpliTaq DNA Polymerase. It provides the benefits of Hot Start PCR (that is, higher specific product yield, increased sensitivity, and success with multiplex PCR) without the extra steps and modifications of experimental conditions that make Hot Start impractical for high throughput applications. AmpliTaq Gold DNA Polymerase is delivered in an inactive state. A prePCR heating step of 10–12 minutes at 95 °C, which can be programmed into the thermal cycling profile, activates the enzyme.[1] For low template copy number amplifications, step-wise activation of AmpliTaq Gold DNA Polymerase, or Time Release PCR, can prove useful.[2]

- AmpliTaq DNA Polymerase, LD

  AmpliTaq DNA Polymerase, LD (Low DNA), is the same enzyme as AmpliTaq DNA Polymerase. However, the LD formulation has undergone a further purification process. The purification step insures that false-positive PCR products will be effectively minimized when amplifying bacterial sequences. AmpliTaq DNA Polymerase, LD is especially useful for low-copy number amplifications.

- AmpliTaq DNA Polymerase, Stoeffel Fragment

  AmpliTaq DNA Polymerase, Stoeffel Fragment, is a modified form of AmpliTaq DNA Polymerase from which the N-terminal 289 amino acids have been deleted. It is approximately twofold more thermostable than AmpliTaq DNA Polymerase allowing higher denaturation temperatures for GC-rich templates or templates with complex secondary structure. It lacks 5´-3´ exonuclease activity making it useful in multiplex PCR. Finally, it is active and specific over a wide range of magnesium ion concentrations (2 –10 mM).

1. A prePCR incubation heating step of 10 minutes at 95 °C activates approximately 40% of the enzyme molecules. This is sufficient to perform efficient amplification during the early cycles when target copy number is low. Because more enzyme is activated during each denaturation step, enzyme activity increases as the number of target molecules increases, providing optimal PCR performance.
2. In Time Release PCR, the prePCR heating step is omitted and the total number of cycles is increased. Because the activation step is omitted, very little active enzyme is present during the first few PCR cycles and many additional cycles are necessary for good results.

UlTma® DNA Polymerase

UlTma DNA Polymerase is obtained by expressing a modified form of the *Thermotoga maritima (Tma)* DNA polymerase gene in an *E. coli* host. It lacks a 5´-3´ exonuclease activity but retains a 3´-5´ exonuclease proofreading activity. It is recommended when a high degree of fidelity is required.

Derivatives of *Tth* DNA Polymerase

Applied Biosystems supplies two modified forms of *Thermus thermophilus (Tth)* DNA Polymerase:

- r*Tth* DNA Polymerase

  r*Tth* DNA Polymerase is obtained by expression of a modified form of the *Tth* gene in an *E. coli* host.

- r*Tth* DNA Polymerase, XL

  r*Tth* DNA Polymerase, XL (Extra Long), provides the same features as r*Tth* DNA Polymerase for target sequences from 5–40 kb in length. An inherent 3´-5´ exonuclease activity allows for the correction of nucleotide misincorporations that might otherwise terminate synthesis prematurely.)

Enzyme Choice Table

If your application has special requirements, the table below will help you choose the best enzyme:

Table 6-2 Application Requirements and Recommended Enzymes

If your application requires...	Use...
High specificity	AmpliTaq Gold DNA Polymerase
High sensitivity	AmpliTaq Gold DNA Polymerase
High fidelity	*UlTma* DNA Polymerase
High temperatures	AmpliTaq DNA Polymerase, Stoeffel Fragment *UlTma* DNA Polymerase
Multiplex PCR	AmpliTaq Gold DNA Polymerase
Amplification of low-copy number template	AmpliTaq Gold DNA Polymerase AmpliTaq DNA Polymerase, LD (for bacterial sequences)
High specificity at high ionic strength	AmpliTaq Gold DNA Polymerase AmpliTaq DNA Polymerase, Stoeffel Fragment
Amplification of extra-long fragments (>5 kb)	r*Tth* DNA Polymerase, XL
PrePCR conversion to cDNA	r*Tth* DNA Polymerase
Extra cycles	AmpliTaq DNA Polymerase, Stoeffel Fragment *UlTma* DNA Polymerase
High Mg^{2+} concentration	AmpliTaq Gold DNA Polymerase AmpliTaq DNA Polymerase, Stoeffel Fragment

Multiplexing PCR

Definition

Multiplex PCR is a technique for simultaneously amplifying multiple DNA targets using multiple primer pairs in the same PCR reaction.

Advantages

Multiplex PCR can:

- Simplify PCR setup
- Increase throughput
- Decrease cost per amplification

Limitations

Potential limitations to multiplex PCR include:

- Primer-oligomer formation
- Loss of specificity
- Decreased yield of specific products

Overcoming these limitations can require a significant amount of optimization.

Enzyme Choice

The high specificity of AmpliTaq Gold DNA Polymerase typically permits amplifying with elevated Mg^{2+} concentrations for increased yield.

Primer Quality

Because reactants (such as dNTPs) are often limiting during multiplex PCR, using high quality primers is particularly important. For example, the decreased specificity (and thus the increased reagent consumption) of one pair of degraded PCR primers can prevent the success of the entire multiplex reaction. Although you can compensate for a degraded pair of primers to some extent by increasing the concentration of the other primer pairs, the increased cost per reaction and the decreased reproducibility over time do not justify this short-term solution.

When buying or making primers make sure that they are length purified and that they are free of contaminants.

Primer-Pair Concentrations

Typically, start out with equal concentrations for all primer pairs.

It will often be necessary to adjust the concentration of primer pairs in the multiplex reaction until the peak heights are relatively even.

- Increase the primer-pair concentration for fragments showing weak amplification.
- Decrease the primer-pair concentration for fragments showing significantly greater than average amplification.

Troubleshooting Multiplex PCR

Consider amplifying separately any primer pair that fails to amplify after its concentration is increased.

To get rid of interfering background peaks, try:

- Swapping primer pairs between different multiplex reactions
- Removing primer pairs from the multiplex reaction

Using RNA Templates

Suitable Templates

RNA templates can be single- or double-stranded. If your starting sample is RNA, then your template can be:

- Total cellular RNA
- Poly (A+) RNA
- Viral RNA
- tRNA
- rRNA

Two-Step RNA-PCR

To synthesize first-strand cDNA prior to performing conventional PCR amplification, you can use a reverse transcriptase such as MuLV or you can use r*Tth* DNA Polymerase.

In the presence of $MnCl_2$, r*Tth* DNA Polymerase will efficiently reverse transcribe RNA to cDNA. After chelating the manganese ions with EGTA and adding $MgCl_2$, r*Tth* DNA Polymerase can act as a thermostable DNA polymerase in a subsequent reaction in the same tube.

Note For RNA templates with a high GC content or complex secondary structure, Applied Biosystems recommends using r*Tth* DNA polymerase because of its high-temperature reverse transcriptase activity and thermostable DNA polymerase activity.

Single-Step RNA-PCR

Using the GeneAmp EZ r*Tth* RNA PCR Kit (P/N N808-0178), you can perform reverse transcription and PCR amplification in successive reactions in the same tube without buffer changes or reagent additions. By combining programmed changes in temperature with the action of Bicine, which is capable of buffering both metal and hydrogen ions, in the EZ Buffer, the GeneAmp EZ r*Tth* RNA PCR Kit enables you to control a complex interaction of factors (metal ion concentration, pH, and ionic strength) affecting enzyme activity.

Note When amplifying multiple samples, making buffer changes is time-consuming and increases the likelihood of contamination. The EZ Buffer affords full dUTP compatibility for AmpErase® UNG-mediated carryover prevention.

For More Information

Refer to the *TaqMan® Gold RT-PCR Kit Protocol* (P/N 402876) and the *TaqMan EZ RT-PCR Kit Protocol* (P/N 402877) for more information.

Preventing Competing Side Reactions—Hot Start PCR

When to Use Hot Start

Consider using the Hot Start technique whenever you need to improve the specificity and sensitivity of your PCR amplifications. Loss of specificity and sensitivity are often caused by competing side reactions, which usually occur during the prePCR setup period while all reactants sit together at permissive temperatures. (A common competing side reaction involves the amplification of nontarget sequences in background DNA either due to mispriming or to primer oligomerization.)

Limitations and Alternatives

The technique is cumbersome. If you have high throughput needs, switching to AmpliTaq Gold DNA Polymerase will give the same benefits as performing the Hot Start technique, without the need for using wax barriers or opening reaction tubes. In particular, if you are already using AmpliTaq Gold DNA Polymerase, performing the Hot Start technique will not improve the specificity and sensitivity of PCR amplification.

How Hot Start Works

In the Hot Start technique, components necessary for amplification are separated so that critical reactants do not mix until reaching a temperature sufficiently high to suppress primer self-annealing or annealing to nontarget sequences.

Hot Start Options

You can perform either a manual Hot Start or an AmpliWax PCR Gem-mediated Hot Start.

Note Although manual Hot Start can increase specificity and yield, it is inconvenient and you can encounter reproducibility and contamination problems.

Performing a Manual Hot Start

Note The manual Hot Start protocol requires the use of mineral oil to prevent evaporation. Thus you cannot perform a manual Hot Start in the GeneAmp PCR systems 2400, 9600, and 9700.

Step	Action
1	Mix all reagents except one key component (choose from dNTPs, $MgCl_2$, or primers) below a mineral oil cap.
2	Load all tubes into the PCR instrument system.
3	Define temperature control parameters so that the temperature rises to 70–80 °C.
4	Add the missing component to each tube, changing pipet tips after each sample.

continued on next page

Performing an AmpliWax PCR Gem-mediated Hot Start

Step	Action
1	At room temperature, add primers, $MgCl_2$, dNTPs, and buffer to reaction tube.
2	Add a single AmpliWax PCR Gem to each reaction tube. **Note** The mass of the PCR Gem necessary for proper performance depends upon the reaction volume and reaction tube geometry.
3	Define temperature control parameters so that the temperature rises to 70–80 °C for 5–10 minutes, then cools to 2–35 °C. **Note** This creates a wax barrier over the aqueous layer.
4	Add the polymerase and sample buffer above the solid wax layer.
5	Create a PCR thermal profile as follows: a. Rapidly heat samples to the first denaturation temperature. This melts the wax layer, denatures the DNA template, and creates enough thermal convection to assure complete mixing of all PCR components under the melted wax. The wax also serves as a vapor barrier during cycling. b. Program temperature control parameters for conventional thermal cycling. c. Cool samples to 2–35 °C at the end of the run.
6	Run the PCR. **Note** After thermal cycling ends, the wax will form a solid shield preventing spillage and evaporation.
7	For post-PCR analysis, you can penetrate the wax layer with a pipet tip and withdraw the PCR product.
8	Reheat the reaction tube to seal for long-term storage.

Modifying Thermal Cycling Parameters

Guidelines

The following table summarizes the effects of modifying temperature control parameters on PCR performance.

Specific Change in Thermal Cycling Parameter	Effect on PCR Performance
Raising denaturation temperatures (up to 96 °C)	Can be necessary to allow denaturation, especially with GC-rich templates Can also cause template degradation by depurination
Lowering annealing temperatures	Can increase yield, but can reduce specificity
Raising annealing temperatures	Increases specificity, but can reduce yield
Setting the denaturation, annealing, and extension step to at least: ♦ 15 seconds (preferably 30 seconds) with the GeneAmp PCR System 9600, 9700, or 2400 ♦ 45 seconds using thin-walled tubes with the DNA Thermal Cycler 480 ♦ 1 minute using thick-walled tubes with the DNA Thermal Cycler 480 or the DNA Thermal Cycler (TC1)	Allows samples to reach thermal equilibrium at each stage
Using the autoextension (or AutoX) function of a thermal cycler to allow longer extension times in later cycles[a]	Increases yield by allowing complete extension of PCR product in later cycles

a. For most applications, an extension temperature of 72 °C is effective and rarely requires optimization. In the two-temperature PCR process, the combined annealing/extension step temperature should range from 60–70 °C.

Temperature Optimization

To find the optimal thermal cycling parameters, perform a series of runs varying the annealing or denaturation temperatures in 2 °C increments.

Note Do not vary more than one parameter at a time.

Avoiding Contamination

Introduction

PCR protocols are extremely sensitive to contaminants in the DNA. Although many protocols that describe “simple” or “fast” extraction or purification methods have been published recently, you should carefully evaluate any changes or improvements in extraction or purification methods. (Also, be sure that the physical and chemical condition of the sample itself are adequate for the intended labeling and assay methods.)

Avoiding Contamination from the Environment

To avoid general contamination, take the following precautionary measures:

- Change pipet tips between samples.
- Use filter-plugged pipet tips.
- Clean any work contaminated surface using a cloth soaked with 50% bleach.

IMPORTANT Before cleaning the sample block of a thermal cycler, refer to the instrument manual for the proper procedure.

- Close sample tubes when not using them.
- Always run a no-DNA negative control.

  A negative control contains no template DNA, only primers and the DNA diluent (usually water or buffer).
- Aliquot reaction reagents so as to minimize the number of times you use a particular stock solution.

Avoiding PCR Product Carryover

Definition

PCR product carryover is the contamination of an unamplified sample with previously amplified DNA.

Why Carryover Is a Particular Concern

PCR product carryover is a particular concern because amplified PCR product serves as an ideal template for subsequent amplifications of that same target.

A single PCR amplification produces a large number of copies (as many as 10^{13}). The inadvertent transfer of even a minute volume or aerosol of amplified product can mean significant contamination. This can result in false-positives and the detection and amplification of the contaminating sequence at the expense of the target sequence.

Precautionary Measures

Adopting these precautionary measures can help you minimize the likelihood of PCR product carryover.

- Use positive displacement pipettes or filter-plugged pipette tips.
- Physically separate reactions prior to and following amplification.
- Handle pre- and post-PCR solutions with separate sets of the following:
  - Pipettes
  - Pipette tips
  - Microcentrifuge tubes

- Gloves

- Use AmpErase® UNG in reaction mixtures to prevent the subsequent reamplification of dU-containing PCR products.

For More Information

Applied Biosystems supplies the GeneAmp PCR Carryover Prevention Kit (P/N N808-0068) and AmpErase UNG (P/N N808-0096) to ensure that PCR products cannot be reamplified in subsequent PCR amplifications.

3´ A Nucleotide Addition

Introduction

The AmpliTaq and AmpliTaq Gold DNA Polymerases, like many other DNA polymerases, catalyze the addition of a single nucleotide (usually an adenosine) to the 3´ ends of the two strands of a double-stranded DNA fragment. This non-template complementary addition results in a denatured PCR product that is one nucleotide longer than the target sequence. A PCR product containing the extra nucleotide is referred to as the "plus-A" form.

Why Incomplete 3´ A Nucleotide Addition Can Cause Problems

Because 3´ A nucleotide addition rarely goes to completion without a long extension step at the end of thermal cycling (*i.e.*, only a fraction of the fragments receive the extra nucleotide), single-base ladders often form (Figure 6-1), creating peak patterns that Genotyper® software might not interpret correctly. The resulting allele calls can be inconsistent, incorrect, or missing entirely, forcing you to inspect all allele calls and to correct erroneous calls manually.

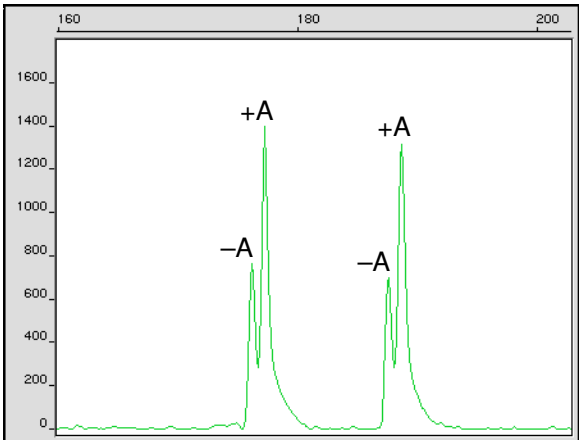


Figure 6-1 Split peaks resulting from incomplete 3´ A nucleotide addition

How to Avoid Problems Caused by Incomplete 3´ A Nucleotide Addition

- Modify the thermal cycling conditions either to promote or to inhibit 3´ A nucleotide addition.
- Modify Mg^{2+} concentration either to promote or to inhibit 3´ A nucleotide addition.
- Modify, or "tail," the 5´ end of the reverse primer either to promote or to inhibit 3´ A nucleotide addition to the (forward) labeled strand.
- Treat PCR products enzymatically to remove the 3´ A overhangs.

In general, the most reliable strategy is to maximize 3´ A nucleotide addition by modifying thermal cycling conditions and Mg^{2+} concentration, and (if necessary) by tailing the reverse primer.

continued on next page

Modifying Thermal Cycling Conditions

Increasing the time spent between 60 and 72 °C promotes 3´ A nucleotide addition. Decreasing the time spent between 60 and 72 °C inhibits 3´ A nucleotide addition.

To use this method effectively, you need to determine the optimal thermal cycling conditions for each marker in each set of reaction conditions.

Promoting 3´ A nucleotide addition has proven to be the more successful strategy. Residual polymerase activity at room temperature (or even at 4 °C) is often sufficient to catalyze enough 3´ A nucleotide addition to create genotyping problems. Many protocols increase the final extension step to 30–45 minutes to promote 3´ A nucleotide addition.

Modifying Mg^{2+} Concentration

Increasing the Mg^{2+} concentration promotes 3´ A nucleotide addition. Decreasing the Mg^{2+} concentration inhibits plus-A addition.

In general, optimizing the Mg^{2+} concentration is best employed in conjunction with other strategies. If you choose to maximize 3´ A nucleotide addition, consider using AmpliTaq Gold DNA Polymerase at 2.5 mM $MgCl_2$.

Reverse-Primer Tailing

What It Is

Brownstein *et al.* (1996) found that adding additional nucleotides (a “tail”) to the 5´ end of the reverse PCR primer either promoted or inhibited 3´ A nucleotide addition to the (forward) labeled strand depending upon the sequence of the added nucleotides.

General Rule

Magnuson *et al.* (1996) noticed a correlation between tail sequence and the amount of 3´ A nucleotide addition. In particular, they found that adding a single G to the 5´ end of the reverse PCR primer generally resulted in almost complete 3´ A nucleotide addition. Therefore, using a tail to promote 3´ A nucleotide addition can yield a consistently callable pattern.

Things to Consider

Reverse-primer tailing has advantages compared to other methods because it:

- Works well under diverse reaction conditions
- Does not require additional experimental steps

Enzymatic Treatment

Ginot *et al.* (1996) used T4 DNA polymerase to remove the 3´ A overhangs from pooled PCR products.

Things to Consider

Although effective, this method has serious limitations because it:

- Requires a post-PCR, enzymatic treatment step
- Requires titrating each lot of T4 DNA polymerase to determine optimal enzyme concentrations and treatment times

IMPORTANT Excess T4 treatment can cause PCR product degradation, whereas insufficient treatment will fail to correct the problem and can even make some alleles more difficult to call.

Specific Suggestion

To begin optimization trials, use 0.5–1 unit of T4 DNA polymerase in 10 µL of pooled PCR product. Incubate at 37 °C for 30 minutes.

For More Information

Refer to the *AmpFℓ*STR Profiler *Plus PCR Amplification Kit User's Manual* (P/N 4303501) for more information on maximizing 3´ A nucleotide addition.

Tailed primers are available through the Applied Biosystems Custom Oligonucleotide Synthesis Service. Call (800)345-5224 for price and availability.

Stutter Products

What is Stutter? During the PCR amplification of di-, tri-, and tetranucleotide microsatellite loci, minor products that are 1–4 repeat units shorter than the main allele are produced. The minor product peaks are referred to as “stutter” peaks. Stutter peaks might be caused by polymerase slippage during elongation.

Stutter Facts You can estimate the percent stutter by calculating the ratio of the combined heights of the stutter peaks with the height of the main allele peak. Some general conclusions about percent stutter follow:

- The longer the length of the repeat unit, the less stutter product made (Figure 6-2).

  In other words, among microsatellite loci with the same number of repeat units, the percent stutter is greater for dinucleotide microsatellite loci than it is for trinucleotide microsatellite loci, and so on (Walsh *et al.*, 1996).

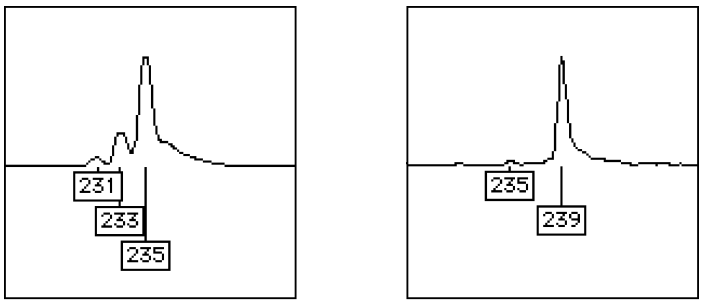


Figure 6-2 Comparison of the amounts of stutter in dinucleotide (left) and tetranucleotide (right) repeat loci. Each locus is homozygous, with the largest peak in each picture representing the “true” allele.

- The percent stutter increases with increasing allele length (*i.e.*, with increasing number of repeat units) as shown in Figure 6-3 on page 6-22.

  This rule is likely to be violated if the repeats are not perfect (*e.g.*, if some of the repeats are partial repeats).

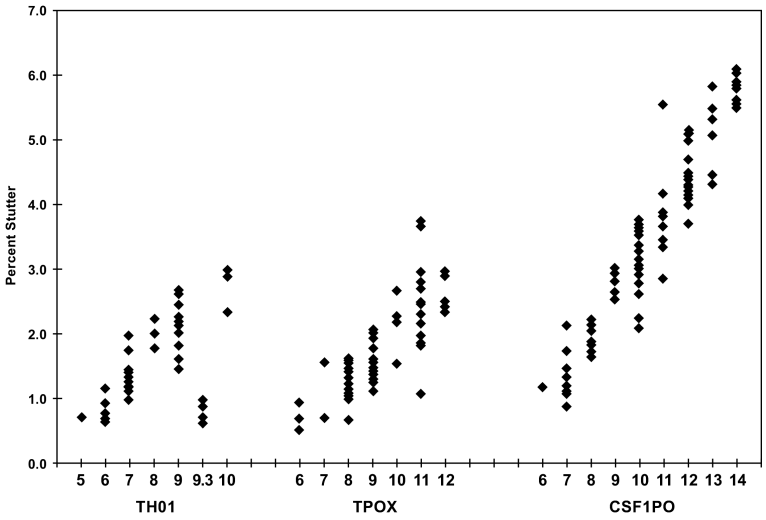


Figure 6-3 Percent stutter observed for the STR loci in the AmpFℓSTR Green™ I PCR Amplification Kit

Evaluating Data with Stutter

The multiband stutter pattern can complicate analysis, particularly of samples with two or more alleles that are close in size. For example, faint bands in a position one repeat unit smaller than the main allele can be interpreted either as a stutter band or as an allele in a minor component of a mixed sample. The possibility of stutter makes precise quantitation especially important to enable the Genotyper software's filtering algorithm to interpret the peak pattern accurately.

Fortunately, the percent stutter for a given allele is reproducible. In particular, the percent stutter does not depend on the quantity of input DNA or the number of loci amplified during multiplex PCR. The relative invariance of percent stutter is important for a few reasons:

- In many cases, you can adjust the Peak Amplitude Threshold in the Analysis Parameters window of the GeneScan software so that the analysis program ignores stutter peaks while recognizing true allele peaks.
- Amplifications with an abnormally high percent stutter can indicate mixed samples (or some other problem with PCR amplification or electrophoresis).

See page 8-25 for examples of stutter patterns in dinucleotide repeat loci.

Preparing PCR Products for Analysis

Dilution

You will almost always need to dilute PCR amplification products before adding them to the sample tube. Typically, the required dilution lies in the range from 1:3–1:80 (PCR product: distilled, deionized H_2O).

A good way to proceed is to begin by optimizing PCR run conditions for your specific application. Then run a dilution series on your ABI PRISM® 310 Genetic Analyzer to determine the optimal dilution. Alternatively, run 1 µL of PCR product on a mini-gel. If, after ethidium bromide staining, the product signal is visible but not oversaturated, try a 1:10 dilution om your ABI PRISM 310 instrument.

After determining the optimal dilution ratio, you can use the same dilutions for subsequent analyses as PCR yields should be fairly consistent.

Purification

If the expected product length is within 50 nucleotides of the primer length, remove unincorporated primers before performing electrophoresis. It is often a good idea to remove unincorporated primers anyway, unless the PCR is run to completion.

SSCP Analysis 7

Overview

About This Chapter

This chapter provides detailed instructions for performing Single-Strand Conformation Polymorphism (SSCP) mutation analysis on the ABI PRISM® 310 Genetic Analyzer.

SSCP analysis is highly sensitive to electrophoresis conditions. The protocol contained in this section is a good starting point for beginning your own optimization trials and includes suggestions for improving performance.

This chapter contains the following topics:

Topic	See Page
Introduction to SSCP Analysis	7-2
Before You Begin	7-3
PCR Amplification, Labeling, and Controls	7-4
To Save Time—Prerun Checklist	7-7
Preparing for a Run	7-8
Analyzing the Data	7-12
Optimizing SSCP Run Conditions	7-14
Troubleshooting	7-17

Introduction to SSCP Analysis

What is SSCP?

Single-strand conformation polymorphism (SSCP) analysis is an application that detects mutations based upon the ability of a single (or multiple) nucleotide change to alter the electrophoretic mobility of a single-stranded DNA molecule under non-denaturing conditions.

Under non-denaturing conditions most single-stranded DNA molecules will assume one or more stable three-dimensional conformations that depend on the nucleotide sequence. In many cases the change of a single nucleotide will cause a conformational change that can be detected as a change in the electrophoretic mobility as compared to the wild type sequence.

Advantages

You can analyze a large number of samples using SSCP technique because the technique is simple and fast. The only step necessary after PCR amplification is a heat denaturation in formamide and NaOH. Moreover, as with any PCR-based technique, you can analyze mutations in a specified DNA region by choosing PCR primers that span that region.

Limitations

SSCP analysis indicates only that a mutation exists. You must perform subsequent DNA sequencing to determine the nature of the mutation that caused an electrophoretic mobility shift in a given sample.

Moreover, not all point mutations in a given sequence will cause a detectable change in electrophoretic mobility. However, by optimizing PCR reactions and run conditions before attempting a large-scale analysis you can increase the sensitivity. (See “Optimizing SSCP Run Conditions” on page 7-14 for more details.)

Changes in relative mobility due to minor variations in electrophoresis conditions limit the ability to compare results obtained on different instrument platforms or even in different laboratories.

Advantages of Using ABI PRISM Technology

Ability to Vary Electrophoresis Temperature

The mobility difference between the wild type and a mutant strand is very sensitive to temperature. Within the temperature range from 25 °C (if your laboratory permits) to 40 °C the temperature for the best differentiation of the two strands will depend upon the particular mutant/wild type strand combination.

With the ABI PRISM 310 Genetic Analyzer, you can automate electrophoresis of the same sample at different temperatures. Running the same samples at different temperatures will maximize your chances of detecting mutations.

Whatever temperature regime you choose, strict temperature control is crucial to ensure consistency because three-dimensional conformation is highly sensitive to changes in temperature.

Rapid Analysis

The ABI PRISM 310 Genetic Analyzer allows extremely rapid separations. Fragments that are 300 bp or less in length can be separated in under 30 minutes. This translates to a throughput of at least 48 samples in a 24-hour period.

Before You Begin

Materials Required

You will need the following materials to perform an SSCP analysis run.

- ABI PRISM Genetic Analyzer Capillary labeled with a green mark (L_t = 47 cm, L_d = 36 cm, i.d. = 50 µm)
- 3% GeneScan Polymer (GSP) with 10% (w/w) glycerol and 1X TBE

Note Optimization might require different polymer concentrations and capillary lengths. See "5% GeneScan Polymer with 10% Glycerol" on page A-1 for details.

- GeneScan Internal Lane Size Standard (recommended: GeneScan-500 or GeneScan-350)
- Deionized formamide
- 1X TBE buffer containing 10% glycerol

Note See "1X TBE with 10% Glycerol" on page A-2 for details.

- Sodium hydroxide (NaOH), 0.3 N, stored in a plastic container
- 4.0-mL Genetic Analyzer Vials (do not reuse)
- 1.0-mL GeneScan Glass Syringe
- 1.5-mL Eppendorf tube with the lid removed

For a 48-well tray:

- 0.5-mL Genetic Analyzer Sample Tubes (do not reuse)
- Genetic Analyzer Septa for 0.5-mL Sample Tubes (do not reuse)

For a 96-well tray:

- 0.2-mL MicroAmp® Reaction Tubes (do not reuse)
- Genetic Analyzer Septa Strips (do not reuse)
- Genetic Analyzer Retainer Clips

Note The 96-well tray used in the GeneAmp PCR System 9700 requires a tray adaptor to be used with the ABI PRISM 310 autosampler.

Software Required/Optional

You will need the following software to perform and analyze an SSCP run:

- ABI PRISM 310 Collection Software, version 1.0.4 or higher
- ABI PRISM Run Module, GS TEMPLATE (A, C, or D)

  Use the module that is compatible with the chosen dye set as a template for creating a dedicated SSCP module.
- GeneScan® Analysis Software, version 2.0.2 or higher

We also recommend Genotyper® software, version 2.0. Using Genotyper software, you can obtain numerical sizing data and use the generated numbers to flag potential mutations automatically.

PCR Amplification, Labeling, and Controls

Primer Choice

Choose primers so that the resulting PCR product is no longer than 400 (preferably 250) base pairs in length.

Note Empirical observation indicates that the efficiency of mutation detection is *optimal* for fragments between 130 and 250 base pairs in length.

Labeling Rules

To use the ABI PRISM™ multicolor fluorescent dye technology for SSCP analysis, follow these general rules.

Use 5´-End Labeled Primers

The success of SSCP analysis depends upon the ability to detect slight mobility shifts. The reproducible sizing and sharp peaks obtained when using the 5´-end labeling method are crucial to the success of this application.

Note Post-PCR end-labeling with [F]dNTPs is an alternative (Iwahana *et al.*, 1995; Inazuka *et al.*, 1996; Inazuka *et al.*, 1997).

Use a Different Dye for Each Strand

When you first perform SSCP analysis on a region of DNA you should label the forward and reverse strands with different dyes. Using different colors for the forward and reverse strands will permit detection of residual double-stranded molecules remaining after denaturation, as indicated by overlapping peaks in the two colors. If you choose not to label both strands, you could mistake a band produced by residual double-stranded product for a band produced by mutant single-stranded product.

Note Under certain circumstances both strands can have identical mobilities.

Using a different color for each strand will also allow you to detect mutations that cause the forward and reverse strands to switch positions without significantly affecting the relative mobilities of the wild type and mutant samples.

Once data interpretation is well established, differential labeling of the two strands will not always be necessary. You can switch to using a single dye in order to increase throughput by running multiple, differently-labeled samples in a single injection.

Dedicate a Color to Each Sample

Because single-stranded DNA molecules can adopt multiple stable conformations, extra peaks will often be present in an electropherogram. Dedicating a color to each sample, *e.g.*, labeling the wild type one color and the mutant another, allows you to confirm the origin of extra peaks.

Label Both Strands

Even if you decide to use a single dye for each sample, it is important that you label both strands. Labeling both strands increases detection sensitivity and can indicate potential false-positive results.

Often, mutations will cause an observable mobility shift in only one of the two strands. By labeling both strands you increase your chances of detecting mutations that affect the mobility of only one strand.

Conversely, if a mutation causes only a slight mobility shift in one of the strands, the likelihood of a false-positive result diminishes if this shift is correlated with a mobility shift, however slight, in the other strand.

Size Standard

A size standard must be defined for each run condition. Many point mutations cause only slight mobility shifts. Internal lane size standards in a dedicated color greatly enhance the sensitivity of mutation detection.

We also recommend adding wild type DNA, labeled with the same dye as the size standard, to the size standard. However, the wild type DNA has to have a similar peak height to those of the size standard fragments to facilitate peak recognition by the GeneScan Analysis Software (Inazuka *et al.*, 1997).

Figure 7-1 shows the GeneScan-500 [TAMRA] Internal Lane Size Standard run under non-denaturing conditions at 25 °C (top panel), 30 °C (middle panel), and 35 °C (bottom panel).

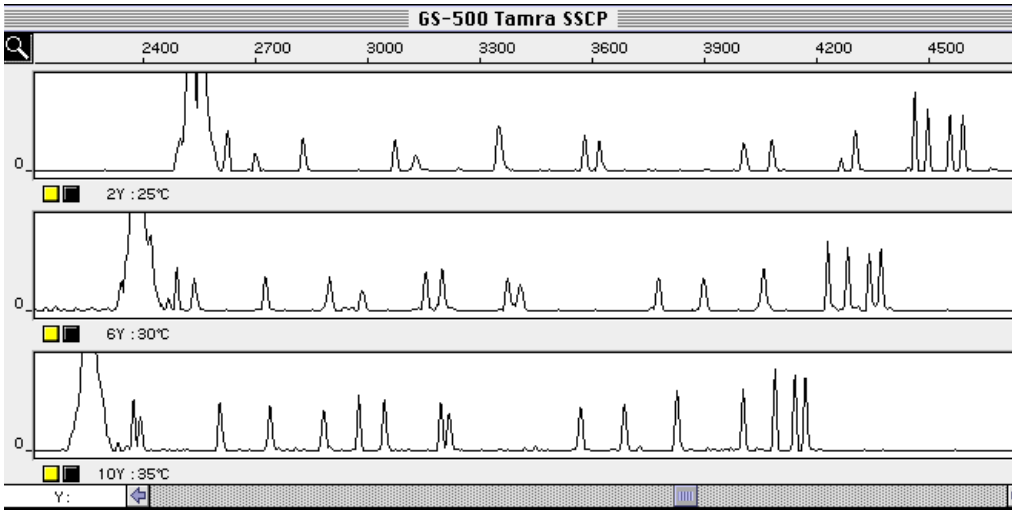


Figure 7-1 GeneScan-500 [TAMRA] run in 3% GeneScan Polymer with 10% glycerol with 1X TBE buffer, L_t = 47 cm, 13 kV electrophoresis voltage

Note The migration rates of the fragments in the size standard do not necessarily conform to their sizes. The standards are denatured and the resulting single-stranded DNA molecules, like the samples for analysis, adopt unpredictable three-dimensional conformations. The goal is to use the size standards to align samples and compare migration rates, not to determine sizes.

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Controls

Determining Run-to-run Variation in the Wild Type Sample

Because the mobility shifts caused by many mutations are slight, you must always run a wild type control to obtain an estimate of injection-to-injection variation in the wild type sample.

Run 3–5 injections of the wild type sample.

Note Running five control lanes will allow you to obtain a satisfactory estimate for the reproducibility under the chosen experimental conditions. Use the values of the five injections to determine the standard deviation of the wild type sample. Any sample that deviates from the wild type mean by more than three standard deviations is 99.7% likely to be caused by a mutation (and not by run-to-run variation in the wild type strand).

Example Control Setup

If you are running 100 injections, run a wild type control every 20th injection. You can inject from the same sample for all control injections.

Estimate Percent Detectable Mutations

If possible, examine known mutant samples to obtain a rough estimate of percent detectable mutations before mounting a large-scale analysis. Using the initial set of electrophoresis conditions described in the following sections, amplify DNA from the wild type and confirmed mutant samples. Tabulate the percent detectable mutations for several repeat experiments. If the detection rate is unacceptably low, see the suggestions for optimizing SSCP run conditions in “Troubleshooting” on page 7-17.

Post-PCR Purification Step

To remove excess primer, purify the amplified PCR product using either a Centricon-100 column (for fragments greater than 130 base pairs in length) or a Centricon-30 column (for fragments less than 130 base pairs in length).

Note Performing this step will simplify the analysis. If you do not intend to sequence putative mutants, this step is not absolutely necessary. However, if you intend to sequence putative mutants isolated during SSCP analysis, you must perform this purification step.

To Save Time—Prerun Checklist

Stock Solutions

Having stocks of the following reagents/buffers saves time during run setup:

- Deionized formamide

  Lasts for three months at –15 to –25 °C. See “Deionized Formamide” on page A-3 for details.

- Sodium hydroxide (NaOH), 0.3 N, stored in a plastic container at room temperature

- Formamide/NaOH/Size Standard Master Mix

  Lasts for one week at 2–6 °C. See step 2 on page 7-8 for details.

- 3% GeneScan Polymer (GSP) with 10% glycerol in 1X TBE

  Lasts for 3 months at room temperature. See “5% GeneScan Polymer with 10% Glycerol” on page A-1 for details.

- 1X TBE buffer with 10% glycerol

  Lasts for 3 month at room temperature. On the instrument, the buffer lasts for 48 hours or 100 injections, whichever comes first. See “1X TBE with 10% Glycerol” on page A-2 for details.

At the Beginning of a Run

Set the instrument run temperature to the desired temperature (30 °C by default) immediately before run setup. Refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* for details.

At Any Time Before a Run

Perform the following at any time before running:

- Complete the Sample Sheet.

  Refer to the *GeneScan Analysis Software User's Manual* for details.

- Create an SSCP analysis matrix file.

  For directions on preparing matrix samples for SSCP analysis, see page 7-9. See Appendix B for instructions on creating a matrix file. Usually, you create and then reuse a single matrix file for each set of run conditions.

Preparing for a Run

Instrument Setup

Refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* for the general procedure.

The specific equipment, polymers, and buffers needed for an SSCP analysis run are listed in "Before You Begin" on page 7-3.

Preparing and Loading Samples

Preparing and Loading Experimental Samples

Note Sometimes you will need to dilute the PCR product in distilled, deionized H_2O before loading 1 µL into the sample tube.

To prepare and load the samples:

Step	Action
1	Label the 0.5-mL (48-well tray) or 0.2-mL (96-well tray) sample tubes with a permanent marker.
2	To each tube add: ♦ 10.5 µL deionized formamide ♦ 1 µL GeneScan Internal Lane Size Standard (GeneScan-350 or GeneScan-500 recommended) ♦ 1 µL PCR product ♦ 0.5 µL 0.3 N NaOH (added to the samples to keep them denatured for extended periods of time) **! WARNING ! CHEMICAL HAZARD. Formamide is a known teratogen. It can cause birth defects. Wash thoroughly after handling formamide. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves. Wash thoroughly after handling formamide.** **! WARNING ! CHEMICAL HAZARD. Sodium hydroxide (NaOH) can cause severe burns to the skin, eyes, and respiratory tract. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.** **Note** For convenience, you can combine the formamide, NaOH, and GeneScan Size Standard into a master mix (1050 µL deionized formamide + 50 µL 0.3 N NaOH + 100 µL GeneScan Size Standard). This mix will last for 1 week at 2–6 °C. Aliquot 12 µL of the mix into each sample tube before adding 1 µL of PCR product.
3	Seal each tube with a septum, taking care to insert the septum completely.
4	Are you using a **48**- or a **96-well** sample tray? ♦ If you are using a **48-well** sample tray, skip to step 5. ♦ If you are using a **96-well** sample tray, insert the tubes into the tray and then go to step 5. You can denature and cool the samples directly in the tray. For directions refer to the *Genetic Analyzer Septa Strip and Retainer Clip User Bulletin* (P/N 904512).
5	Place the samples in a heat block or thermal cycler for 5 minutes at 95 °C.
6	Remove the tubes from the heat source and place immediately in an ice-water bath.

To prepare and load the samples: *(continued)*

Step	Action
7	Are you using a **48**- or a **96-well** sample tray? ♦ If you are using a **48-well** sample tray, insert the tubes into the tray and then go to step 8. ♦ If you are using a **96-well** sample tray, skip to step 8.
8	Place the sample tray on the autosampler. **Note** The 96-well tray used in the GeneAmp PCR System 9700 requires a tray adaptor to be used with the ABI PRISM 310 autosampler.

Preparing the Matrix Samples

Step	Action
1	Prepare the matrix samples by adding 10.5 µL of deionized formamide, 0.5 µL of 0.3 N NaOH, and 1 µL of one of the matrix standards (Dye Primer or Fluorescent Amidite, see page 7-12) to each of four sample tubes. **! WARNING ! CHEMICAL HAZARD. Formamide is a known teratogen. It can cause birth defects. Wash thoroughly after handling formamide. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves. Wash thoroughly after handling formamide.** **! WARNING ! CHEMICAL HAZARD. Sodium hydroxide (NaOH) can cause severe burns to the skin, eyes, and respiratory tract. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.**
2	Place the samples in a heat block or thermal cycler for 5 minutes at 95 °C.
3	Remove the tubes from the heat source and place immediately in an ice-water bath.
4	Run these four injections either alone or in the first four injections of an experimental run.

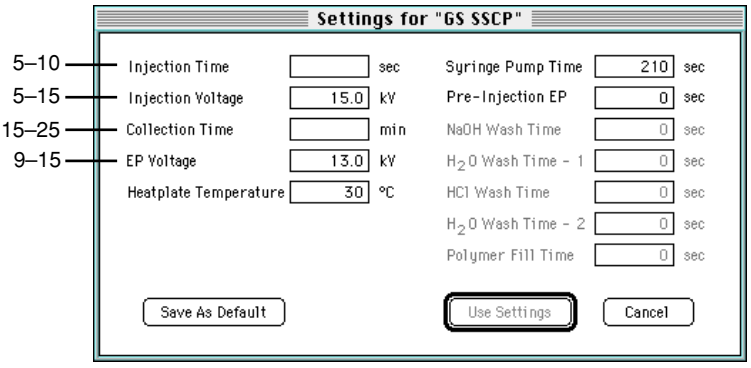
Creating the Run Module

Currently no dedicated SSCP module exists for the ABI PRISM 310 Genetic Analyzer. You will need to duplicate and edit one of the template modules.

To create the run module:

Step	Action
1	Open the Modules folder (located in the ABI PRISM 310 folder).
2	Duplicate one of the GS TEMPLATE modules and rename the new module GS SSCP A (or C or D), as appropriate. **Note** The proper template module depends upon the dye set you are using. Use module GS TEMPLATE A with dye primers and module GS TEMPLATE C or GS TEMPLATE D with fluorescent amidites (see Table 4-3 on page 4-6 and “Creating a Matrix File” on page 7-12).
3	Double-click the ABI PRISM 310 Collection icon if the program is not currently open.
4	Select your module from step 2 in the Manual Control window.

To create the run module: *(continued)*

Step	Action
5	Specify the settings as shown below.  **Note** Choose injection times between 5 and 10 seconds and collection times between 15 and 25 minutes depending upon the polymer concentration, electrophoresis voltage, and size of your samples. **Note** The Syringe Pump Time setting is for a 47-cm capillary filled with 3% GeneScan Polymer. Other capillary length and polymer concentration combinations might require different pump times (see Table 7-1 on page 7-17).
6	Click on Save As Default to save the GS SSCP module settings.

Starting the Run

To start the run:

Step	Action
1	If you have installed a new capillary and have not already reset the injection counter to zero, select Change Capillary from the Instrument window. Then click OK in the Reset window.
2	Set up the Sample Sheet as described in the *ABI PRISM 310 Genetic Analyzer User's Manual*. **Note** You can prepare the Sample Sheet at any time prior to the run and save it to the Sample Sheet folder.
3	Select New from the File pull-down menu and click the GeneScan™ Injection List icon.
4	Set up the Injection List as described in the *ABI PRISM 310 Genetic Analyzer User's Manual*. Be sure to do the following: ♦ Select the appropriate Sample Sheet from the Sample Sheet pop-up window. ♦ Select module GS SSCP from the Module pop-up menu for every injection. ♦ If available, select the appropriate matrix file from the Matrix pop-up window for every injection. **Note** If you have not already created a matrix file, see "Preparing the Matrix Samples" on page 7-9 and Appendix B. You can still start the run and assign the matrix to the sample files later.

To start the run: *(continued)*

Step	Action
5	Click the Start button. **Note** If you have not preheated the heat plate, the module has an initial step in which the plate is heated to the selected run temperature before the first sample is run.

Analyzing the Data

Creating a Matrix File

You must create a matrix file before analyzing SSCP data for the first time. For more information on creating matrices, see Appendix B.

For directions on preparing matrix samples for SSCP analysis, see page 7-9. Make sure that you denature the matrix samples before loading.

To create the matrix file, use one of the following:

- Dye Primer Matrix Standards Kit (5-FAM, JOE, TAMRA, and ROX, P/N 401114) and module GS SSCP A
- Fluorescent Amidite Matrix Standards Kit (6-FAM, TET, HEX, and TAMRA, P/N 401546) and module GS SSCP C
- Fluorescent Amidite Matrix Standards Kit (6-FAM, HEX, and ROX, P/N 401546), the NED Matrix Standard (P/N 402996), and module GS SSCP D

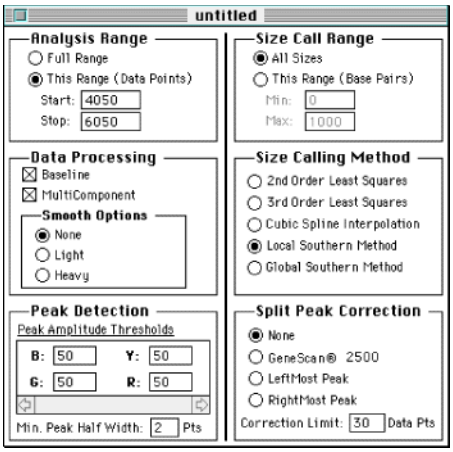
Name the new matrix SSCP Matrix A, SSCP Matrix C, or SSCP Matrix D as appropriate.

Note Usually, you create and save a single matrix file for each set of run conditions. However, if you experience persistent problems, such as spectral overlap in the analyzed data, you should remake the matrix file even if you have not altered the run conditions.

Setting the Analysis Parameters

IMPORTANT If you choose to use different analysis parameters from those recommended here, be sure to define the Analysis Range to exclude the primer peak.

To set the analysis parameters:

Step	Action
1	Open the GeneScan Analysis Software (version 2.0.2 or higher).
2	Choose New from the File menu and click the Analysis Parameters icon.
3	Specify the settings as shown below.  **IMPORTANT** The Analysis Range will vary with polymer concentration and temperature. The setting shown is for 3% GeneScan Polymer at 30 °C.
4	Choose Save As... from the File menu.

To set the analysis parameters: *(continued)*

Step	Action
5	Save the settings as “SSCP Analysis Parameters.”
6	Click OK when done.

Analyzing Sample Files

For brief directions on analyzing sample files, see page 3-2.

Refer to the *GeneScan Analysis Software User's Manual* for detailed protocols.

Optimizing SSCP Run Conditions

Sensitivity to Run Conditions

SSCP analysis is more sensitive to electrophoresis conditions than many ABI PRISM applications. You should optimize run conditions for your particular system.

Of the factors affecting electrophoretic mobility under non-denaturing conditions, only capillary length and polymer concentration have a predictable effect. Increasing either capillary length or polymer concentration will increase the separation between the wild type and mutant strands.

The following factors have an unpredictable or undetermined effect on electrophoretic mobility under non-denaturing conditions. Therefore many of the optimization suggestions contained here are empirically derived.

- Glycerol (or other cosolvent) percentage in polymer
- Fragment size
- Mutation position within the DNA region of interest
- Buffer pH
- Electrophoresis voltage
- Run temperature

Capillary Length

Because many of the mobility shifts in mutants are slight, increasing the length of the capillary increases the detection power. Increasing the capillary length also increases both the capillary fill time and the run time.

Polymer Concentration

Within the range of 1–5% GeneScan Polymer concentration, experimental results indicate that increasing polymer concentration increases detection power (Inazuka *et al.*, 1997).

- Begin your trials using 3% GeneScan Polymer:
- If your results are poor, increase the polymer percentage to as much as 5%.
- If your results are good, to save time decrease the polymer concentration to as little as 1% as long as the percentage of detectable mutations continues to be acceptable.

Increasing the polymer concentration also increases both the capillary fill time and the run time.

continued on next page

Glycerol Percentage

Glycerol at concentrations of 5–10% usually stabilizes three-dimensional DNA conformations and thus enhances mutation detection.

Begin your trials using 10% glycerol. If you decide to alter glycerol concentration, try concentrations in the range from 5–10%.

Other possible cosolvents you might want to consider include the following (Glavac and Dean, 1993):

- Urea
- Formamide
- Sucrose
- Glucose
- DMSO

Fragment Size

If your results are poor and the fragment length is well outside the optimal size range of 130–250 bp, consider choosing new primers so that the fragment length is within the optimal size range.

Hayashi *et al.* (1992) found that at least 90% of single base-pair substitutions can be detected if the PCR products are kept under 200 bp in length and that 80% of single base-pair substitutions can be detected if the PCR products are kept under 400 bp in length.

Inazuka *et al.* (1997) found that the ABI PRISM 310 Genetic Analyzer can be used to analyze fragments up to 741 bp.

Mutation Position

A given point mutation will interact differently with the various regions in the surrounding DNA. Changing the primer positions to amplify different regions of the surrounding DNA can change the relative mobility difference between the mutant and the wild type fragments.

Buffer pH

Kukita *et al.* (1997) suggest that low pH greatly increases the sensitivity of mutation detection in a slab gel format, enabling effective analysis of fragments as large as 800 bp in length.

Electrophoresis Voltage

Within the range from 9–15 kV, a slight drop-off in detectability is apparent at or above 13 kV (perhaps due to a destabilizing effect of high electric-field strengths on three-dimensional conformation).

Begin your trials with an electrophoresis voltage of 15 kV. If your results are poor, consider decreasing the voltage to as little a 9 kV.

Note Decreasing electrophoresis voltage increases run time.

continued on next page

Run Temperature

The ABI PRISM 310 Genetic Analyzer has no mechanism for capillary cooling. In general, cooler temperatures stabilize three-dimensional conformation and thus enhance detectability. If your lab permits, try run temperatures below 30 °C.

Sometimes, however, raising the temperature above 30 °C improves results. 40–45 °C appears to be an empirical upper limit to improving performance (Atha *et al.*, 1998).

Figure 7-2 shows mobility differences between p53 mutant and wild type SSCP samples at 25 °C, 30 °C, and 35 °C for both forward (blue) and reverse strands (green). The Namalwa sample (left) gives the best differentiation between wild type and mutant at 25 °C, the H596 sample (middle) gives the best differentiation at 30 °C, and the Colo320 sample (right) gives the best differentiation at 35 °C.

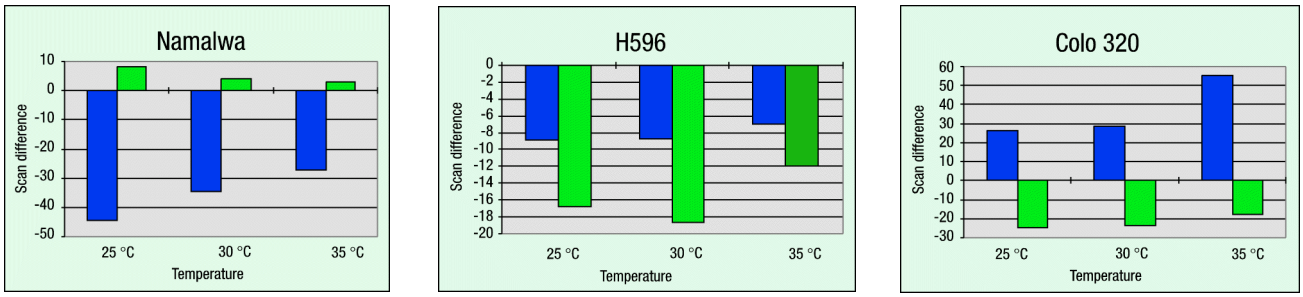


Figure 7-2 Temperature dependence of p53 mutant and wild type SSCP mobility profiles. Mutant sample mobilities are given as differences in number of data points from the wild type sample mobilities (defined as zero). Forward strands are shown in blue and reverse strands are shown in green.

Troubleshooting

Preventing Problems

As with any high-resolution application, it is advisable to display the migration time of at least one size standard peak for every injection. Verify that the migration times of the size standard peaks are similar from injection to injection before flagging a potential mutant. This will decrease the number of false-positive results.

Size Standards

Because of the complex peak patterns of the internal lane size standards that are often seen in SSCP runs, it can be difficult for the GeneScan Analysis Software's peak-detection algorithm to recognize peaks reproducibly.

- Examine your data for each injection to ensure that the correct peaks are being called.
- Adjust the Peak Height Threshold in the Analysis Parameters window to get the most consistent data.
- Define the size standard so that each unknown peak is flanked by at least two size standard peaks on either side.
- Adjust the values in the Analysis Range section of the Analysis Parameters window to include only the peaks of interest.

Sodium Hydroxide

Using poor-quality or old sodium hydroxide solutions can lead to decreased capillary lifetimes.

IMPORTANT Do not store sodium hydroxide in glass bottles. NaOH etches glass.

Syringe Pump Times

When you change any of the following parameters, you must also change the Syringe Pump Time (in the GS SSCP module settings) accordingly:

- Capillary length
- Polymer concentration
- Glycerol (or other co-solvent) percentage

Table 7-1 illustrates the effect of capillary length, polymer concentration, and glycerol presence on fill time at 30 °C.

Table 7-1 Syringe Pump Times

L_t (cm)	10% Glycerol	Fill Time (sec.) for various GeneScan Polymer Concentrations		
		1%	3%	5%
47	no	10	20	50
47	yes	25	30	60
61	yes	30	40	100

- When using low polymer concentrations, if the Syringe Pump Time is too long, you may run out of polymer.
- When using high polymer concentrations, if the Syringe Pump Time is too short, the capillary is not completely filled. Runs get progressively slower (*i.e.*, size standard peaks come off at higher and higher scan numbers).

continued on next page

Laboratory Temperature and Humidity

The laboratory temperature should be maintained between 15 and 30 °C. Once the ABI PRISM 310 Genetic Analyzer is set up and in operation, the laboratory temperature should not fluctuate more than ±2 °C.

The instrument can tolerate up to 80% non-condensing relative humidity. Avoid placing it near heaters, cooling ducts, or heat-producing instruments.

8 Microsatellite Analysis

Overview

In This Chapter

This chapter provides detailed instructions for performing microsatellite analysis with the Performance Optimized Polymer 4 (POP-4™) on the ABI PRISM® 310 Genetic Analyzer.

This chapter contains the following topics:

Topic	See Page
Introduction to Microsatellite Analysis	8-2
Before You Begin	8-3
PCR Amplification, Labeling, and Controls for Microsatellite Analysis	8-4
To Save Time—Prerun Checklist	8-6
Preparing for a Run	8-7
Analyzing the Data, Part I—Using GeneScan	8-10
Analyzing the Data, Part II—Allele Binning Using Genotyper 2.0	8-12
Troubleshooting Microsatellite Analysis	8-25

Introduction to Microsatellite Analysis

Definition

Microsatellite markers, also called short tandem repeat (STR) markers, are polymorphic DNA loci that contain a repeated nucleotide sequence. The repeat unit can be from 2–7 nucleotides in length. The number of nucleotides per repeat unit is the same for a majority of repeats within a microsatellite locus.

What is Microsatellite Analysis?

Microsatellite loci are PCR amplified and the PCR products are then analyzed by electrophoresis to separate the alleles according to size. PCR-amplified microsatellite alleles can be detected using various methods, such as fluorescent dye labeling, silver staining, or fluorescent dye staining.

Background

The number of repeat units at a microsatellite locus may differ, so alleles of many different lengths are possible. Microsatellite loci occur throughout the genome of most organisms and therefore have been used as markers to establish linkage groups in crosses and to map genetically identified mutations to chromosomal positions.

If allele frequencies are known, highly polymorphic microsatellite loci are very useful for identifying individuals in a population and for determining the probability that two individuals are related. Their even distribution in the genome makes them very good markers for constructing genetic maps (Edwards *et al.*, 1992).

Advantages of PCR-Based Microsatellite Analysis

PCR-based microsatellite analysis has the following advantages over conventional genotyping methods, *e.g.*, Restriction Fragment Length Polymorphism (RFLP):

- The small size of microsatellite loci improves the chance of obtaining a result, particularly for samples containing minute amounts of DNA and/or degraded DNA.
- The small size range of microsatellite loci makes them ideal candidates for co-amplification while keeping all amplified alleles smaller than 350 base pairs. Many microsatellite loci can therefore be typed from a single PCR.
- Microsatellite alleles have discrete sizes, allowing for simplified interpretation of results.
- PCR-based tests are rapid, giving results in 24 hours or less.
- PCR-based tests are easy to standardize and automate, ensuring reproducible results.

Advantages of Using ABI PRISM Technology

To exploit the potential for increased throughput using ABI PRISM™ multicolor fluorescent dye technology, you can multiplex electrophoresis by co-loading the products of multiple PCR reactions during the same capillary injection.

The ABI PRISM 310 Genetic Analyzer allows extremely rapid separations: fragments that are 450 base pairs or less in length can be separated in under 30 minutes. This translates to a throughput of up to 48 samples in a 24-hour period.

Before You Begin

Materials Required

You will need the following materials to perform a microsatellite analysis run.

- Performance Optimized Polymer 4 (POP-4)
- ABI PRISM Genetic Analyzer Capillary labeled with a green mark (L_t = 47 cm, L_d = 36 cm, i.d. = 50 µm)
- GeneScan Internal Lane Size Standard (recommended: GeneScan-350, GeneScan-400 HD, or GeneScan-500)
- Genetic Analyzer Buffer with EDTA
- 4.0-mL Genetic Analyzer Vials (do not reuse)
- 1.0-mL or 2.5-mL GeneScan Glass Syringe
- 1.5-mL Eppendorf tube with the lid removed

For a 48-well tray:

- 0.5-mL Genetic Analyzer Sample Tubes (do not reuse)
- Genetic Analyzer Septa for 0.5-mL Sample Tubes (do not reuse)

For a 96-well tray:

- 0.2-mL MicroAmp® Reaction Tubes (do not reuse)
- Genetic Analyzer Septa Strips (do not reuse)
- Genetic Analyzer Retainer Clips

Note The 96-well tray used in the GeneAmp PCR System 9700 requires a tray adaptor to be used with the ABI PRISM 310 autosampler.

Software Required

You will need the following software to perform and analyze a microsatellite analysis run:

- ABI PRISM 310 Collection Software, version 1.0.4 or higher
- ABI PRISM Run Module, GS STR POP4 (A,C, D, or F) (1.0 or 2.5 mL).

  Be sure to use the version that is compatible with the chosen dye set.
- GeneScan® Analysis Software, version 2.0.2 or higher
- Genotyper® Software, version 2.0

PCR Amplification, Labeling, and Controls for Microsatellite Analysis

PCR Amplification

Reactant Concentrations and Volumes

The following table provides guidelines for beginning multiplex PCR in your system. In many systems, the 7.5-µL reaction volume produces sufficient product without extensive optimization.

Table 8-1 Preparing PCR Reaction Mixtures

Reaction Component	Volume (µL) (15-µL rxn)	Volume (µL) (7.5-µL rxn)
DNA[a]	1.20 (50 ng/µL stock)	1.20 (25 ng/µL stock)
PCR Primer Mix (5 µM each primer)	1.00	0.50
10X GeneAmp® PCR Buffer II	1.50	0.75
GeneAmp dNTP Mix (250 µM each dNTP)	1.50	0.75
AmpliTaq Gold™ DNA Polymerase (5 U/µL)	0.12	0.06
$MgCl_2$ (25 mM)	1.50	0.75
Distilled, deionized H_2O	8.18	3.49

a. Applied Biosystems recommends adding the same volume of template DNA to the 15-µL and 7.5-µL reactions because manual pipetting of volumes smaller than 1 µL is inaccurate.

IMPORTANT To avoid the inaccuracies associated with pipetting small volumes, you should combine all reaction components except sample DNA in a PCR Master Mix. Using the ratios in the preceding table, prepare sufficient mix for at least one extra reaction volume. Aliquot 13.8 µL of the mix into each 15-µL reaction or 6.3 µL of the mix into each 7.5-µL reaction. PCR Master Mix lasts for 1–2 weeks at 2–6 °C.

Thermal Cycling Profile

The following table lists recommended thermal cycling times and temperatures on a GeneAmp® PCR System 2400, 9600, or 9700 for both the 15- and 7.5-µL reactions.

Initial Incubation Step	Each of 10 Cycles			Each of 20 Cycles			Final Extension	Final Step
	Melt	Anneal	Extend	Melt	Anneal	Extend		
HOLD	CYCLE			CYCLE			HOLD	HOLD
95 °C 12 min.	94 °C 15 sec.	55 °C 15 sec.	72 °C 15 sec.	89 °C 15 sec.	55 °C 15 sec.	72 °C 15 sec.	72 °C 10 min.	4 °C (forever)

If some loci amplify poorly, see “Multiplexing PCR” on page 6-10 and “Troubleshooting PCR Amplification” on page 11-1 for suggestions on improving PCR performance.

continued on next page

Labeling Rules

Use 5´-end labeled primers. The success of microsatellite analysis depends upon the ability to detect small mobility differences. The reproducible sizing and sharp peaks obtained when using the 5´-end labeling method are crucial to the success of this application.

Size Standard

Always use a GeneScan Internal Lane Size Standard.

Control DNA

In this context, control DNA satisfies the following criteria:

- The DNA comes from a single individual with known genotype.
- The DNA sample is in sufficiently good condition to serve as a positive control for PCR amplification.

Applied Biosystems recommends using control DNA (such as the CEPH 1347-02 standard used to generate the Généthon map of the human genome) to monitor several stages of the experimental process. Control DNA:

- Serves as a positive control for troubleshooting PCR amplification

  For example, if the sample DNA amplifies poorly, knowing whether the control DNA amplifies will allow you to distinguish between problems with the sample DNA (control DNA amplifies) and problems with reagents, instruments, or protocols (control DNA does not amplify).
- Serves as a sizing reference for monitoring injection-to-injection and capillary-to-capillary variation

  Because the control DNA is not used to calculate the sizing curve, the size obtained for the control DNA across gels will alert you to potential problems with sizing precision.
- Facilitates allele binning

  Allele binning is a simple statistical method for converting peak sizes to alleles. Briefly, the method involves generating a frequency histogram of called sizes. Refer to the *Genotyper User's Manual* for a detailed discussion of allele binning.

IMPORTANT To convert a fragment's called size to an allele, you cannot simply round the called size to the nearest allele size.

If you choose a commonly-used standard such as CEPH 1347-02 (P/N 403062), you can correlate the allele sizes that you obtain with the allele sizes obtained by others, such as the CEPH Genotype Database (http://www.cephdb.fr/cephdb).

For more information on the CEPH Genotype Database, refer to page 7-33 of the *ABI PRISM Linkage Mapping Set Version 2 User's Manual*.

You can also use an allelic ladder to genotype analyzed samples (see page 9-23).

Guidelines for Using Control DNA

Amplify at least one control DNA sample for every round of PCR amplification.

Run at least one injection of amplified control DNA for every set of microsatellite markers used. Run at least one injection of amplified control DNA whenever you change the capillary or electrophoresis conditions.

To Save Time—Prerun Checklist

Stock Solutions

Having stocks of the following reagents/buffers saves time during run setup:

- Deionized formamide

  Lasts for 3 months at –15 to –25 °C. See “Deionized Formamide” on page A-3 for details.

- Formamide/Size Standard Master Mix

  Lasts for 2 weeks at 2–6 °C. See step 2 on page 8-7 for details.

- 1X Genetic Analyzer Buffer with EDTA

  Lasts for 2 weeks at 2–6 °C and for 48 hours or 100 injections, whichever comes first, on the instrument.

At the Beginning of a Run

Perform the following immediately before run setup:

- Allow the POP-4 polymer to warm to room temperature.
- Set the instrument run temperature to 60 °C.

  Refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* for details.

At Any Time Before a Run

Perform the following at any time before running:

- Complete the Sample Sheet.

  Refer to the *GeneScan Analysis Software User's Manual* for details.

- Create a microsatellite analysis matrix file.

  For directions on preparing matrix samples for microsatellite analysis, see page 8-8. See Appendix B for instructions on creating a matrix file. Usually, you create and then reuse a single matrix file for each set of run conditions.

Preparing for a Run

Instrument Setup

Refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* for the general procedure.

The specific equipment, polymers, and buffers needed for microsatellite analysis run are listed in "Before You Begin" on page 8-3.

Preparing and Loading Samples

Note Sometimes you will need to dilute the PCR product 1:10 in distilled, deionized H_2O before loading 1 µL into the sample tube.

Step	Action
1	Label the 0.5-mL (48-well tray) or 0.2-mL (96-well tray) sample tubes with a permanent marker.
2	To each tube add the following: ♦ 12 µL deionized formamide ♦ 0.5 µL GeneScan Internal Lane Size Standard ♦ 1 µL PCR product **! WARNING ! CHEMICAL HAZARD. Formamide is a known teratogen. It can cause birth defects. Wash thoroughly after handling formamide. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves. Wash thoroughly after handling formamide.** **Note** For convenience, you can combine the formamide and GeneScan Size Standard into a master mix (1200 µL deionized formamide + 50 µL GeneScan Size Standard). Put 12.5 µL of the mix and 1 µL of PCR product into each sample tube. This mix will last for 2 weeks at 2–6 °C.
3	Seal each tube with a septum, taking care to insert the septum completely.
4	Are you using a **48**- or a **96-well** sample tray? ♦ If you are using a **48-well** sample tray, skip to step 5. ♦ If you are using a **96-well** sample tray, insert the tubes into the tray and then go to step 5. You can denature and cool the samples directly in the tray. For directions refer to the *Genetic Analyzer Septa Strip and Retainer Clip User Bulletin* (P/N 904512).
5	Place the samples in a heat block or thermal cycler for 5 minutes at 95 °C.
6	Remove the tubes from the heat source and place immediately in an ice-water bath.
7	Are you using a **48**- or a **96-well** sample tray? ♦ If you are using a **48-well** sample tray, insert the tubes into the tray and then go to step 8. ♦ If you are using a **96-well** sample tray, skip to step 8.
8	Place the sample tray on the autosampler. **Note** The 96-well tray used in the GeneAmp PCR System 9700 requires a tray adaptor to be used with the ABI PRISM 310 autosampler.

Preparing the Matrix Samples

Step	Action
1	Prepare the matrix samples by adding 12 µL of deionized formamide and 1 µL of one of the matrix standards (Dye Primer or Fluorescent Amidite) to each of four sample tubes. **! WARNING ! CHEMICAL HAZARD. Formamide is a known teratogen. It can cause birth defects. Wash thoroughly after handling formamide. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves. Wash thoroughly after handling formamide.**
2	Denature the samples for 5 minutes at 95 °C.
3	Cool the samples by placing directly on ice.
4	Run these four injections, either alone or in the first four injections of an experimental run.

Starting the Run

Note Run module GS STR POP4 permits detection of the 400-bp peak of the GeneScan-500 size standard. By increasing the run time from 24 minutes to 26 minutes, you can also detect the 500-bp peak of the GeneScan-500 size standard.

To start the run:

Step	Action
1	Double-click the ABI PRISM 310 Collection icon if the program is not currently open.
2	If you have installed a new capillary and have not already reset the injection counter to zero, select Change Capillary from the Instrument window. Then click OK in the Reset window.
3	Set up the Sample Sheet as described in the *ABI PRISM 310 Genetic Analyzer User's Manual*. **Note** You can prepare the Sample Sheet at any time before the run and save it to the Sample Sheet folder.
4	From the File pull-down menu, select New and click the GeneScan Injection List icon.
5	To increase the run time from 24 minutes to 26 minutes: a. Select run module GS STR POP4 from the Manual Control window. b. Type 26 in the Collection Time data field. c. Click Use Settings. **Note** The Use Settings button will store the settings for the current Injection List only.
6	Set up the Injection List as described in Chapter 4 of the ABI PRISM 310 *Genetic Analyzer User's Manual*. Be sure to do the following: a. Select the appropriate Sample Sheet from the Sample Sheet window. b. Select run module GS STR POP4 from the Module pop-up menu for every injection. c. If available, select the appropriate matrix file from the Matrix window for every injection. **Note** If you have not already created a matrix file, see page 8-8 and Appendix B. You can still start the run and assign the matrix to the sample files later.

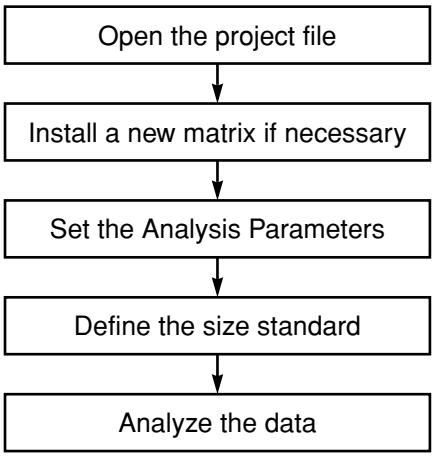
To start the run: *(continued)*

Step	Action
7	Click Start. **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 the first sample is run.

Analyzing the Data, Part I—Using GeneScan

After A Run: Process Overview

The following diagram summarizes the data analysis process using the GeneScan Analysis software. Setting the analysis parameters is covered in more detail on page 8-11. For brief directions on analyzing sample files, see page 3-2.



Creating a New Matrix File

You must create a matrix file before analyzing microsatellite data for the first time. For more information on creating matrix files, see Appendix B.

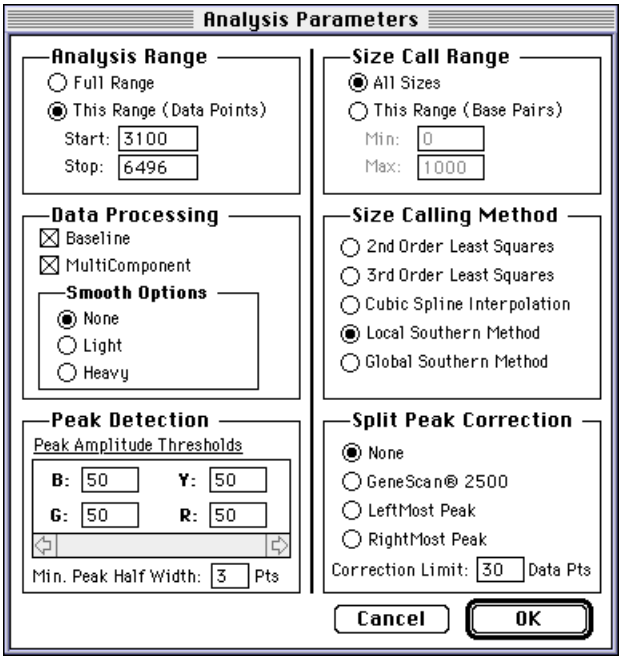
For directions on preparing matrix samples for microsatellite analysis, see "Preparing the Matrix Samples" on page 8-8. To create the matrix file, use one of the following:

- Dye Primer Matrix Standards Kit (5-FAM, JOE, TAMRA, and ROX, P/N 401114) and module GS STR POP4 A
- Fluorescent Amidite Matrix Standards Kit (6-FAM, TET, HEX, and TAMRA, P/N 401546) and module GS STR POP4 C
- Fluorescent Amidite Matrix Standards Kit (6-FAM, HEX, and ROX, P/N 401546), the NED Matrix Standard (P/N 402996), and module GS STR POP4 D
- Dye Primer Matrix Standards Kit (5-FAM, JOE, and ROX, P/N 401114), the NED Matrix Standard (P/N 402996), and module GS STR POP4 F

Note Usually, you create and save a single matrix file for each set of run conditions. However, if you experience persistent problems, such as spectral overlap in the analyzed data, you should remake the matrix file even if you have not altered the run conditions.

continued on next page

Setting the Analysis Parameters

Step	Action
1	Open the GeneScan Analysis Software (version 2.0.2 or higher).
2	From the File menu, choose New and click the Analysis Parameters icon.
3	Specify the settings as shown below.  **Note** The primer peak is usually detected in the 2600–3000 scan number range. You should start the analysis range immediately after the primer peak in order to see only the size standard and microsatellite data. Examine the raw data to determine the exact position of the primer peak.
4	Choose Save As... from the File menu.
5	Save the settings as Microsatellite Analysis Parameters.
6	Click OK when done.

Analyzing Sample Files

For brief directions on analyzing sample files, see page 3-2.

The *GeneScan Analysis Software User's Manual* contains detailed protocols.

Analyzing the Data, Part II—Allele Binning Using Genotyper 2.0

What is Allele Binning?

Allele definitions for microsatellite markers are based on the fragment length (size) of the PCR products as estimated by gel or capillary electrophoresis. Experimental variation in sizing has led to the practice of "binning" alleles—grouping allele fragments belonging to a particular size into a range (bin) centered around the average size with a tolerance limit. A typical allele definition would look like this: 101.5 ± 0.5 bp.

Benefits of Allele Binning

Allele binning has several benefits:

- As the sample size increases for a particular marker or set of markers, new or previously undefined alleles can appear in the sample. Allele binning helps you to accommodate undefined alleles.
- Allele sizes tend to vary among electrophoresis runs due to subtle differences in gels or capillaries and electrophoresis conditions. Allele binning allows you to define appropriate tolerances for this variance.
- You can define alleles more precisely by binning alleles based on sample size.
- If using the same control DNA (*e.g.*, CEPH 1347-02) on every run, you can adjust allele definitions against the reference alleles automatically by binning alleles.

Methods Used to Bin Alleles

You can perform allele binning using any of the following Genotyper 2.0 features:

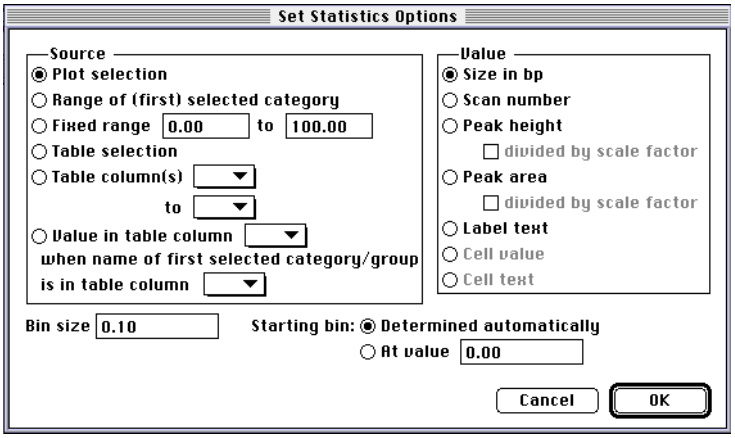
- Histogram window
- Plot window
- Make from Labels…
- Add Multiple Categories…
- Offset/Calculate Offset…

In general, we recommend using the Histogram window for binning alleles. This method works best when the full data set from a study is available for each marker before the allele bins are determined.

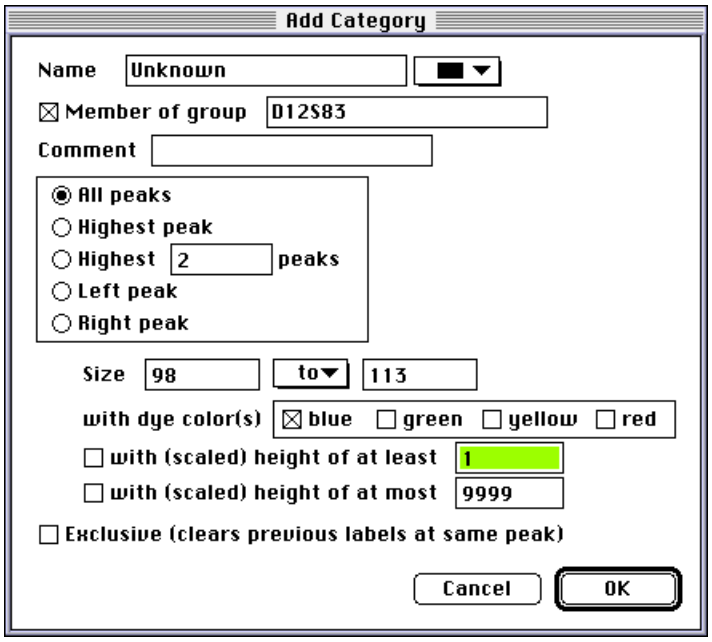
Note For users of the ABI PRISM Fluorescent Genotyping Demonstration Kit—To familiarize yourself with the allele binning methods described in this section, you can use the sample files from the Fluorescent Genotyping Demonstration Kit. The Tutorial disk supplied with the installation disks for Genotyper 2.0 software contains the data files.

Using the Histogram Window

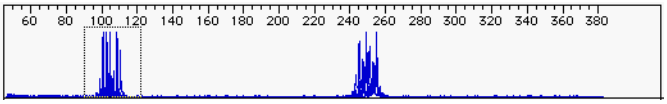
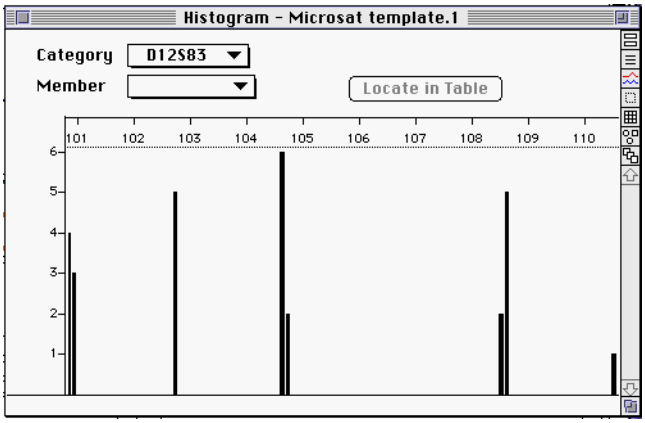
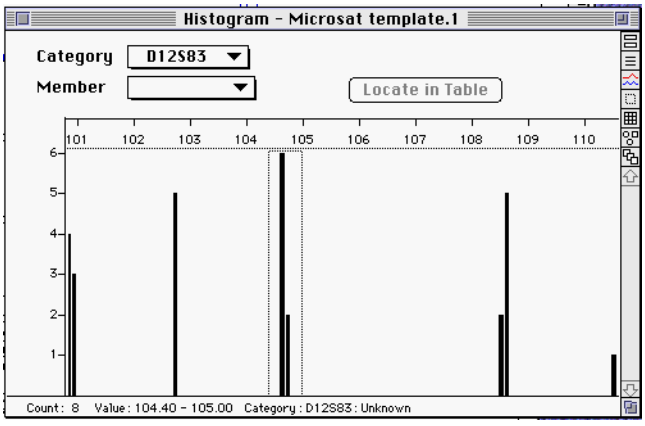
To bin alleles using the Histogram window:

Step	Action
1	Define the bin size as follows: a. From the Analysis menu, choose Set Statistics Options… b. Select the following buttons as shown below: – Plot selection – Size in bp – Starting bin: determined automatically c. Enter 0.10 in the Bin size field.  **Note** A bin size of 0.1 bp gives the most precise allele binning. If insufficient data is available, however, the Genotyper software displays an error message stating that the bin size is too small. If this occurs, increase the bin size.
2	From the View menu, choose Show Categories Window (or type ⌘ K).

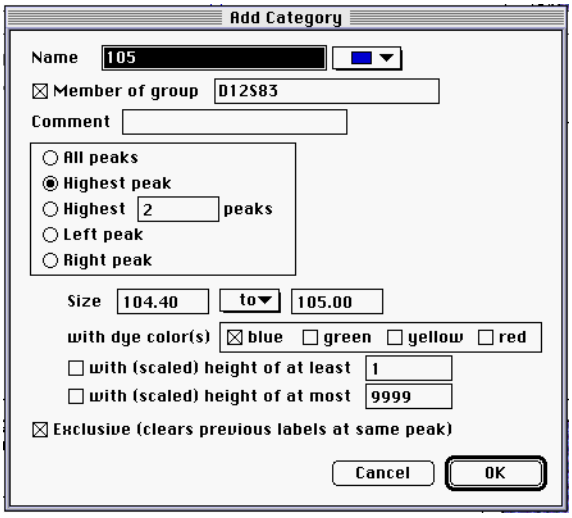
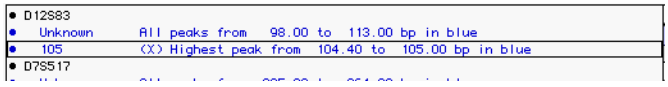
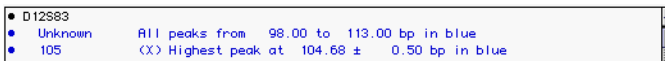
To bin alleles using the Histogram window: *(continued)*

Step	Action
3	Follow these steps to set up a Category (Group) for each marker. a. From the Analysis menu, choose Clear Category List. b. From the Category menu, choose Add Category. c. Enter the marker name, size range, and dye color for the first marker as shown below. d. Click OK. e. Repeat these steps for the remaining markers: – From the Category menu, choose Add Category. – Enter the marker name, size range, and dye color. – Click OK. 
4	From the Analysis menu, choose Label Peaks… Label peaks with Size in bp only.
5	From the Analysis menu, choose Filter Labels… Filter labels using the default settings (best for dinucleotide repeat markers).

To bin alleles using the Histogram window: *(continued)*

Step	Action
6	Working with one dye color at a time in the Main window: a. Click B to choose all blue dye/lanes. b. Draw a box in the plot window that covers all of the peaks associated with a single marker.  c. From the Views menu, choose Show Histogram Window.  d. Make sure the correct marker name is displayed in the Category field. Leave the Member field blank. **Note** All labeled peaks in the selected range for a given marker display as vertical bars in the Histogram window. Each bar represents a particular size (x-axis, value). The height represents the number of labeled peaks found for that size (y-axis, counts). If you place the cursor on a particular peak/bar, Genotyper software displays the corresponding value and counts.
7	Draw a box around a bar or group of bars that represent one allele. The area inside the box is the allelic bin. Genotyper software displays the size range for the bin and the number of peaks found in that range in the status box at the bottom of the window. 

To bin alleles using the Histogram window: *(continued)*

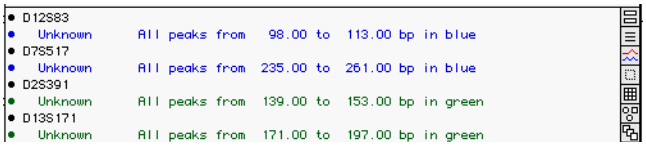
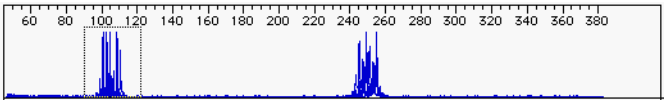
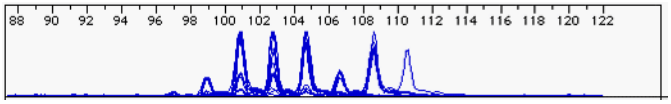
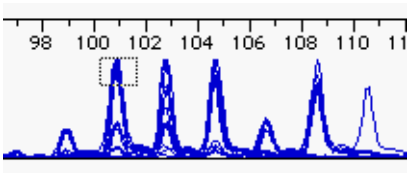
Step	Action
8	From the Category menu, choose Add Category… Genotyper software automatically: ♦ Enters the allele (member) set name to the rounded size in bp ♦ Enters the "Member of group" name (*i.e.*, the marker name) ♦ Selects the Highest peak button ♦ Enters the allele size range (from x to y bp) ♦ Selects the dye color ♦ Selects the Exclusive checkbox  **Note** Check the information entered automatically for accuracy. **Note** Genotyper software will not allow the addition of a new group if a group or category with the same name already exists.
9	Click OK to create a category member (allele bin) such as the one shown here. 
10	**Optional** The bin shown in Step 9 was created with the size as a range. You can also create a bin centered around the median size of the range with a set tolerance (for example, 104.68 ± 0.5 bp) as follows: a. Hold down the Shift key while choosing Add Category… from the Category menu. b. Edit the bin tolerance as desired. The size is displayed in the dialog box as shown here.  **Note** The category member generated appears as follows: 

To bin alleles using the Histogram window: *(continued)*

Step	Action
11	Repeat these steps to continue adding categories for each allele. Remember to: ♦ Select the markers by color ♦ Verify that the Histogram window displays the correct marker name

Using the Plot Window

To bin alleles directly using the individual allele plots:

Step	Action
1	From the Views menu, choose Show Categories Window (or type ⌘ K) and set up the main categories (Groups) for each marker as shown here. 
2	From the Analysis menu, choose Label Peaks… Label peaks with Size in bp only.
3	From the Analysis menu, choose Filter Labels… Filter labels using the default settings (best for dinucleotide repeat markers).
4	In the Main window, working with one dye color at a time: a. Choose all blue dye/lanes by clicking on the Blue color button to the left of the dye/lanes window. b. Draw a box in the plot window that covers all of the peaks associated with a single marker.  c. From the Views menu, choose Zoom In (Selected Range), or type ⌘ R, to display the plots for the individual alleles. 
5	Draw a box around the first tall peak from the left. 

To bin alleles directly using the individual allele plots: *(continued)*

Step	Action
6	From the Category menu, choose Add Category… Genotyper software automatically enters the size information for the category definition. Enter the following information: ♦ Name of the allele (member) set to the rounded size in bp ♦ Member of group name (marker name) ♦ Highest peak button ♦ Size range of the allele (from x to y bp) ♦ Color of dye ♦ Exclusive checkbox **Note** You cannot add a new group if a group or category with the same name already exists.
7	Click OK to add the category (bin).
8	Continuing to move from left to right, repeat step 4 through step 6 for the remaining peaks.
9	**Optional** To create a bin centered around the median size of the range with a set tolerance (for example, 104.68 ± 0.5 bp), follow these steps: a. Hold down the Shift key while choosing Add Category… from the Category menu. b. Edit the bin tolerance as desired. The size is displayed in the dialog box as shown here. 

Binning Alleles Using the Make from Labels… Feature

You can use the Make from Labels… feature in Genotyper 2.0 software to generate category members (allele bins) automatically. This method is ideal for the following types of linkage mapping/ genotyping projects:

♦ A single family/pedigree typed with a number of markers

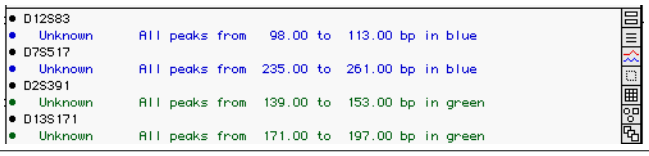
♦ Small studies in which all markers for all individuals fit on a single gel

Unlike the other binning methods presented in this manual, using this method requires:

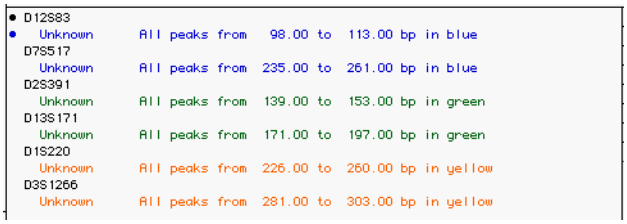
♦ Working with one marker at a time to make categories from labels

♦ Clearing all labels between markers/categories

To bin alleles using the Make from Labels… feature:

Step	Action
1	From the Views menu, choose Show Categories Window (⌘ K) and set up the main categories (groups) for your markers as follows: 

To bin alleles using the Make from Labels… feature: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>2</td><td>From the Edit menu, choose Select All (⌘ A) to select all categories.</td></tr>
<tr><td>3</td><td>From the Edit menu, choose Unmark (⌘ U) to unmark the categories.</td></tr>
<tr><td>4</td><td>Select the first category in the list. From the Edit menu, choose Mark (⌘ M).
 
</td></tr>
<tr><td rowspan="3">5</td><td>If the first category…</td><td>Then…</td></tr>
<tr><td>is currently being defined</td><td>proceed directly to step 6.</td></tr>
<tr><td>is defined already</td><td>from the Analysis menu, choose Clear All Labels.</td></tr>
<tr><td>6</td><td colspan="2">Select the appropriate dye/lanes by clicking on the appropriate color button.</td></tr>
<tr><td>7</td><td colspan="2">From the Analysis menu, choose Label Peaks… Label peaks with Size in bp only.</td></tr>
<tr><td>8</td><td colspan="2">From the Analysis menu, choose Filter Labels… Filter labels using the default settings (best for dinucleotide repeat markers).</td></tr>
<tr><td>9</td><td colspan="2">From the Category menu, choose Make from Labels… to display the Make Categories from Labels dialog box. Set the parameters as follows:
a. Select Unmark overlapping categories and deselect Skip overlapping categories.
To enable you to correct for overlaps, Genotyper software automatically unmarks two or more category members that overlap in size based on the tolerance.
b. In the Name box:
– Either leave the Prefix field blank (Figure 8-1 on page 8-20) or enter a name for the allele in the Prefix field (Figure 8-3 on page 8-21) which will become part of the name of the alleles (Figure 8-2 on page 8-20 and Figure 8-4 on page 8-21).
– In the First number box, enter the number of the first allele (the smallest allele expected in the data, for example, 101) for the marker, or the starting number (for example, 1) if using a prefix.
– In the Number increment box, enter a numeric value. This is the value by which software automatically increases each successive allele number. For example, enter 2 for dinucleotide markers if alleles are expected every 2 bp. Enter 1 to number alleles sequentially (for example, A1, A2, A3, etc.).
c. Select the With checkbox and the group name button.
d. Enter the group/marker name in the field to the right of the group name parameter. This indicates that the category members created belong to the group/marker that you are currently working with.
e. The appropriate dye color box should have been selected automatically by Genotyper software. If not, check the appropriate box.
f. Select the Exclusive checkbox if not automatically selected.
g. Click OK.</td></tr>
<tr><td>10</td><td colspan="2">Return to step 3 to define the remaining categories.</td></tr>
</table>

To bin alleles using the Make from Labels… feature: *(continued)*

Step	Action
11	When all the categories (markers) have been defined, choose Select All from the Edit menu (⌘ A) to select all categories. From the Edit menu, choose Mark (⌘ M) to mark all the categories.
12	From the Analysis menu, choose Clear All Labels. You can now label the alleles with the newly defined bin names.

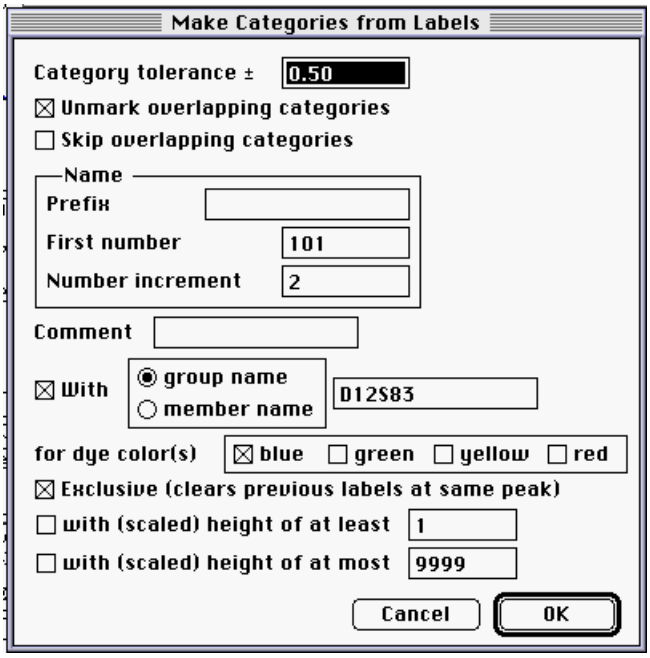


Figure 8-1 The Make from Labels…dialog box configured to use allele sizes as allele names

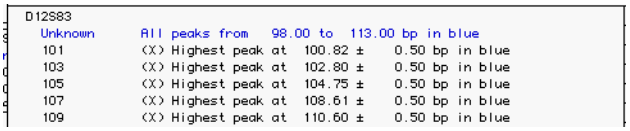


Figure 8-2 Example of allele bin names generated from the Make from Labels…dialog box configured as shown in Figure 8-1

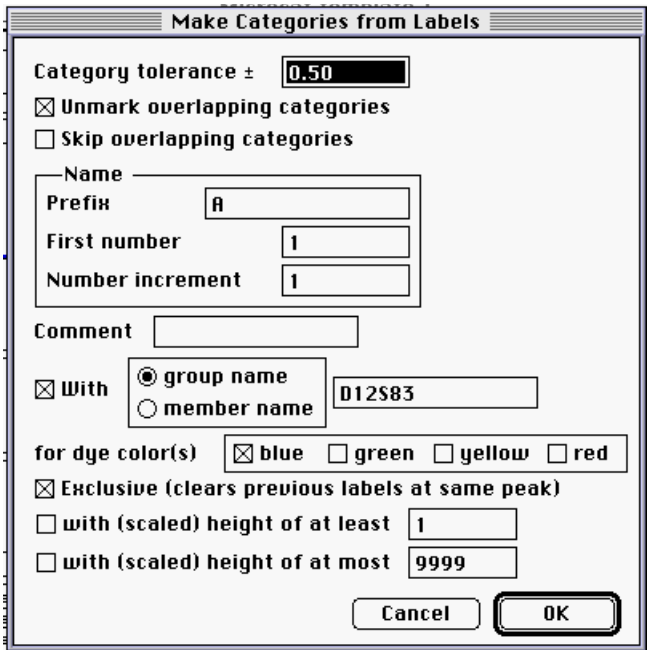


Figure 8-3 The Make from Labels…dialog box configured to use a prefix for the allele name

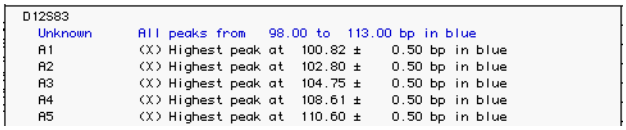


Figure 8-4 Example of allelic bin names generated from the Make from Labels…dialog box configured as shown in Figure 8-3

Using the Add Multiple Categories… Feature

You can use the Add Multiple Categories… feature to create a defined set of category members (allele bins) that are equally spaced with a fixed tolerance.

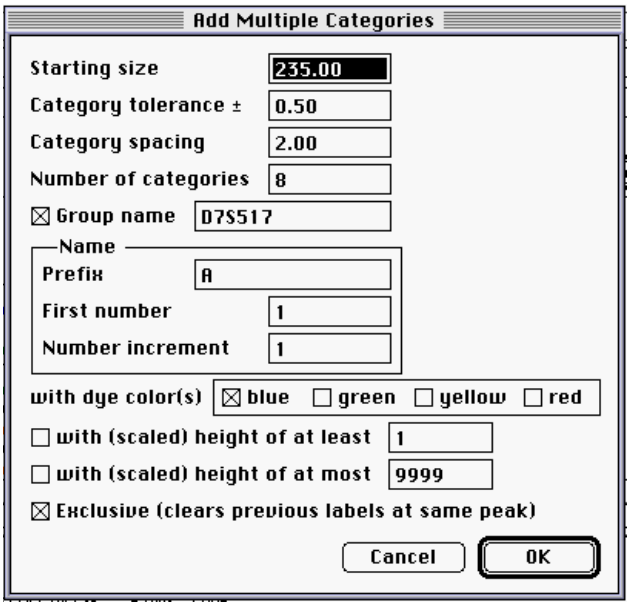
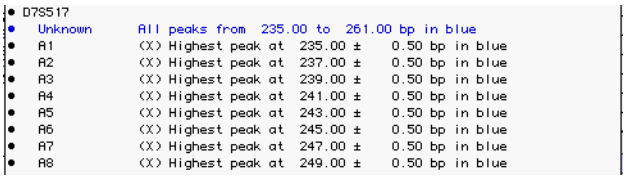
Once Genotyper software creates the categories, you can:

- Label and filter peaks
- Use the Histogram window to fine tune the category definitions

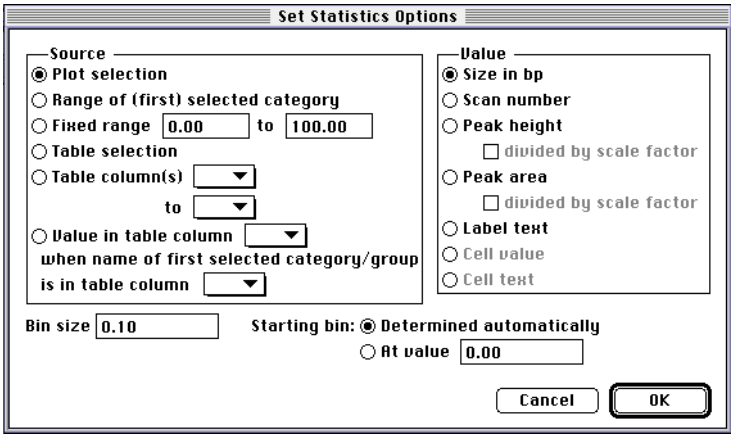
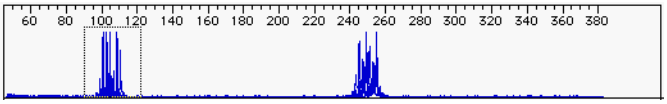
To define a set of equally spaced allelic bins with a fixed tolerance:

Step	Action
1	Set up the main categories (groups) for your markers as follows: 

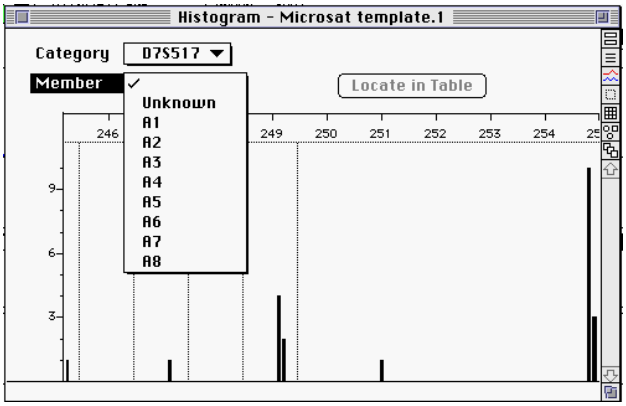
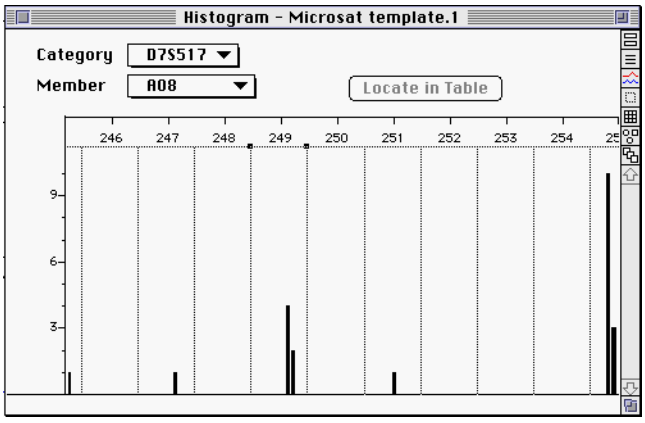
To define a set of equally spaced allelic bins with a fixed tolerance: *(continued)*

Step	Action
2	From the Category menu, choose Add Multiple Categories… Choose the appropriate settings for the first marker as follows: 
3	Click OK to generate a set of categories for the marker as follows: 
4	Repeat step 2 and step 3 for the rest of the markers making sure to enter the appropriate starting size, dye color, and marker name in the Add Multiple Categories dialog box.
5	From the Analysis menu, choose Label Peaks… Label peaks with Size in bp only.
6	From the Analysis menu, choose Filter Labels… Filter labels using the default settings (best for dinucleotide repeat markers).

To fine tune the category definitions using the Histogram window:

Step	Action
1	Define the bin size as follows: a. From the Analysis menu, choose Set Statistics Options… b. Select the following buttons as shown below: – Plot selection – Size in bp – Starting bin: determined automatically c. Enter 0.10 in the Bin size field.  **Note** A bin size of 0.1 bp gives the most precise allele binning. If insufficient data is available, however, Genotyper software displays an error message stating that the bin size is too small. If this occurs, increase the bin size.
2	Click B to choose all blue dye/lanes.
3	In the Plot window, draw a box around all the peaks associated with a single marker. 
4	From the Views menu, choose Show Histogram Window.

To fine tune the category definitions using the Histogram window: *(continued)*

Step	Action
5	Check the marker name displayed by the Category pop-up menu. If incorrect, select the correct marker from the Category pop-up menu.  **Note** Leave the Member pop-up field blank.
6	Select the Member pop-up menu to see a list of all the defined allele bins (category members). The boundaries for each bin are displayed as vertical dotted lines in the histogram. All the peaks/alleles belonging to a particular bin should fall between the two outer boundaries of that bin. If they do not, proceed to the next step to adjust the bin size.
7	From the Member pop-up menu, and choose the name of the bin you want to adjust. Two “handles” are displayed on the corresponding bin boundaries. 
8	Move the cross-hair cursor onto one of the handles until the cursor becomes a cross-hairs in a circle. Click and drag the handle in or out as appropriate to redefine the bin. Genotyper software automatically updates the bin size (category definition) as you move the handles.

Troubleshooting Microsatellite Analysis

Common Problems

The most commonly encountered problems during microsatellite analysis are:

- Poor or non-specific amplification

  See Chapter 6, “Optimizing PCR,” and “Troubleshooting PCR Amplification” on page 11-1 for suggestions.

- Incomplete 3´ A nucleotide addition

  See page 6-18 for a discussion of the “plus-A” phenomenon and for suggestions.

- Stutter

  See page 6-21 for a discussion of the stutter phenomenon and for suggestions. See below for examples of stutter patterns in dinucleotide repeat loci.

Examples of Stutter—Dinucleotide Repeats

Successful amplification of dinucleotide repeat markers yields allele peaks and associated PCR stutter bands within a maximum range of eight base pairs from the allele peak. The number of allele peaks depends on whether the individual tested is a heterozygote or homozygote.

Dinucleotide repeats give specific stutter patterns that are illustrated in Figure 8-5 through Figure 8-9 on pages 8-25 through 8-27.

Example 1

The GeneScan electropherogram of a dinucleotide repeat marker from a homozygous individual (118.6 bp, 118.6 bp) is shown in Figure 8-5.

The peaks at 116.6 bp, 114.6 bp and 112.6 bp are the typical 2-bp stutter pattern seen with dinucleotide repeats. They represent the –2 bp, –4 bp, and –6 bp stutters from the true 118.6-bp allele.

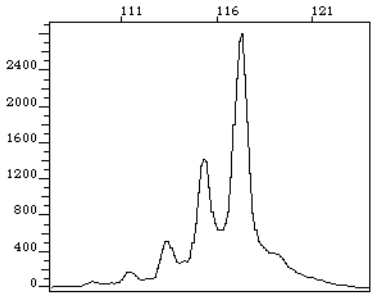


Figure 8-5 Typical pattern for dinucleotide repeat homozygote

Example 2

The GeneScan electropherogram of a dinucleotide repeat marker from a heterozygous individual (90 bp, 98 bp) is shown in Figure 8-6. Allele sizes differ by 8 bp.

The 2-bp stutter peak to the left of each allele peak is always of lower intensity than the allele peak itself. The larger 98-bp allele peak is of lower intensity than the smaller 90-bp allele. In heterozygotes, the higher molecular weight allele often produces a fluorescent signal of lower intensity than the lower molecular weight peak, suggesting a less efficient amplification of the larger fragment.

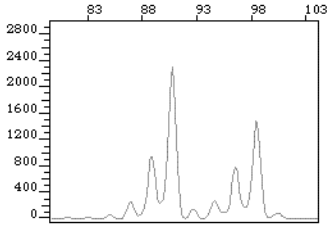


Figure 8-6 Typical pattern for dinucleotide repeat heterozygote. Alleles differ by 8 bp.

Example 3

The GeneScan electropherogram from a dinucleotide repeat marker of a heterozygous individual (86 bp, 90 bp) is shown in Figure 8-7 on page 8-26. Allele sizes differ by 4 bp.

When the difference between the allele sizes is 4 bp or less, a shift occurs in the height ratio between the two allele peaks (compare with Figure 8-6). The fluorescent signal from the –4 bp stutter of the 90-bp allele is added to the signal from the 86-bp allele.

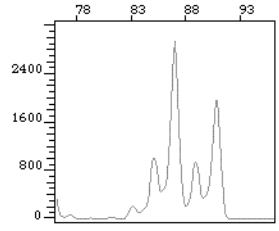


Figure 8-7 Typical pattern for dinucleotide repeat heterozygote. Alleles differ by 4 bp.

Example 4

The GeneScan electropherogram from a dinucleotide repeat marker of a heterozygous individual (92 bp, 94 bp) is shown in Figure 8-8. Allele sizes differ by 2 bp.

The fluorescent signal from the –2 bp stutter of the 94-bp allele is added to the signal of the 92-bp allele. The signal from the –4 bp stutter band of the 94-bp allele is added to the signal from the –2 bp stutter band of the 92-bp allele.

A dinucleotide repeat marker for a heterozygous individual shows this typical triangle pattern when the alleles differ by 2 bp.

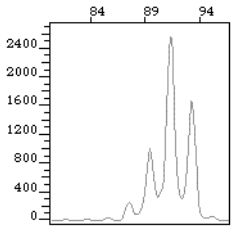


Figure 8-8 Typical pattern for dinucleotide repeat heterozygote. Alleles differ by 2 bp.

Example 5

A GeneScan electropherogram for a dinucleotide repeat marker, displaying peaks at 1-bp intervals, is shown in Figure 8-9. AmpliTaq Gold and other DNA polymerases can add a non-templated A to the end of a PCR product during amplification. When both the true allele and allele-plus-A products show 2-bp stutter bands, a ladder of peaks differing by 1 bp may be seen for PCR products. This phenomenon tends to be locus-specific.

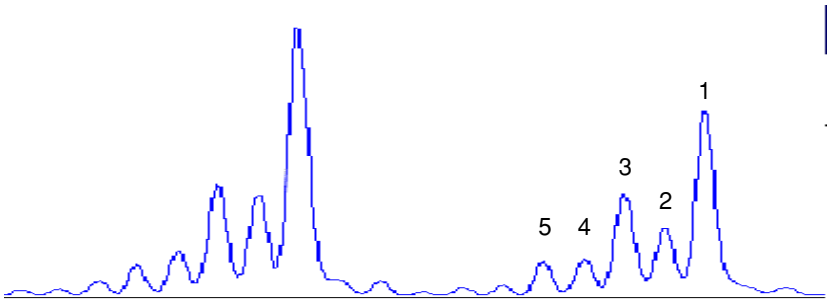


Figure 8-9 Typical pattern for dinucleotide repeat heterozygote showing 1-bp stutter. See Table 8-2 for explanation of peak labels.

One allele in Figure 8-9 is labeled to indicate the origin of the peaks. The pattern produced is a combination of both the 2-bp stutter peaks from the true allele and the allele plus the non-templated A. The resulting peaks differ by 1 bp (Table 8-2).

Table 8-2 Origin of 1-bp peak pattern in dinucleotide repeat marker

Peak	Origin
1	+A product of allele peak
2	True allele peak based on DNA sequence
3	–2 bp stutter of +A peak
4	–2 bp stutter to true allele peak
5	–4 bp stutter of +A peak

continued on next page

Is Stutter a Real Problem?

Stutter, once understood, does not pose a real problem for microsatellite analysis. In fact, stutter can actually aid in allele calling in two cases:

- Distinguishing true allele peaks from non-specific PCR products

  Non-specific PCR products are not associated with stutter bands.

- Identifying alleles that fall far outside the reported allele range

  The percent stutter is often specific to a particular locus. You can sometimes identify alleles that fall far outside the previously reported range on the basis of percent stutter.

Microsatellite Analysis Applications

9

Overview

In This Chapter

This chapter provides instructions for performing specialized applications of microsatellite analysis on the ABI PRISM® 310 Genetic Analyzer.

- Performing microsatellite analysis using the ABI PRISM™ Linkage Mapping Set Version 2 (LMS V2)
- Screening for the loss of heterozygosity (LOH) of microsatellite markers linked to known oncogenes or tumor-supressor genes
- Screening for evidence of replication error (RER) using microsatellite markers
- Determining animal paternity with the StockMarks® kits
- Determining human identity with the AmpFℓSTR™ kits

This chapter contains the following topics:

Topic	See Page
Microsatellite Analysis Using the LMS V2	9-2
Troubleshooting the LMS V2	9-4
LOH Screening	9-5
RER Screening	9-13
Troubleshooting LOH and RER Screening	9-16
Animal Paternity	9-17
Human Identification	9-19

Microsatellite Analysis Using the LMS V2

What is the LMS V2?

The ABI PRISM Linkage Mapping Set Version 2 (LMS V2) contains 400 fluorescently labeled PCR primers which amplify a highly informative subset of the microsatellite loci from the Généthon human linkage map (Weissenbach *et al.*, 1992; Gyapay *et al.*, 1994).

The LMS V2 consists of 28 panels, each containing 10–18 primer pairs. It expands the capabilities of the original ABI PRISM Linkage Mapping Set with:

- New markers for improved resolution
- Tailed reverse primers for improved automatic allele calling
- A new dye set (containing NED) for improved spectral resolution of allele peaks
- True Allele™ PCR Premix (P/N 403061) with AmpliTaq Gold™ DNA Polymerase

  This premix contains an optimized solution of AmpliTaq Gold DNA Polymerase, dNTPs, and magnesium in a buffer for simpler, more reliable PCR amplification.

Advantages

Product Performance and Quality Control

The primer pair combinations in all panels have been optimized for maximum reliability. All have been tested on CEPH family 1347 and various sample DNAs to confirm PCR conditions and to verify allele size ranges. In addition, Applied Biosystems performs a final use test on all manufactured lots of primers using the CEPH 1347-02 control DNA (P/N 403062).

New patented reverse-primer tailing (pig-tailing) chemistry improves allele calling efficiency by eliminating problems associated with non-templated nucleotide addition.

ABI PRISM Technology

In addition to the high throughput afforded by the Applied Biosystems multicolor fluorescence technology, the ABI PRISM 310 Genetic Analyzer allows extremely rapid separations. Fragments that are 300 bp or less in length can be separated in under 30 minutes. This translates to a throughput of at least 48 samples in a 24-hour period.

The PCR conditions and optimized protocols described in this section were developed using the following:

- GeneAmp® PCR System 9600
- True Allele PCR Premix with AmpliTaq Gold DNA Polymerase
- CEPH family 1347 DNA

When using other instruments, reagents, or DNA, some optimization might be required.

continued on next page

Data Collection and Analysis

Data collection and analysis are performed as for any microsatellite application. For more information, refer to the *ABI PRISM Linkage Mapping Set Version 2 User's Manual* (P/N 904999).

Troubleshooting the LMS V2

If No Amplification Occurs

For PCR failures, repeat PCR on the CEPH 1347-02 control DNA using the recommended protocol in the *ABI PRISM Linkage Mapping Set Version 2 User's Manual*, Applied Biosystems reagents, consumables, and thermal cyclers. Make sure that pipettes are calibrated, and that reagents have been stored properly.

If the control DNA is amplified, the problem may lie with the sample DNA. We suggest you try the following:

- Use the PureGene DNA Isolation Kit (Gentra Systems, Inc., P/N D-5500A).
- Increase the pooling ratio of that marker.
- Perform a DNA titration with:
  - 1/5 less DNA than the original concentration
  - 1/2 less DNA than the original concentration
  - Twice as much DNA as the original concentration
  - Five times as much DNA as the original concentration
- Increase the number of PCR cycles from 30 to 33–35 by increasing the second set of melt/anneal/extend cycles.

If amplification occurs using samples containing less DNA, inhibitors might be present. Washing the samples in a Centricon-100 may help remove inhibitors.

If amplification occurs using samples containing more DNA, the original concentration of DNA in the sample may not have been high enough, or the sample may be degraded.

Optimizing Marker Performance

Increasing Signal Strength

- Increase the amount of a particular marker in your sample by adjusting the pooling ratios for that marker.
- Increase the number of PCR cycles from 30 to 33–35 by increasing the second set of melt/anneal/extend cycles.
- Increase the magnesium chloride concentration by performing a titration as described on page 6-6. Background may increase as well.
- Decrease the annealing temperature 2–3 °C at a time. Background may increase.

Decreasing Background (Non-specific Amplification)

- Decrease the amount of the marker used by adjusting the pooling ratios if background is interfering with allele calls of other markers.
- Increase the annealing temperature 2–3 °C at a time. Overall signal may decrease.

LOH Screening

What is LOH?

The body has many tumor-suppressing genes. These genes are functional unless one or both of the alleles is lost or inactive, the remaining allele contains recessive mutations, or both alleles contain recessive mutations. This allele loss is called loss of heterozygosity (LOH). Analysis of DNA for LOH is a useful tool for the detection of cancer.

The microsatellite markers used in LOH screening map either to known oncogenes or to tumor-suppressor genes. Therefore, the loss of the DNA region containing the linked oncogene or tumor-supressor gene often correlates both with the loss of LOH markers and with the onset or susceptibility to certain types of cancer.

LOH is the second “hit” in the two-hit model. One needs to inherit a mutant oncogene or tumor suppressor gene for LOH to cause cancer (Mulligan *et al.*, 1990).

LOH screening has demonstrated reliability in the early detection of nonpolyposis colon cancer, as well as for prognosis in confirmed cases (Aaltonen *et al.*, 1993; de la Chapelle and Peltomaki, 1995; Canzian *et al.*, 1996). The application of this technique to other tumor types only awaits further characterization of the relevant genes and linked polymorphic markers.

Advantages

The original LOH studies (Dryja *et al.*, 1984; Mannens *et al.*, 1988) employed DNA probes that recognize RFLPs throughout the genome (Southern analysis). PCR-based detection of LOH has the following advantages over Southern-based LOH:

- It is faster than Southern-based detection of LOH.
- It requires only minute (nanogram) amounts of tumor DNA.
- It is suitable for formalin-fixed and paraffin-embedded archival tissue.

  The third feature of PCR-based LOH will prove extremely useful in developing tests to diagnose and predict the course of new types of cancer. Existing archival tissue contains a wealth of genetic material along with complete patient histories.

Limitations

- Because LOH often appears in the same types of tumors as RER (replication error, see page 9-13), in some instances you will need to perform LOH screening in conjunction with RER screening. (The undetected presence of RER can mask the presence of LOH, leading to a false-negative LOH diagnosis.)
- Extraction of high-quality DNA from formalin-fixed and paraffin-embedded tissue can be difficult.
- If you cannot perform accurate DNA quantitation before PCR amplification, interpretation of results can be difficult.

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Advantages of Using ABI PRISM Technology

In one study of cervical cancer, fluorescent detection had several key advantages over radioactive detection:

- Quantitative results were repeatable for a given locus.
- Results from contiguous loci were entirely consistent.

  When two contiguous loci indicated LOH, the calculated LOH ratios were similar.

Rapid Screening

Analysis is rapid. Under ideal circumstances, you can analyze entire chromosomes at a 20–30 cM resolution for 12 tumor/normal pairs in a single experiment. For any given sample, you can detect LOH in 1 day.

The ABI PRISM 310 Genetic Analyzer allows extremely rapid separations. Fragments that are 300 bp or less in length can be separated in under 30 minutes. This translates to a throughput of at least 48 samples in a 24-hour period.

Microsatellite RER/LOH Assay

Any microsatellite markers can be used in LOH and replication error (RER) screening. Applied Biosystems offers two options:

- The Applied Biosystems Microsatellite RER/LOH Assay (P/N K0015) is a fluorescent microsatellite assay that detects RER and loss of heterozygosity (LOH) in genomic DNA isolated from microdissected normal and tumor tissue pairs. This assay is for research purposes only. It can be used to help determine if tumor cells are positive for the RER or the LOH phenotype.

  The Microsatellite RER/LOH Assay includes reagents used for the polymerase chain reaction (PCR) amplification of ten microsatellite markers located on several chromosomes near known and suspected cancer genes. The assay also includes control DNA.

  Refer to the *Microsatellite RER/LOH Assay User's Manual* (P/N L0089) for more information.

- The ABI PRISM Linkage Mapping Set Version 2 (see page 9-2), a 10-cM microsatellite map, has 400 markers that can also be used for LOH and RER screening. However, this kit may require optimization for your application because of the quantitative nature of LOH/RER screening.

Performing LOH Screening

Overview

Microsatellites are amplified using one fluorescently labeled and one unlabeled primer for each locus. Two amplifications are run for each microsatellite, one using sample genomic DNA isolated from normal cells, and one using sample DNA isolated from tumor cells from the same individual.

An GeneScan Internal Lane Size Standard is added to each sample before denaturation and loading. The amplification products are separated and detected using the ABI PRISM 310 Genetic Analyzer and the data is analyzed using GeneScan® Analysis Software. Further analysis can be done using Genotyper® software.

Run Setup

LOH run setup and operation is the same as for the basic microsatellite protocol given in Chapter 8, with the following minor modifications:

- Run two DNA samples from each individual:
  - One from normal tissue (N)
  - One from tumor tissue (T)

Note Some normal tissue contaminating the tumor tissue sample is typical.

- Run 3–4 independent injections for each sample (N and T).

  This allows you to obtain sufficiently accurate quantitative estimates for subsequent data analysis.
- In addition to the controls suggested for basic microsatellite analysis (see “Control DNA” on page 8-5), obtain 8–10 confirmed N/T sample pairs to test the ability to detect LOH.

Handling Samples

Preventing Contamination

When using the PCR process, certain laboratory practices are necessary in order to avoid false positive amplifications (Kwok and Higuchi, 1989). This is because the PCR process is capable of amplifying single DNA molecules (Saiki *et al.*, 1985; Mullis and Faloona, 1987).

- Wear a clean lab coat (one never worn while handling amplified PCR products or doing sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever contamination is possible.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification and analysis of PCR products
- Use aerosol-resistant (filter-plugged) PCR pipet tips.
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Clean lab benches and equipment regularly with 10% bleach solution.

Precautions for Working with Human Tissue

! WARNING ! BIOHAZARD. Tissue samples have the potential to transmit infectious diseases. Follow the latest guidelines published by the Centers for Disease Control (CDC) and National Institutes of Health (NIH) concerning the principles of risk assessment, biological containment, and safe laboratory practices for activities involving clinical specimens. These principles can be found in the U.S. Department of Health and Human Services (HHS) publication, *Biosafety in Microbiological and Biomedical Laboratories* (publication number 93-8395, stock number 017-040-523-7). The biosafety Level 2 containment elements are consistent with the Occupational Health and Safety Administration (OSHA) requirements contained in the HHS *OSHA Bloodborne Pathogen Standard 29 CFR*, part 1910.1030.

Preparing DNA

Extract DNA

Genomic DNA for the RER/LOH Assay can be extracted from fresh, frozen, or paraffin-embedded tissue.

For successful results, particularly when extracting DNA from paraffin-embedded tissue, you can use the following:

- Nucleon Genomic DNA Kit (Scotlab, P/N SL-8501)
- PureGene DNA Isolation Kit (Gentra Systems, Inc., P/N D-5500A)
- QIAamp Tissue Kit (Qiagen, Inc., P/N 29304)

Good yields of DNA have been obtained in Applied Biosystems laboratories using any of these kits and have also found that they work well to eliminate PCR inhibitors from the DNA. Extract the DNA as described in the protocol provided with these kits.

Note The quality of genomic DNA isolated from paraffin-embedded tissue will vary with the type of fixative used, the time in formalin, the age of the sample, and the method of DNA isolation.

Evaluate DNA Quality and Quantity

After DNA extraction it is critical to evaluate DNA quantity. This can be done using the QuantiBlot® Human DNA Quantitation Kit (P/N N808-0114) or spectrophotometrically using Picogreen (Molecular Probes, P/N P-7581).

DNA quality and quantity can also be evaluated by electrophoresing 5 µL of genomic DNA through a 1% Seakem GTG agarose gel with 0.8 µg/mL ethidium bromide.

Note The genomic DNA must be quantitated accurately for the assay to be successful.

Estimate DNA yields by comparing the isolated DNA to DNA standards of known molecular weight and concentration. A 5-µm section of 1 cm^2 paraffin-embedded tissue generally yields 100–500 ng of DNA. The size of the DNA typically ranges from 200 base pairs (bp) to one kilobase pair (kb) or more, depending on how the tissue was processed.

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PCR Amplification

The protocols described here use the Microsatellite RER/LOH Assay, which is optimized for LOH/RER assays and includes appropriate controls. This kit is a convenient way to become familiar with these assays, even if your loci of interest are not in the kit.

Preparing PCR Samples

This procedure describes how to prepare DNA samples for amplifying specific loci within the human genome. Refer to the *Microsatellite RER/LOH Assay User's Manual* for more information.

Step	Action
1	Label ten (one for each marker) 0.2-mL MicroAmp® tubes for each control sample and ten for each tumor sample.
2	Thaw and gently vortex the PCR Mix.
3	In an area free of PCR products, aliquot 15 µL of the PCR Mix into each MicroAmp tube.
4	Dilute the DNA to a final concentration of 10 ng/µL using sterile-distilled water or DNA Diluent Buffer. **Note** If 1X TE buffer is used to dilute genomic DNA, the EDTA in the buffer will alter the magnesium concentration in the PCR Mix, and potentially influence the amplification results.
5	Combine the following to prepare 60 µL of 12X Master Mix: ♦ DNA Diluent Buffer, 27.6 µL ♦ AmpliTaq Gold DNA Polymerase (5 U/µL), 2.4 µL ♦ Normal or tumor genomic DNA (10 ng/µL), 30 µL
6	Aliquot 5 µL of the Master Mix into each MicroAmp tube containing the PCR Mix.
7	Close the MicroAmp tubes.
8	Place the MicroAmp tray with samples into a centrifuge with a 96-well adapter. Spin the tubes for 20 seconds at 150 × *g*.
9	Place the MicroAmp tray with samples into the thermal cycler.
10	Program the GeneAmp® PCR System 9600 as described in "Thermal Cycling" on page 9-10. **Note** Upon completion of thermal cycling, you may store the samples at 2–6 °C.

Thermal Cycling

The thermal cycling conditions in Table 9-1 and Table 9-2 are optimized for the GeneAmp 9600 PCR System. Other thermal cyclers may require reoptimization of cycling conditions.

IMPORTANT DNA from paraffin-embedded tissue requires more amplification cycles than DNA from fresh or frozen tissue.

Table 9-1 Thermal Cycling Parameters for Samples Isolated from Fresh or Frozen Tissue

Step	AmpliTaq Gold Activation	PCR			Final Extension	Preserve Sample
	Hold	30 Cycles			Hold	Hold
		Denature	Anneal	Extend		
Time	10 minutes	10 seconds	30 seconds	3 minutes	30 minutes	Forever
Temperature	95 °C	96 °C	55 °C	70 °C	70 °C	4 °C

Table 9-2 Thermal Cycling Parameters for DNA Samples Isolated from Paraffin-embedded Tissue

Step	AmpliTaq Gold Activation	PCR			Final extension	Preserve Sample
	Hold	45 Cycles			Hold	Hold
		Denature	Anneal	Extend		
Time	10 minutes	10 seconds	30 seconds	3 minutes	30 minutes	Forever
Temperature	95 °C	96 °C	55 °C	70 °C	70 °C	4 °C

Pooling the Markers

For specific information on pooling markers, refer to Chapter 2 of the *ABI PRISM Linkage Mapping Set Version 2 User's Manual* (P/N 904999).

Why Pool the Markers

It is necessary to pool markers in different volumes because:

- Primers amplifying markers are labeled with fluorescent dyes that vary in intensity.
- The yields of the PCR products from the few markers are different.
- DNA varies in amplification efficiency, especially DNA extracted from paraffin-embedded tissue.

Optimizing Pooling Ratios

Occasionally one marker may amplify either more or less than expected. If the fluorescent intensity is too high or too low:

- Load and run that one marker sample individually, or
- Adjust the pooling ratios

Note Loading the gel with a volume of sample that gives a fluorescent signal of approximately 500–1000 relative fluorescent units (RFU) will produce the most consistently interpretable data. Although the dynamic range of the DNA sequencing instruments is

50–6000 RFU, a target of approximately 1000 RFU will ensure that data remains within this range, even if there is slight sample-to-sample and run-to-run variation.

Analyzing LOH Data

Preliminary Data Analysis

Follow the protocols for creating a matrix, setting Analysis Parameters, and analyzing sample files provided in Chapter 8, “Microsatellite Analysis.”

For detailed instructions on using Genotyper to assess LOH see “Using Analyze and Calculate in Table Commands—An LOH Example” on page 8-26 of the *Genotyper User’s Manual* or Chapter 6 of the *Microsatellite RER/LOH Assay User’s Manual*.

Average Across Independent Injections

Once GeneScan software determines the peak height and area for all alleles of all relevant microsatellite loci, use Genotyper software to pool the data from all the independent injections of each N or T sample to obtain an average peak height and area for every allele in every sample.

IMPORTANT Do not combine N data with T data to obtain this average.

IMPORTANT In almost any microdissection there are contaminating normal cells, so normal DNA is present. Your results will depend upon the amount of contaminating normal DNA in your sample.

Calculate the LOH Value

LOH can be defined mathematically as follows:

$$\mathrm{LOH} = \frac{\dfrac{(\text{height of normal allele two})}{(\text{height of normal allele one})}}{\dfrac{(\text{height of tumor allele two})}{(\text{height of tumor allele one})}} \qquad \text{(Eq. 1)}$$

An LOH value ≤0.5 indicates that the tumor sample shows significant loss of the longer allele whereas an LOH value ≥1.5 indicates that the tumor sample shows significant loss of the shorter allele.

Note If a particular locus in an N sample is homozygous, you cannot use that locus to diagnose LOH for the corresponding N-T pair.

Peak Height vs. Peak Area

Although a number of examples in the literature (*e.g.*, Canzian *et al.*, 1996; Cawkwell *et al.*,1993) calculate LOH using peak area, we find peak height to be a more reliable metric.

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Sample Calculation

Here is an example of a LOH calculation for the TP53-Penta marker, which is located near the p53 gene.

Using Equation 1:

$$LOH = \frac{\frac{1343}{1723}}{\frac{2315}{480}} = \frac{0.2073}{1.283} = 0.162$$

An LOH value of 0.16 clearly indicates loss of heterozygosity in the tumor sample.

In Figure 9-1, the first electropherogram corresponds to the normal tissue sample and the second electropherogram to the tumor tissue sample. Peak heights are as shown in Figure 9-1.

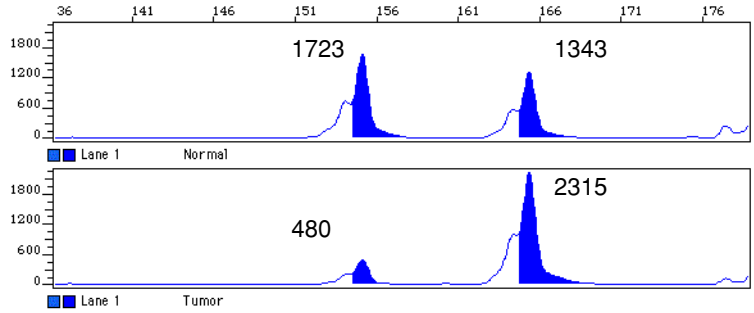

Figure 9-1 Example of LOH at TP53-Penta

Preferential Amplification

When alleles differ in size by ten or more base pairs you will likely observe preferential amplification of shorter PCR products over longer ones (Walsh *et al.,* 1992). This will also occur when amplifying low copy number DNA or DNA isolated from paraffin embedded tissues. Be aware that preferential amplification can make LOH measurements less accurate.

Figure 9-2 shows an electropherogram example of preferential amplification of the D5S346 marker. In both the normal (top panel) and tumor (bottom panel) samples, the peak height of the larger 124-bp fragment is much less than that of the smaller 110-bp fragment.

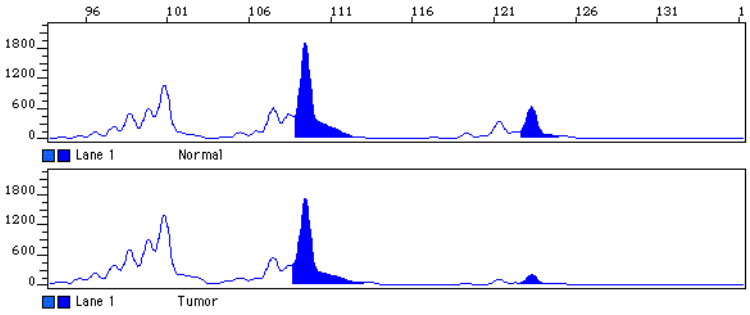

Figure 9-2 Example of preferential amplification of the shorter PCR product at D5S346

RER Screening

What is RER? Replication error (RER), also called microsatellite instability, describes the reduced fidelity during the replication of repetitive DNA often occurring in tumor cells. RER leads to the appearance of multiple alleles at microsatellite loci. It is thought to be caused by strand slippage during DNA replication due to mutations in DNA mismatch repair genes.

The technique for detecting RER involves comparing microsatellite alleles after PCR amplification in normal and tumor samples from the same host. You calculate a raw "RER score" using an algebraic formula that quantifies the relative strength of the stutter bands in the two samples after normalizing for differences in PCR efficiency.

Advantages RER is a simple, inexpensive, and reliable tool for the analysis of tumors.

Limitations The RER phenotype can be variable, ranging from a simple increase in the strength of the stutter bands to the presence of extra bands on top of variable strength stutter bands.

Although the formula for determining the raw RER score partially corrects for differences in the amplification efficiency of normal and tumor samples, extreme discrepancies in amplification efficiency can lead to false-positive or false-negative results. For example, if the amplification of the normal sample is unusually poor, Genotyper might only recognize the relatively strong peaks corresponding to the main alleles. In an efficiently-amplified tumor sample, both the main-allele peaks and the stutter peaks would be recognized. In this hypothetical case, you would obtain a false-positive determination of RER.

Because RER often appears in the same types of tumors as LOH, RER screening should be performed in conjunction with LOH screening. A false-negative RER diagnosis can be obtained in LOH-positive samples.

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Advantages of Using ABI PRISM Technology

Increasing Reliability of RER Results

Traditional approaches using radioactivity are labor-intensive, time-consuming, and difficult to automate. For example, multiple exposures of films are often required and if normal and tumor DNA do not amplify with equal efficiency, interpretation becomes difficult. Moreover, automation is highly desirable to reduce the arbitrariness in analysis encouraged by the variability of the RER phenotype.

Co-electrophoresis To Increase Throughput

One of the primary advantages of using multiple dyes in RER screening is valid for any microsatellite application: you can increase throughput by co-loading multiple different reactions covering many relevant microsatellite loci for a single individual in one capillary injection. Co-loading allows you to screen hundreds of individuals in a single day.

Rapid Screening

The ABI PRISM 310 Genetic Analyzer allows extremely rapid separations. Fragments that are 300 bp or less in length can be separated in under 30 minutes. This translates to a throughput of at least 48 samples in a 24-hour period.

Protocol

RER screening uses the same protocols as LOH screening (see page 9-7).

Examples of RER

This section presents two classic examples of RER. Refer to them when interpreting your own data.

Figure 9-3 shows the electropherogram of the dinucleotide repeat marker D18S35 from a homozygous individual. The appearance of numerous extra alleles at lower molecular weights in the tumor sample (bottom panel) indicates significant genomic instability.

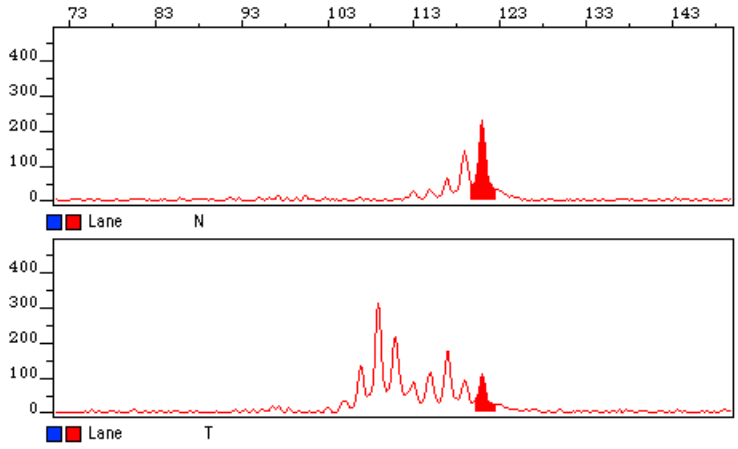

Figure 9-3 An example of RER for a homozygote allele at D18S35

Figure 9-4 on page 9-15 shows the electropherogram of two dinucleotide repeat markers (NM23 and D5S346) from one individual, heterozygous in both alleles. The appearance of numerous extra alleles at both higher and lower molecular weight in the tumor (bottom panel) sample indicates significant genomic instability.

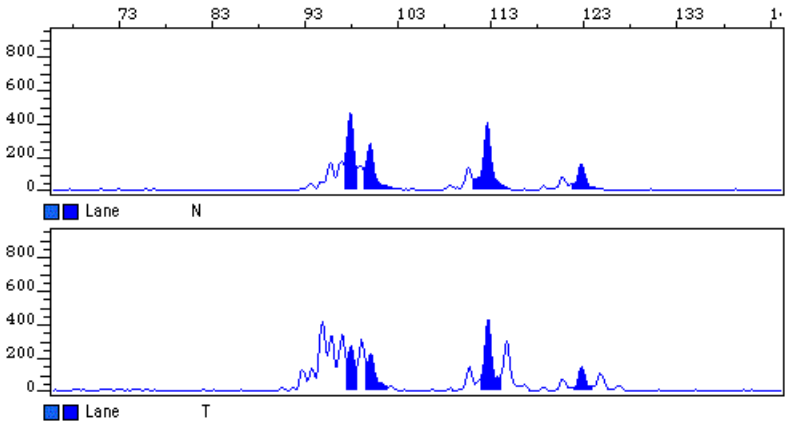

Figure 9-4 An example of RER for heterozygote alleles at markers NM23 and D5S346

RER is often more subtle than is shown in these examples. Virtually all difference between normal and tumor samples that are not LOH are interpreted as replication error. In general, you should see RER in more than one marker to be sure that it is really genomic instability that you are observing and not merely an artifact.

Note While both microsatellite instability and loss of heterozygosity are indicative of cancerous tissue, if an electropherogram shows RER at a given marker location, an LOH calculation for that allele region is complicated or even invalid (Canzian *et al*. 1996). We do not recommend LOH calculations in regions that show clear signs of RER.

Troubleshooting LOH and RER Screening

Common LOH and RER Problems

Most problems in LOH and RER screening are common to all microsatellite applications. See Chapter 11 for more information. A few problems that are more specific to LOH and RER screening are described in Table 9-3.

Table 9-3 Troubleshooting LOH and RER Screening

Observation	Possible Cause	Recommended Action
No bands on GeneScan gel	PCR amplification failed due to presence of PCR inhibitors.	Run DNA sample on an agarose gel. If DNA is present, repurify the sample using the QIAamp Tissue Kit or other commercial kit to remove PCR inhibitors.
	DNA degraded.	If no DNA is observed on an agarose gel, prepare new DNA from the tissue sample.
	Incorrect DNA loading or mispipetting.	
Preferential amplification of shorter PCR products over longer ones	Alleles are separated by ten base pairs or more.	Make sure the DNA is quantitated accurately for both the normal (N) and tumor (T) samples.

Animal Paternity

StockMarks Kits

The StockMarks for Cattle® Bovine Paternity PCR Typing Kit uses 11 microsatellite loci to automate the genotyping of cattle for breeding purposes.The StockMarks® for Horses Equine Paternity PCR Typing Kit uses 12 microsatellite loci to automate the genotyping of horses for breeding purposes.

Improved Breeding

Humans have been breeding cattle and horses selectively for centuries. Animals that exhibit superior production traits, such as high milk production, lean carcasses, speed, or strength are used as breeding stock for subsequent generations. This classical method requires the measurement of quantitative production traits and maintenance of ancestral records by the breeding and racing associations. Recently, researchers have turned to microsatellite markers to identify genetically desirable animals much more quickly, reliably, and inexpensively.

The StockMarks kits have been used to perform the following:

- Parental identification for accurate pedigree analysis
- Quantitative trait loci (QTL) research to find markers linked to desirable traits

Advantages of DNA-based Tests

The amplification capability of PCR, combined with the information content of microsatellites, provides the following advantages:

- PCR-based tests are easy to standardize and automate, ensuring reproducible results.
- PCR-based tests can be run on a variety of samples, including blood, semen, and hair. Obtaining samples from hair eliminates the expense of having a veterinarian draw blood. In addition, hair is easier to transport than blood.
- Very little sample is required for a positive result, unlike traditional serological (blood) assays.
- Quicker, more accurate parentage identification can be obtained from DNA analysis than from serological testing.
- DNA analysis allows inclusion as well as exclusion. An animal can be identified positively as the parent rather than merely being eliminated as a possibility.

Advantages of Using ABI PRISM Technology

One of the primary advantages of using multiple dyes in StockMarks analysis is valid for any mapping or identification application: you can increase throughput by co-loading multiple different reactions covering all relevant microsatellite loci for a single individual in one capillary injection. Co-loading allows you to genotype hundreds of animals in a single day.

You can also automate genotyping by analyzing your results with GeneScan Analysis and Genotyper software (Figure 9-5 on page 9-18).

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Allele Frequencies

StockMarks for Cattle

Allele frequency information is currently available for the Holstein dairy cattle breed. Similar frequency information is being collected for Aberdeen Angus, Red Angus, Simmental, Gelbvieh, Salers, and South Devon. To test non-Holstein breeds with the StockMarks for Cattle kit, you will first need to genotype approximately 20 unrelated animals to determine if the Holstein frequencies apply to the breed of interest.

StockMarks for Horses

Allele frequency information is currently available for a number of horse breeds (Warmblood 1 and 2, Standardbred, Fjord, Friesian, Throroughbred, and Tennessee Walkers).

Genotyping Example

A Genotyper plot of GeneScan results from the equine control DNA is shown in Figure 9-5. All 12 alleles amplified by the StockMarks for Horses Equine Paternity PCR Typing Kit are plotted.

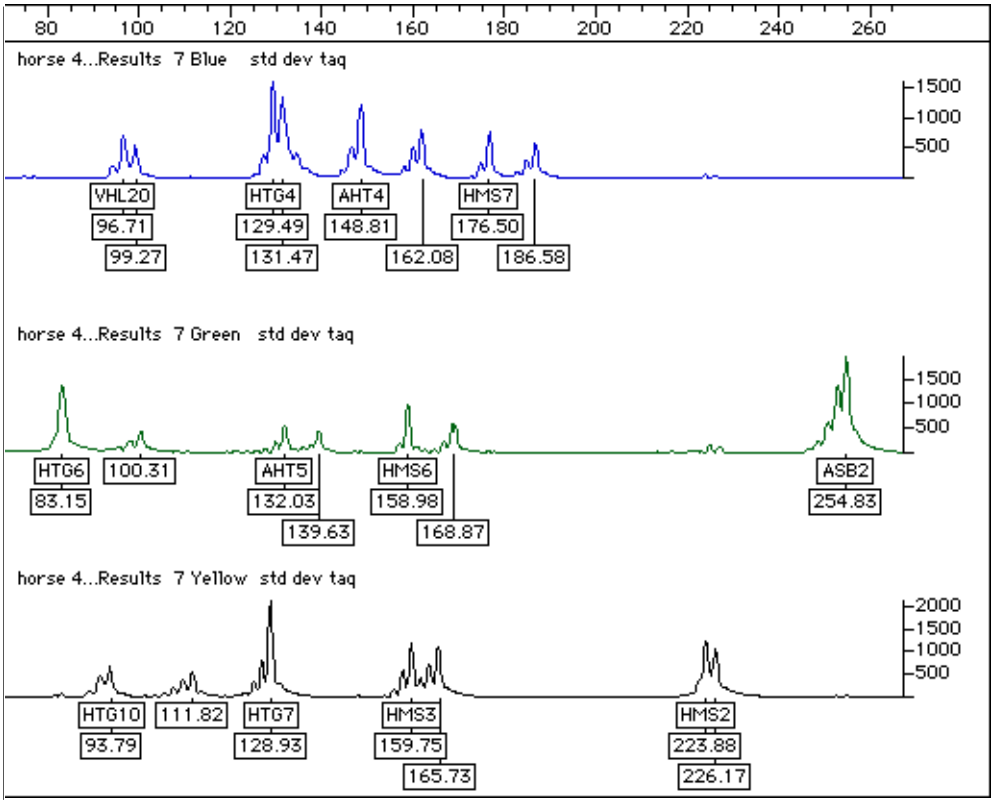

Figure 9-5 Genotyper plot of the equine control DNA

For More Information

Refer to the *StockMarks for Cattle Bovine Paternity PCR Typing Kit Protocol* (P/N 401917) and the *StockMarks for Horses Equine Paternity PCR Typing Kit Protocol* (P/N 402828) for more information. You can also find information about animal paternity testing on the Agriculture page of the Applied Biosystems Web site (www.appliedbiosystems.com/techsupport).

Human Identification

Human Identification with STRs

Short tandem repeat (STR) markers, also referred to as microsatellites, are polymorphic DNA loci that contain a repeated nucleotide sequence. The STR repeat unit can be from two to seven nucleotides in length. The number of nucleotides per repeat unit is the same for a majority of repeats within an STR locus. The number of repeat units at an STR locus may differ, so alleles of many different lengths are possible. Polymorphic STR loci are therefore very useful for human identification purposes (Edwards *et al.*, 1992).

STR loci can be amplified using the polymerase chain reaction (PCR) process and the PCR products are then analyzed by electrophoresis to separate the alleles according to size. PCR-amplified STR alleles can be detected using various methods, such as fluorescent dye labeling, silver staining, or fluorescent dye staining.

The analysis of short tandem repeat loci is an important complement to the length- and sequence-based DNA typing systems already in use for human identification. A majority of the STRs that have been evaluated by the forensic community are composed of four-nucleotide repeat units (Frégeau and Fourney, 1993; Kimpton *et al.*, 1993; Urquhart *et al.*, 1995).

Advantages of STR Analysis

PCR-based STR analysis has the following advantages over conventional methods of DNA analysis such as Restriction Fragment Length Polymorphism (RFLP):

- PCR-based tests are rapid, giving results in 24 hours or less.
- The small size of STR loci improves the chance of obtaining a result, particularly for samples containing minute amounts of DNA and/or degraded DNA.
- The small size range of STR loci makes them ideal candidates for co-amplification while keeping all amplified alleles smaller than 350 base pairs. Many STR loci can therefore be typed from a single PCR.
- STR alleles have discrete sizes, allowing for simplified interpretation of results.
- The STR alleles can be combined to form an allelic ladder, which is used to genotype individuals.
- PCR-based STR tests can be automated, increasing laboratory throughput while decreasing analysts' hands-on time.

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Applied Biosystems Fluorescent Dye Technology

Applied Biosystems fluorescent multicolor dye technology allows multiple loci, including loci that have alleles with overlapping size ranges, to be analyzed in a single gel lane or capillary injection. Alleles for overlapping loci are distinguished by labeling locus-specific primers with different color dyes.

Because only one primer of each pair is labeled, the ABI PRISM® instruments detect only one strand for each amplified DNA fragment. The detection of only one strand eliminates doublets arising from the different mobilities of complementary strands that are often observed when using gel staining detection methods.

Automated Sizing and Genotyping

Amplified samples can be analyzed in a slab gel format or can be injected into a capillary. An internal lane size standard is loaded with each sample to allow for automatic sizing of the PCR products and to normalize differences in electrophoretic mobility between gel lanes or injections (Ziegle *et al.*, 1992). GeneScan software automatically analyzes the collected data, which can then be imported into Genotyper software for automatic genotyping of alleles.

High Throughput

Laboratories can analyze hundreds of loci in a single day using four-dye fluorescent labeling. This is a dramatic increase in productivity compared with gel staining techniques, which visualize all PCR products in the same color, or with other systems that are limited to one or two colors.

continued on next page

AmpFℓSTR Loci

The AmpFℓSTR™ PCR Amplification Kits co-amplify the repeat regions of various short tandem repeat loci (Table 9-4). In some kits, a segment of the X-Y homologous gene amelogenin is also amplified. Amplifying a segment of the amelogenin gene with a single primer pair can be used for gender identification because different length products from the X and Y chromosomes are generated (Sullivan *et al.*,1993).

Table 9-4 AmpFℓSTR loci

Locus Designation	Chromosome Location	Common Sequence Motif	Size Range (bp)[a]	Dye Label
D3S1358	3p	TCTA $(TCTG)_{1-3}$ $(TCTA)_n$	114–142	5-FAM
vWA	12p12-pter	TCTA $(TCTG)_{3-4}$ $(TCTA)_n$	157–197	5-FAM
FGA	4q28	$(TTTC)_3$ TTTT TTCT $(CTTT)_n$ CTCC $(TTCC)_2$	219–267	5-FAM
Amelogenin	X: p22.1–22.3 Y: p11.2	– –	107 113	JOE
D8S1179[b]	8	$(TCTR)_n$[c]	128–168	JOE
TH01	11p15.5	$(AATG)_n$	169–189	JOE
D21S11	21	$(TCTA)_n$ $(TCTG)_n$ [$(TCTA)_3$ TA $(TCTA)_3$ TCA $(TCTA)_2$ TCCA TA] $(TCTA)_n$	189–243	JOE
TPOX	2p23–2per	$(AATG)_n$	218–242	JOE
D18S51	18q21.3	$(AGAA)_n$	273–341	JOE
CSF1PO	5q33.3–34	$(AGAT)_n$	281–317	JOE
D5S818	5q21–31	$(AGAT)_n$	135–171	NED
D13S317	13q22–31	$(GATA)_n$	206–234	NED
D7S820	7q	$(GATA)_n$	258–294	NED

a. The size range is the actual base pair size of sequenced alleles contained in the AmpFℓSTR Allelic Ladders. The sizes in the table include the 3´ A nucleotide addition.

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

c. R can represent either an A or G nucleotide.

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AmpFℓSTR Kits

The following AmpFℓSTR kits are currently available from Applied Biosystems:

- AmpFℓSTR Blue™ PCR Amplification Kit (P/N 402800)
- AmpFℓSTR Green™ I PCR Amplification Kit (P/N 402902)
- AmpFℓSTR Profiler™ PCR Amplification Kit (P/N 403038)
- AmpFℓSTR Profiler Plus™ PCR Amplification Kit (P/N 4303326)

Each AmpFℓSTR kit contains preformulated AmpFℓSTR PCR Reaction Mix, blended primer set, AmpliTaq Gold™ DNA Polymerase, control DNA of known genotype, mineral oil, and AmpFℓSTR Allelic Ladders. The PCR license rights for forensic testing and research use are also included in the kit.

The AmpFℓSTR loci contained in the AmpFℓSTR kits are shown in Table 9-5.

Table 9-5 Loci in the AmpFℓSTR Kits

Locus	AmpFℓSTR Blue	AmpFℓSTR Green I	AmpFℓSTR Profiler	AmpFℓSTR Profiler Plus
D3S1358	X		X	X
vWA	X		X	X
FGA	X		X	X
Amelogenin		X	X	X
TH01		X	X	
TPOX		X	X	
CSF1PO		X	X	
D8S1179				X
D21S11				X
D18S51				X
D5S818			X	X
D13S317			X	X
D7S820			X	X

continued on next page

Genotyping Using the AmpFℓSTR Allelic Ladders

The AmpFℓSTR Allelic Ladders contain the most common alleles of the loci and are used to genotype the analyzed samples. The AmpFℓSTR Allelic Ladders in the AmpFℓSTR Profiler Plus PCR Amplification Kit are shown in Figure 9-6.

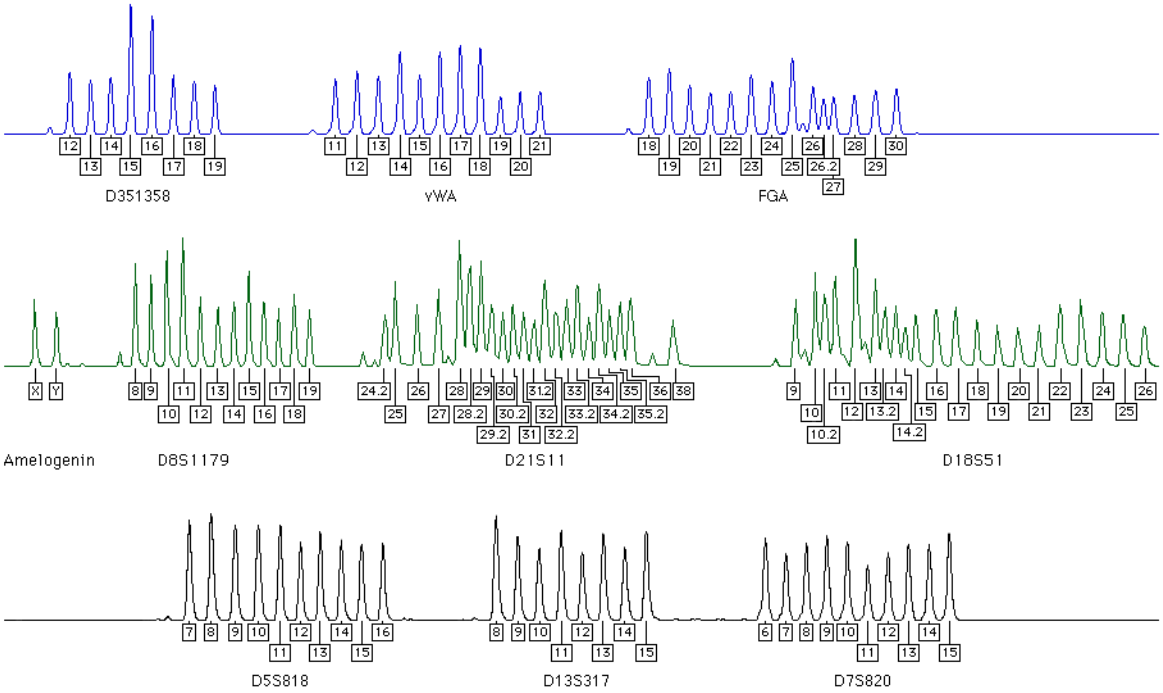

Figure 9-6 Genotyper plot of the AmpFℓSTR Allelic Ladders contained in the AmpFℓSTR Profiler Plus PCR Amplification Kit

When interpreting AmpFℓSTR kit results, genotypes are assigned to sample alleles by comparison of their sizes to those obtained for the known alleles in the AmpFℓSTR Allelic Ladders. Genotypes, not sizes, are used for comparison of data between runs, instruments, and laboratories. We strongly recommend that laboratories use the AmpFℓSTR Allelic Ladders on each gel or set of capillary injections to convert the allele sizes to genotypes. The main reasons for this approach are outlined below:

- The size values obtained for the same sample can differ between instrument platforms (ABI PRISM 310 Genetic Analyzer versus ABI PRISM® 377 DNA Sequencer) because of differences in the type and concentration of the gel/polymer matrices and in electrophoresis 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 capillaries result in greater size variation than that found between samples on the same gel or between samples injected in the same capillary.

The internal lane size standard is run with every sample and 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 and genotypes to the samples. The genotyping of samples by comparison to the AmpFℓSTR Allelic Ladder

can be automated using Genotyper 2.0 software and the custom AmpFℓSTR template files.

Reliable Results

The AmpFℓSTR kit reagents and protocols have been optimized to produce the quality of results necessary for human identification applications. Primers have been designed to give a high degree of target specificity, maximum 3´ A nucleotide addition, and balance in intensity between loci labeled with the same color dye (Figure 9-7).

Component concentrations of the reagents combined for PCR amplification (including PCR reaction mix, primer set, and enzyme), are set in the middle of a range of concentrations that give acceptable performance. To increase the success of obtaining a result in the presence of enzyme inhibitors, bovine serum albumin (BSA) has been included in the AmpFℓSTR PCR Reaction Mix.

GeneAmp PCR Instrument times and temperatures have been developed to produce specific amplification while providing the necessary degree of sensitivity. The recommended range of input DNA is 1.0–2.5 ng. Furthermore, instrument detection protocols have been optimized to provide reproducible results with excellent resolution and precision in sizing alleles.

Each AmpFℓSTR Kit has been validated according to the Technical Working Group on DNA Analysis Methods (TWGDAM) guidelines. Validation was performed by scientists at Applied Biosystems in conjunction with forensic laboratories. These validation studies demonstrate that the AmpFℓSTR kits can be used reliably with Applied Biosystems instruments and software to analyze samples commonly encountered in forensic casework.

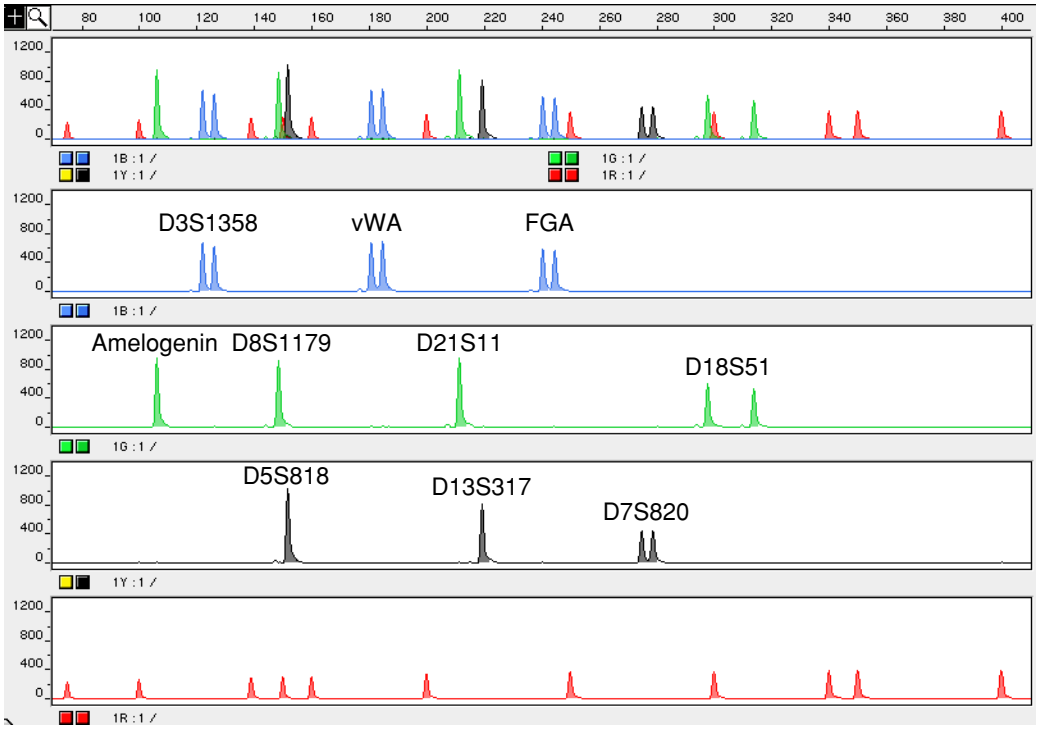

Figure 9-7 GeneScan electropherogram of AmpFℓSTR Profiler Plus alleles in AmpFℓSTR Control DNA 9947A

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Discrimination Power

The Probability of Identity (P_I) and Probability of Paternity Exclusion (P_E) values have been determined for each AmpFℓSTR kit and are shown in Table 9-6. Allele frequencies for each population database are provided in the respective user's manuals. Existence of random association (linkage equilibrium) among all 12 loci was established.

Table 9-6 Discrimination Power of the AmpFℓSTR Kits

AmpFℓSTR Kit	African-American		U.S. Caucasian	
	P_I	P_E	P_I	P_E
AmpFℓSTR Blue	0.00021	N/A	0.00020	N/A
AmpFℓSTR Green I	0.00058	N/A	0.0024	N/A
AmpFℓSTR Profiler	1.23×10^{-10}	0.9996	2.79×10^{-10}	0.9994
AmpFℓSTR Profiler Plus	1.48×10^{-11}	0.999989	1.04×10^{-11}	0.999982

For More Information

Refer to the following manuals:

- *AmpFℓ*STR *Blue PCR Amplification Kit User's Manual* (P/N 402827)
- *AmpFℓ*STR Green I *PCR Amplification Kit User's Manual* (P/N 402944)
- *AmpFℓ*STR *Profiler PCR Amplification Kit User's Manual* (P/N 402945)
- *AmpFℓ*STR *Profiler Plus PCR Amplification Kit User's Manual* (P/N 4303501)

These manuals contain sections describing the following:

- background information on the respective AmpFℓSTR kits
- guidelines for setting up a laboratory for PCR DNA analysis
- recommended protocols for DNA extraction
- the importance of DNA quantitation prior to STR analysis
- protocols for PCR amplification of the kit loci
- information on the multicomponent analysis of fluorescence data
- protocols for detection and analysis of PCR products
- guidelines for interpretation of results
- the use of Genotyper software for automated genotyping of alleles
- guidelines for troubleshooting results
- summaries of the validation work for the respective kits according to the guidelines established by the Technical Working Group on DNA Analysis Methods (TWGDAM)
- population genetics data for the kit loci

The sections detailing how to set up a laboratory for PCR DNA analysis and how to perform the recommended protocols for DNA extraction can be used by all laboratories that perform PCR analysis. These sections are updated from the Applied Biosystems*AmpliType® User's Guide* (Versions 1 and 2).

AFLP Mapping 10

Introduction

What is AFLP?

The AFLP™ amplified fragment polymorphism technique is used to visualize hundreds of amplified DNA restriction fragments simultaneously. The AFLP band patterns, or fingerprints, can be used for many purposes, such as monitoring the identity of an isolate or the degree of similarity among isolates. Polymorphisms in band patterns map to specific loci, allowing the individuals to be genotyped or differentiated based on the alleles they carry.

AFLP technology combines the power of restriction fragment length polymorphism (RFLP) with the flexibility of PCR-based technology by ligating primer-recognition sequences (adaptors) to the restricted DNA.

Advantages of AFLP

The advantages of the AFLP technique include the following:

- Only small amounts of DNA are needed.
- Unlike randomly amplified polymorphic DNAs (RAPDs) that use multiple, arbitrary primers and lead to unreliable results, the AFLP technique uses only two primers and gives reproducible results.
- Many restriction fragment subsets can be amplified by changing the nucleotide extensions on the adaptor sequences. Hundreds of markers can be generated reliably.
- High resolution is obtained because of the stringent PCR conditions.
- The AFLP technique works on a variety of genomic DNA samples.
- No prior knowledge of the genomic sequence is required.

Applications of AFLP

Applications for AFLP in microbial fingerprinting include:

- differentiation and tracking of highly related microbes at the species or strain level
- high-resolution genotyping for taxonomic applications
- detection of DNA polymorphisms in genome evolution studies
- determining the relatedness of pathogenic organisms in epidemiological studies
- mapping of cloned fragments in bacterial and yeast artificial chromosomes (BACs and YACs)

An example of AFLP microbial fingerprints is shown in Figure 10-1 on page 10-2. The first 24 lanes show six samples each of four different *Escherichia coli* strains (each of the six samples represents a different growth phase of the organism). The final 11

lanes show different growth phases of a single strain of *Legionella pneumophila*. Note that the *E. coli* fingerprints are similar to each other and different from the *Legionella* fingerprint. Within a strain, all of the bands are reproducible. Large population studies provide data for the linkage of a band with a given phenotype, such as pathogenicity.

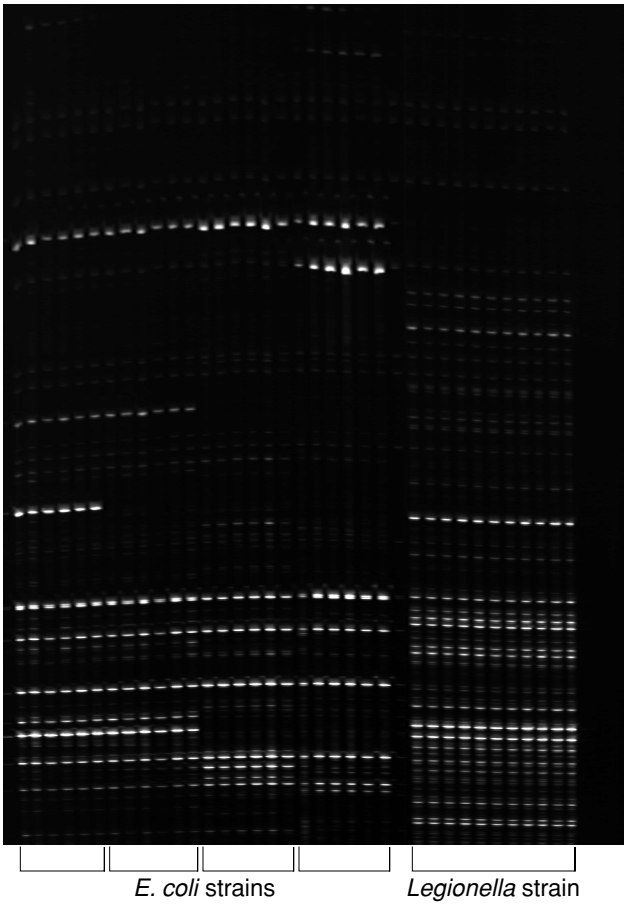


Figure 10-1 AFLP fingerprints of four *E. coli* strains and one *Legionella* strain

Applications for AFLP in plant mapping include:

- establishing linkage groups in crosses
- saturating regions of introgression with markers for gene landing efforts
- assessing the degree of relatedness or variability among cultivars

Examples of AFLP plant mapping are shown in Figure 10-2 and Figure 10-3 on page 10-3 and Figure 10-4 on page 10-4.

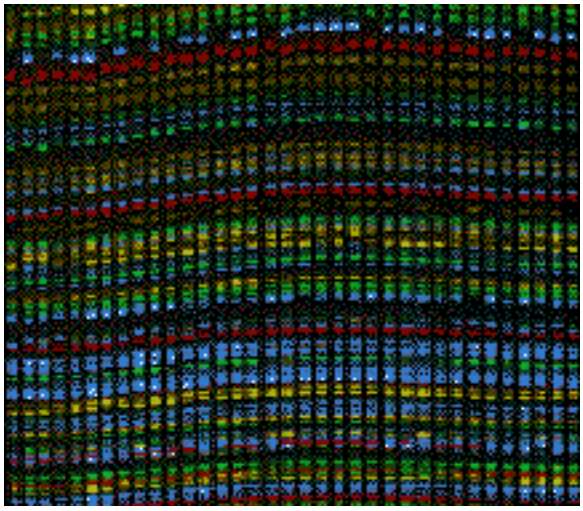


Figure 10-2 AFLP plant mapping gel. Note that the pattern is much more complicated for plants than for bacteria.

You can build a genetic map of markers showing Mendelian inheritance from AFLP data such as that shown in Figure 10-3. The four electropherogram panels in Figure 10-3 contain data from tomato DNA samples prepared using the AFLP technique. Samples were run on an ABI™ 373 DNA Sequencer and the resulting data analyzed using GeneScan® Analysis Software.

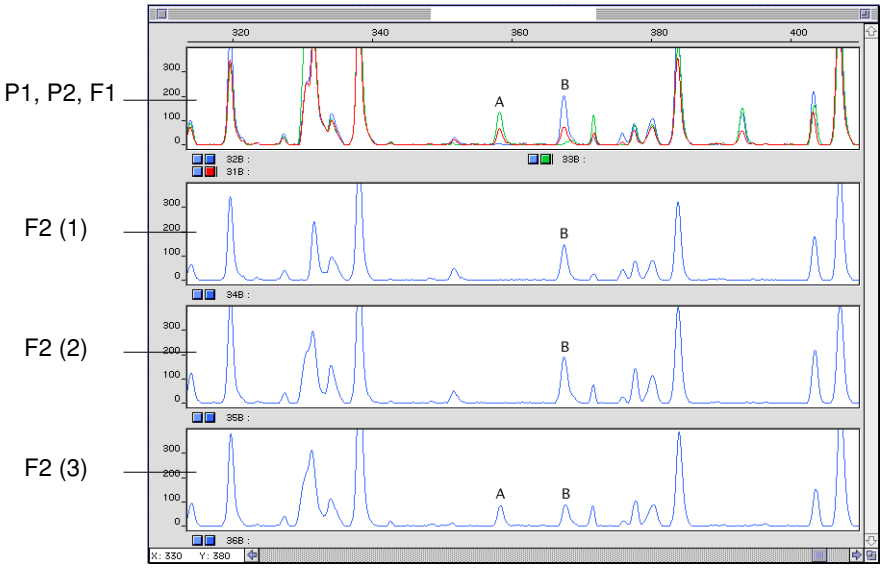
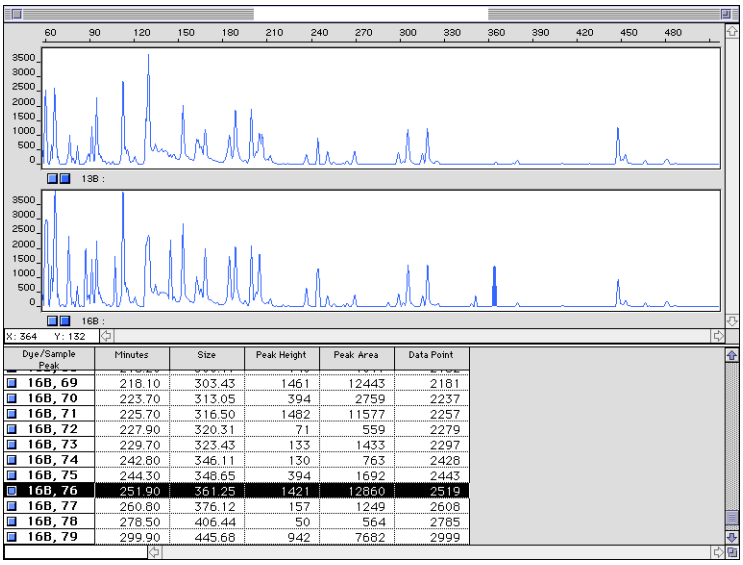


Figure 10-3 Tomato AFLP samples showing Mendelian segregation

The overlapping electropherograms in the top panel are AFLP results of sample DNA from three individuals: parent one (P1), parent two (P2), and F1 from a cross. A and B are the two significant peaks on this panel and appear only in P2 and F1.

The lower three electropherogram panels are AFLP results of sample DNA from three F2 generations. Peak A appears in F2 (3), but does not appear in either F2 (1), or F2 (2). Peak B is inherited in all three F2 individuals. The remaining non-polymorphic

peaks appear in all three F2 electropherograms and show that the overall AFLP patterns are reproducible.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
16B, 69	218.10	303.43	1461	12443	2181
16B, 70	223.70	313.05	394	2759	2237
16B, 71	225.70	316.50	1482	11577	2257
16B, 72	227.90	320.31	71	559	2279
16B, 73	229.70	323.43	133	1433	2297
16B, 74	242.80	346.11	130	763	2428
16B, 75	244.30	348.65	394	1692	2443
16B, 76	251.90	361.25	1421	12860	2519
16B, 77	260.80	376.12	157	1249	2608
16B, 78	278.50	406.44	50	564	2785
16B, 79	299.90	445.68	942	7682	2999

Figure 10-4 Rice AFLP samples showing near-isogenic regions

The two electropherogram panels shown in Figure 10-4 contain data from rice DNA samples prepared using the AFLP technique. Samples were run on an ABI 373 DNA Sequencer and the resulting data analyzed using GeneScan Analysis Software.

The rice DNA was isolated from near-isogenic lines (almost identical genetic material). It was selected for an introgressed region carrying a disease-resistance gene. By comparing peak patterns in the two electropherograms, you will find that the rice lines differ by only 1–2%. One of the peaks distinguishing the two lines has been highlighted in both the electropherogram display and the related tabular data beneath the electropherogram panels.

For examples of other applications, refer to the literature cited in Appendix D.

For More Information

Refer to the *AFLP Microbial Fingerprinting Protocol* (P/N 402977) and the *AFLP Plant Mapping Protocol* (P/N 4303146) for more information.

The AFLP Technique

Template Preparation and Adaptor Ligation

The first step of the AFLP technique is to generate restriction fragments by using two restriction endonucleases (EcoRI and MseI in the AFLP Microbial Fingerprinting and AFLP Plant Mapping Kits). Double-stranded adaptors supplied with each kit are ligated to the ends of the DNA fragments, generating template DNA for subsequent polymerase chain reaction (PCR) amplification.

Restriction and ligation may take place in a single reaction if the buffers are compatible (Figure 10-5). Adaptor sequences have been designed such that ligation of the adaptor oligonucleotide to the restricted DNA does not regenerate the recognition site. If the buffers are not compatible, the reactions must be run sequentially.

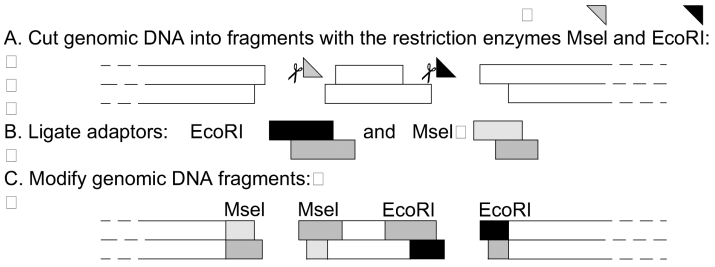


Figure 10-5 Example of template preparation and AFLP adaptor ligation

Preselective Amplification—Microbial Fingerprinting

The sequences of the adaptors and the restriction site serve as primer binding sites for a subsequent low-level selection or “preselective” amplification of the restriction fragments.

Only those genomic fragments that have an adaptor on each end amplify exponentially during PCR amplification (Figure 10-6). This step effectively “purifies” the target away from sequences that amplify only linearly, *i.e.*, those with one modified end.

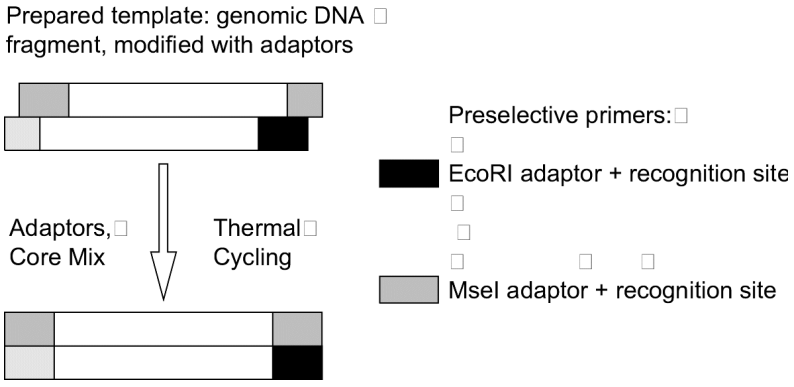


Figure 10-6 Preselective amplification of the prepared template

In the microbial genomes targeted by the AFLP Microbial Fingerprinting Kit (P/N 402948), the core primer sequence is used. In larger genomes, such as plants

and some fungi, this amplification would create too many fragments. In those cases, the preselective amplification is performed with additional nucleotides on the end of each primer (see page 10-7). Each added nucleotide reduces the number of sequences by a factor of four.

The thermal cycling conditions of the preselective amplification step have been optimized to generate a constant final mass of fragments. Band intensity in subsequent reactions can therefore be correlated with relative differences in representation of the fragments within the genome, and not to the overall amount of genomic DNA that went into the initial restriction-ligation mix.

It is not necessary to perform this step if:

- relative peak height information is not desired
- methods are available to normalize the final signal
- very accurate quantitation of the input DNA is performed routinely

Selective Amplification—Microbial Fingerprinting

Additional PCR amplifications are run to reduce the complexity of the mixture further so that the fragments can be resolved on a polyacrylamide gel. These amplifications use primers chosen from the 18 available AFLP Microbial Fingerprinting Kit Selective Primers (nine EcoRI fluorescent dye-labeled primers and nine unlabeled MseI primers). After PCR amplification with these primers, a portion of the samples is analyzed on a Applied Biosystems DNA Sequencer.

Selective amplification with an EcoRI and an MseI primer amplifies primarily EcoRI-MseI-ended fragments. The EcoRI-EcoRI fragments do not amplify well. The MseI-MseI fragments are not visualized because they do not contain fluorescent dye labels. Only the EcoRI-containing strands are detected (Figure 10-7).

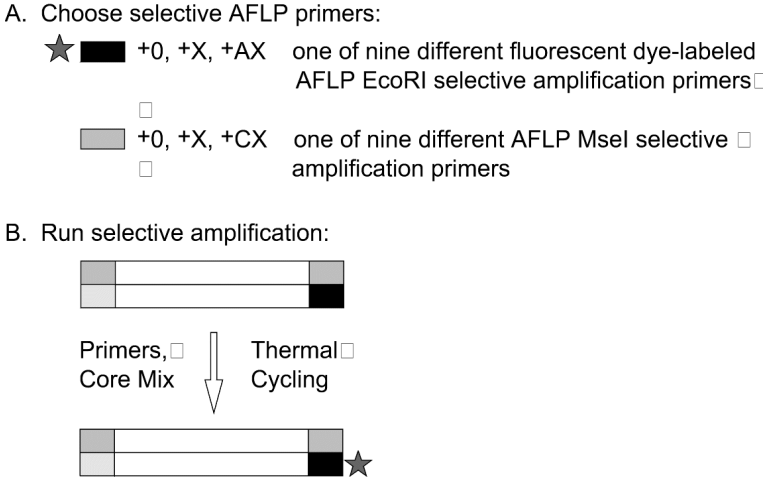


Figure 10-7 Selective amplification with fluorescent dye-labeled primers

continued on next page

Simplifying Complex Patterns

Figure 10-8 shows examples of AFLP fingerprint patterns that were prepared using different selective primers. Note that the EcoRI selective primers with one-nucleotide extensions (EcoRI-A, EcoRI-T, and EcoRI-G) give simpler patterns than that obtained using the primer with no extra nucleotide (EcoRI-0).

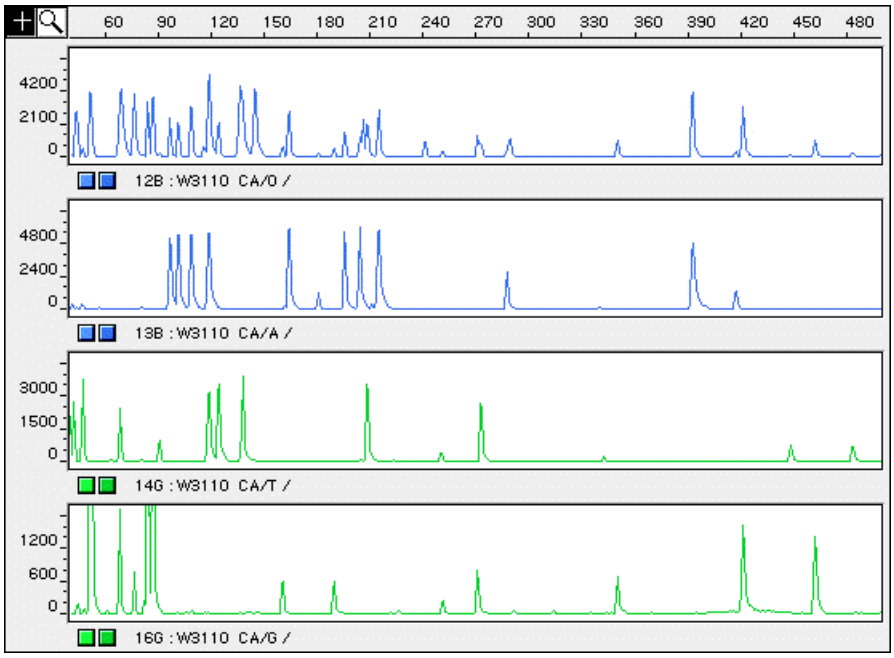


Figure 10-8 AFLP fingerprints of *E. coli* W3110 Reference DNA. The MseI-CA and fluorescent dye-labeled EcoRI-0, EcoRI-A, EcoRI-T, and EcoRI-G selective primers (shown here top to bottom, respectively) were used.

If the complexity of the AFLP pattern is still too high at the +2/+2 level, we recommend reamplifying the preselective amplification sample with the preselective primers from the AFLP Ligation and Preselective Amplification Modules of the AFLP Regular and Small Plant Genome Mapping Kits (P/N 402004 and 402273, respectively).

Preselective Amplification—Plant Mapping

The sequences of the adaptors and the restriction site serve as primer binding sites for a subsequent low-level selection or “preselective” amplification of the restriction fragments. The MseI complementary primer contains a 3´ C. The EcoRI complementary primer contains a 3´ A (Regular Plant Genome Kit modules) or no base addition (Small Plant Genome Kit modules).

Only those genomic fragments that have an adaptor on each end amplify exponentially during PCR amplification (Figure 10-9 on page 10-8). This step effectively “purifies” the target away from sequences that amplify only linearly, *i.e.*, those with one modified end.

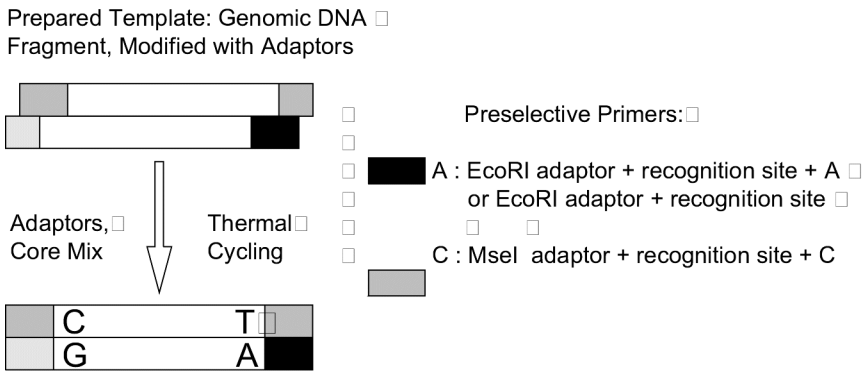


Figure 10-9 Preselective amplification of the prepared template

Selective Amplification—Plant Mapping

Additional PCR amplifications are run to further reduce the complexity of the mixture so that it can be resolved on a polyacrylamide gel. These amplifications use primers chosen from the 24 available AFLP Selective Primers (eight MseI and sixteen EcoRI primers). After PCR amplification with these primers, a portion of each sample is analyzed on a Applied Biosystems DNA Sequencer.

Selective amplification with an EcoRI and an MseI primer amplifies primarily EcoRI-MseI-ended fragments. The EcoRI-EcoRI fragments do not amplify well. The MseI-MseI fragments are not visualized because they do not contain fluorescent dye labels. Only the EcoRI-containing strands are detected (Figure 10-10).

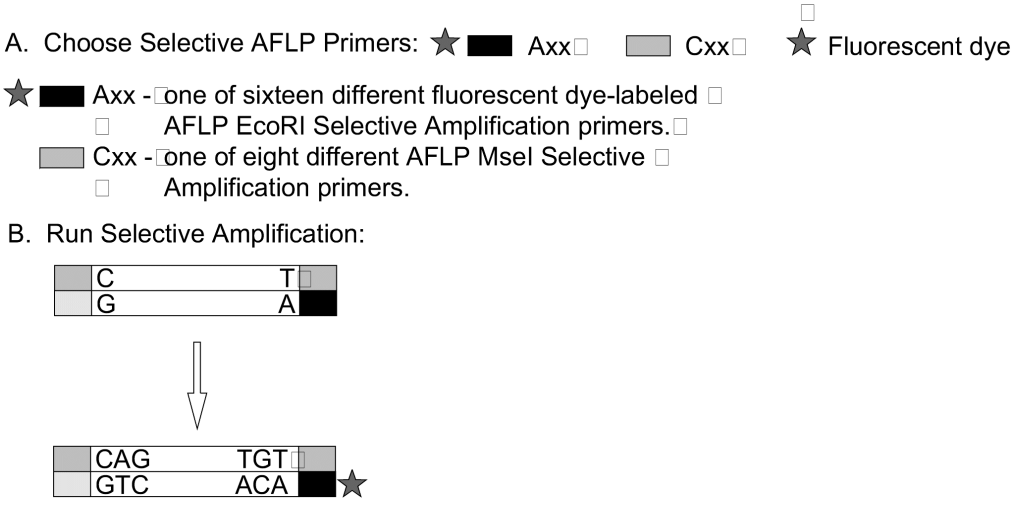


Figure 10-10 Selective amplification with fluorescent dye-labeled primers

Use the AFLP Selective Amplification Start-Up Modules (Regular Plant Genomes, P/N 4303050; Small Plant Genomes, P/N 4303051) or individual AFLP Selective Primers (see Table 10-3 on page 10-11).

continued on next page

Testing New Genomes

When testing novel genomes, you must be sure that the DNA restriction digest with EcoRI and MseI generates enough fragments for comparison of samples. There is a large variability in the number of restriction sites within microbial genomes. No assurances of kit performance are made for organisms not listed.

Empirical guidelines suggest that if the G-C content of the genome is >65%, MseI will not give a significant number of fragments. Optimal results are obtained with MseI when the G-C content is <50%. EcoRI also tends to produce more fragments in G-C-poor genomes. In cases where an organism's G-C content is unknown, the effectiveness of the restriction enzymes must be determined empirically.

Primer Selection Guidelines—Microbial Fingerprinting

For genomes that restrict well with the EcoRI and MseI restriction endonuclease combination, some general recommendations can be made in terms of the genome size and the selective nucleotides to choose for subsequent amplification (Table 10-1).

Table 10-1 Guide to Choosing Selective Primers for Microbial Fingerprinting

Application	Nucleotide Addition	EcoRI Primers	MseI Primers
Cosmids, BACs, P1 mapping	+0/+0	EcoRI-0 FAM	MseI-0
YACs, some larger BACs	+0/+1	EcoRI-0 FAM	MseI-A MseI-C MseI-G MseI-T
	+1/+0	EcoRI-A FAM EcoRI-C NED EcoRI-G JOE EcoRI-T JOE	MseI-0
Bacteria	+0/+2	EcoRI-0 FAM	MseI-CA MseI-CC MseI-CG MseI-CT
	+1/+1	EcoRI-A FAM EcoRI-C NED EcoRI-G JOE EcoRI-T JOE	MseI-A MseI-C MseI-G MseI-T
	+2/+0	EcoRI-AA JOE EcoRI-AC FAM EcoRI-AG JOE EcoRI-AT NED	MseI-0
Yeast, small fungi genomes	+2/+2	EcoRI-AA JOE EcoRI-AC FAM EcoRI-AG JOE EcoRI-AT NED	MseI-CA MseI-CC MseI-CG MseI-CT
Large fungi genomes	+2/+3 +3/+2	Use the primers from the AFLP Regular and Small Plant Genome Mapping Kits. See Table 10-3 on page 10-11 for the primers available.	

continued on next page

Genome Analysis Guide

Some bacterial and fungal genomes that have been analyzed successfully using EcoRI, MseI, and the primers in the AFLP Microbial Fingerprinting Kit are shown in Table 10-2.

Table 10-2 Genomes Analyzed with EcoRI and MseI Primer Pairs

Organism	Primer Pairs Used Successfully[a]	Primer Pairs to Avoid[b]
Acinetobacter sp.	EcoRI-C/MseI-T	–
Aeromonas sp.	EcoRI-A/MseI-T	–
Aspergillus sp.	EcoRI-A/MseI-G EcoRI-A/MseI-CA EcoRI-C/MseI-CA EcoRI-T/MseI-A	–
Bacillus sp.	EcoRI-0/MseI-A	–
Candida utilis	EcoRI-G/MseI-A	–
Clostridium sp.	EcoRI-C/MseI-C	–
Vancomycin-resistant *Enterobacter*	EcoRI-A/MseI-T EcoRI-G/MseI-A EcoRI-T/MseI-C	–
Escherichia coli	EcoRI-0/MseI-C EcoRI-A/MseI-C EcoRI-G/MseI-A EcoRI-T/MseI-C	EcoRI-0/MseI-A EcoRI-0/MseI-G
Eutypa sp.	EcoRI-A/MseI-CA EcoRI-AC/MseI-C	–
Legionella pneumophila	EcoRI-A/MseI-G EcoRI-AC/MseI-C	EcoRI-0/MseI-A
Nensenula anomola	EcoRI-A/MseI-T EcoRI-G/MseI-A	–
Paenibacillus larvae	EcoRI-C/MseI-A	–
Pichia membrefaciens	EcoRI-AC/MseI-C	
Saccharomyces sp.	EcoRI-A/MseI-CA EcoRI-AC/MseI-C	–
Schizosaccharomyces pombe	EcoRI-AC/MseI-C	–
Xanthomonas sp.	EcoRI-0/MseI-C	–

a. Producing 25–130 bands evenly dispersed from 50–500 bases with intensities of 100–2000 relative fluorescent units

b. Too few or too many bands or uneven size distribution

Note The list in Table 10-2 is not exhaustive. Refer to the publications listed in Appendix D for in-depth discussion of primer choices.

continued on next page

Primers Available—Plant Mapping

If you want to use a specific primer combination for the AFLP Selective Amplification reactions, you can order primer pairs in any combination of one EcoRI primer and one MseI primer. This gives you 128 possible primer pair combinations from which you can choose, for either regular or small plant genomes. Order the AFLP Amplification Core Mix Module (P/N 402005) and the desired AFLP Selective Amplification Primers from Table 10-3.

Table 10-3 AFLP Selective Amplification Primers for Plant Mapping

EcoRI Primers, Regular Plant Genomes

Primer	Part Number (250 reactions)	Part Number (500 reactions)
EcoRI-ACT FAM	402045	402037
EcoRI-ACA FAM	402038	402030
EcoRI-AAC NED	4303053	4303054
EcoRI-ACC NED	4303055	4303056
EcoRI-AGC NED	4303057	4303058
EcoRI-AAG JOE	402042	402034
EcoRI-AGG JOE	402043	402035
EcoRI-ACG JOE	402044	402036

EcoRI Primers, Small Plant Genomes

Primer	Part Number (250 reactions)
EcoRI-TG FAM	402264
EcoRI-TC FAM	402265
EcoRI-AC FAM	402269
EcoRI-TT NED	4304352
EcoRI-AT NED	402955 (500 reactions)
EcoRI-TA JOE	402267
EcoRI-AG JOE	402268
EcoRI-AA JOE	402271

MseI Primers, Regular and Small Plant Genomes

Primer	Part Number (250 reactions)	Part Number (500 reactions)
MseI-CAA	402021	402029
MseI-CAC	402020	402028
MseI-CAG	402019	402027
MseI-CAT	402018	402026
MseI-CTA	402017	402025
MseI-CTC	402016	402024
MseI-CTG	402015	402023
MseI-CTT	402014	402022

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Plant Genomes Mapped Using AFLP

Ten different crop species genomes were analyzed using the AFLP technique. For each crop species, primer combinations that produce the best DNA fingerprints were determined.

The names of each crop species tested and corresponding primer combination tables are given in Table 10-4. Those combinations of EcoRI and MseI Selective Amplification primers that are best suited for amplification screening of the designated crop genomes are shown in Table 10-5 through Table 10-14.

Table 10-4 Primer Combination Tables for Crop Species

Crop Species	Primer Combination Table
Regular Plant Genomes	
Sunflower	Table 10-5 on page 10-12
Pepper	Table 10-6 on page 10-13
Barley	Table 10-7 on page 10-13
Maize	Table 10-8 on page 10-14
Sugar beet	Table 10-9 on page 10-14
Tomato	Table 10-10 on page 10-15
Lettuce	Table 10-11 on page 10-15
Small Plant Genomes	
Arabidopsis	Table 10-12 on page 10-16
Cucumber	Table 10-13 on page 10-16
Rice	Table 10-14 on page 10-17

⊘ The following symbol indicates **unacceptable** primer combinations for amplification screening of designated species:

Table 10-5 Primer Combinations for Sunflower Species

		MseI Primers							
		-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
EcoRI Primers	-AAC		⊘						
	-AAG	⊘				⊘			
	-ACA	⊘			⊘	⊘			
	-ACC								
	-ACG		⊘			⊘			
	-ACT								
	-AGC						⊘		
	-AGG						⊘		

Table 10-6 Primer Combinations for Pepper Species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC			⊘	⊘		⊘		⊘
-AAG				⊘				
-ACA				⊘				
-ACC								⊘
-ACG								
-ACT	⊘							
-AGC								
-AGG							⊘	

Table 10-7 Primer Combinations for Barley Species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC								
-AAG			⊘	⊘				
-ACA								
-ACC								
-ACG								
-ACT			⊘					
-AGC						⊘		
-AGG								

Table 10-8 Primer Combinations for Maize Species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC	⊘							
-AAG					⊘			
-ACA	⊘							
-ACC								
-ACG						⊘		⊘
-ACT		⊘		⊘			⊘	⊘
-AGC								⊘
-AGG								

Table 10-9 Primer Combinations for Sugar Beet Species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC								
-AAG								⊘
-ACA								
-ACC								
-ACG								
-ACT	⊘	⊘						
-AGC								
-AGG								

Table 10-10 Primer Combinations for Tomato Species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC								
-AAG				⊘			⊘	
-ACA								
-ACC								
-ACG								
-ACT								
-AGC								
-AGG								

Table 10-11 Primer Combinations for Lettuce Species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC								
-AAG				⊘			⊘	
-ACA								
-ACC								
-ACG				⊘				⊘
-ACT								
-AGC								
-AGG				⊘				

Table 10-12 Primer Combinations for Arabidopsis Species (Small Plant Genome)

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AA								
-AC								
-AG								
-AT	⊘							
-TA		⊘						
-TC								⊘
-TG								
-TT								

Table 10-13 Primer Combinations for Cucumber Species (Small Plant Genome)

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AA							⊘	
-AC	⊘							
-AG								
-AT								
-TA	NOT DETERMINED							
-TC								
-TG								
-TT								

Table 10-14 Primer Combinations for Rice Species (Small Plant Genome)

		MseI Primers							
		-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
EcoRI Primers	-AA								
	-AC								
	-AG								
	-AT								
	-TA								
	-TC								
	-TG								
	-TT								

Fluorescent Dye-labeling and Marker Detection

Applied Biosystems has adapted the AFLP technique for use with our ABI PRISM™ fluorescent dye-labeling and detection technology. PCR products are dye labeled during amplification using a 5´ dye-labeled primer. For high throughput, you can co-load up to three different reactions labeled with different colored dyes in a single injection on the ABI PRISM® 310 Genetic Analyzer. Load a GeneScan Internal Lane Size Standard in a fourth color in every lane or injection to size all amplification fragments accurately.

You can automate the scoring of the large numbers of markers that are typically generated by analyzing your results with GeneScan Analysis and Genotyper® software.

11 Troubleshooting

Troubleshooting PCR Amplification

Topics This section offers troubleshooting suggestions for the following problem areas:

- Problems with poor amplification (page 11-1)
- Problems with extra peaks (page 11-5)
- Problems with missing peaks (page 11-6)

Table 11-1 Problems with Poor Amplification

Observation	Possible Causes	Recommended Actions
Faint or no signal from sample DNA and from positive control	Insufficient enzyme in reactions	Use the recommended amount of enzyme.
	Incomplete activation of AmpliTaq Gold™ DNA Polymerase	Repeat amplification, making sure to: ♦ Hold reactions initially at 95 °C for 10–15 minutes. ♦ Use the recommended buffer. **Note** Both buffer pH and buffer composition affect enzyme activation. **Note** At temperatures >95 °C, the enzyme is susceptible to irreversible denaturation. If you suspect insufficient activation, increase the incubation time, not the incubation temperature.
	Too little sample DNA added to reaction **Note** This is especially critical in human identification experiments because sample quality is often poor.	Quantitate DNA and use the amount recommended in the protocol. **Note** For accurate quantitation of human DNA samples, use the QuantiBlot® Human DNA Quantitation Kit (P/N N808-0114).

Table 11-1 Problems with Poor Amplification *(continued)*

Observation	Possible Causes	Recommended Actions
Faint or no signal from sample DNA and from positive control	Incorrect or suboptimal thermal cycler parameters	Check protocol for correct thermal cycler parameters. If the correct parameters were used, they may need to be optimized for your specific application. (For example allow a linear increase in extension time with increasing cycle number or increase time at the denaturation plateau.)
	PCR Master Mix not well mixed before aliquoting	Vortex PCR Master Mix thoroughly.
	Primer concentration too low	Use the recommended primer concentration.
	Primers degraded	Use new primers. **Note** Preincubation at 95 °C for 5–10 minutes should inactivate proteases or nucleases. **Note** To prevent primer degradation during storage, store primers at –15 to –25 °C, either lyophilized or in TE. Avoid excessive (more than 3–4) freeze-thaw cycles.
	Too little free Mg^{2+} in reaction	Check that you added sufficient total Mg^{2+} given concentration of the dNTPs and EDTA. **Note** [Free Mg^{2+}] = [Total Mg^{2+}] – [Total dNTP] – 2[EDTA]
	Incorrect pH	Verify buffer pH and buffer concentration.
	Wrong PCR tube	Use: ♦ Applied Biosystems GeneAmp® Thin-Walled Reaction Tubes for the DNA Thermal Cycler 480 ♦ MicroAmp® Reaction Tubes with Caps for the GeneAmp PCR Systems 9600 and 2400
	MicroAmp Base used with tray/retainer set and tubes in GeneAmp® PCR System 9600 or 2400	Remove MicroAmp Base from tray/retainer set and repeat amplification.
	Verify GeneAmp PCR System protocols and programmed parameters	Refer to the thermal cycler user's manual and check instrument calibration.
	Tubes not seated tightly in the thermal cycler during amplification (DNA Thermal Cycler 480)	Push reaction tubes firmly into contact with block after first cycle. Repeat amplification.
	GeneAmp PCR System 9600 heated cover misaligned	Align the heated cover so that white stripes align after twisting the top portion clockwise.

Table 11-1 Problems with Poor Amplification *(continued)*

Observation	Possible Causes	Recommended Actions
Faint or no signal from sample DNA and from positive control	Poor thermal cycler performance	Check instrument calibration.
		Use a Applied Biosystems thermal cycler.
Good signal from positive control but faint or no signal from sample DNA	Sample contains PCR inhibitor (for example, heme compounds, EDTA, or certain dyes)	Quantitate DNA. Dilute if possible in order to add minimum necessary volume. Repeat amplification.
		Wash the sample in an Amicon Centricon-100 column and repeat amplification. **Note** For fragments smaller than 130 bp, use the Amicon Centricon-30 column instead.
		Add bovine serum albumin (BSA) to the PCR reaction mixture. (Use 8–16 µg BSA for every 50 µL PCR reaction volume.)
	Sample DNA is degraded	Evaluate the quality and concentration of the DNA sample by: ♦ Using the QuantiBlot Human DNA Quantitation Kit (for human DNA) ♦ Running an agarose yield gel If DNA is degraded or inaccurately quantitated, reamplify with an increased amount of DNA.
	Insufficient sample DNA added because of inaccurate quantitation	Evaluate the quality and concentration of the DNA sample by: ♦ Using the QuantiBlot Human DNA Quantitation Kit (for human DNA) ♦ Running an agarose yield gel If DNA is degraded or inaccurately quantitated, reamplify with an increased amount of DNA.
	Incorrect pH	Verify buffer pH and concentration. If correct, quantitate sample DNA. Too little or too much DNA can alter the pH.
	Primer choice not optimal (for example, primers may be annealing to sites of template secondary structure or may have internal secondary structure)	Use different primers. See “Designing Custom Primers” on page 6-3 for more information.
	T_m of primers is lower than expected	Decrease the annealing temperature by 2 °C increments.

Table 11-1 Problems with Poor Amplification *(continued)*

Observation	Possible Causes	Recommended Actions
Poor yield for multiplex PCR	Non-optimal thermal cycling parameters	Between the denaturation and annealing stages, add a 2 minute down-ramp time to thermal cycling profile. **Note** For multiplex PCR, a short down-ramp time is not necessarily optimal.
	Competition from mispriming and other competing side reactions	Use AmpliTaq Gold DNA Polymerase. See "Designing Custom Primers" on page 6-3, "Multiplexing PCR" on page 6-10, and "Preventing Competing Side Reactions—Hot Start PCR" on page 6-13 for additional suggestions.
	Problems with primer choice, concentration, or degradation	See "Designing Custom Primers" on page 6-3, "Determining Reagent Concentrations" on page 6-6, and "Multiplexing PCR" on page 6-10 for additional suggestions.
Yield gets progressively poorer for successive PCR amplifications performed over time	Expired or mishandled reagents	Check expiration dates on all reagents. If not expired, verify that reagents are being stored and used according to manufacturer's instructions. Compare with PCR performance using fresh reagents.
Inconsistent yields with control DNA	Combined reagents not spun to bottom of PCR sample tube	Place all reagents in apex of tube and spin briefly after combining.
	Combined reagents left at room temperature or on ice for extended periods of time (encouraging mispriming and other primer artifacts)	Put tubes in block immediately after combining reagents.
	Combined reagents not thoroughly mixed	Vortex all primers, reagents, and reaction mixes (minus enzyme) thoroughly to ensure uniform concentration.
	Primers not uniformly suspended before adding to reaction mixture. (Primers can aggregate and settle to the bottom of the tube.)	
	Pipetting errors.	Follow all these precautionary measures: ♦ Calibrate pipettes ♦ Attach tips firmly ♦ Check all phases of pipetting technique ♦ Whenever possible minimize pipetting small volumes (for example, make master mixes) **Note** You may also want to consider using a 2-µL or other high-precision pipette

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Table 11-2 Problems with Extra Peaks

Observation	Possible Causes	Recommended Actions
Extra peaks appear with no discernible pattern	Presence of exogenous DNA	Use appropriate techniques to avoid introducing foreign DNA during laboratory handling.
	Nonspecific priming (*i.e.*, primer-template mismatch)	Check for good primer design. See "Designing Custom Primers" on page 6-3 for more information.
		Add less template DNA. **Note** High DNA concentrations promote nonspecific annealing.
		Optimize Mg^{2+} concentration.
		Add less primer DNA **Note** High primer concentrations promote nonspecific annealing.
		If you are not using AmpliTaq Gold DNA Polymerase, consider using a Hot-Start Technique.
		Increase annealing temperature in 2–5 °C increments.
		Decrease annealing and/or extension times.
		Increase primer length.
		Perform a second amplification with nested primers.
		Perform Touchdown PCR.
	Primer-dimer and primer-oligomer artifacts (likelihood increases with multiplex PCR)	Check primers for 3´ complementarity.
		Design longer primers.
		Reduce primer concentration.
		Reduce number of cycles.
		Raise the annealing temperature in 2–5 °C increments.
		Increase amount of target DNA.
	Incomplete restriction (and/or ligation if performing AFLP)	Repeat restriction (and/or ligation).
	If performing AFLP, too much DNA in reaction so that insufficient adaptor is present	Use the recommended amount of template DNA.
	Mixed sample	Verify quality and integrity of sample.

Table 11-2 Problems with Extra Peaks *(continued)*

Observation	Possible Causes	Recommended Actions
Presence of split peaks differing in size by one base pair (Extra peak of size n+1)	Partial nontemplate addition of an extra nucleotide (usually adenosine) to the blunt end of the PCR product	Add the correct amount of Mg^{2+} to the reaction mix. **Note** Increasing Mg^{2+} concentrations can increase the frequency of nontemplated nucleotide addition and vice versa. Increasing the extension time at 72 °C will increase the frequency of nontemplate nucleotide addition. For more suggestions see "3´ A Nucleotide Addition" on page 6-18.
Presence of peaks differing in size by two, three, or four base pairs (Extra peak of size n–2, n–3, or n–4)	Stutter product formed during amplification of di-, tri-, or tetranucleotide STR loci	See "Stutter Products" on page 6-21 for suggestions.

Table 11-3 Problems with Missing Peaks

Observation	Possible Causes	Recommended Actions
Some but not all loci visible on electropherogram	Sample DNA is degraded (indicated if shorter amplicons are favored)	Quantitate DNA and add more template. Repeat amplification.
		Wash the sample in an Amicon Centricon-100 column and repeat amplification. **Note** For fragments smaller than 130 bp the Amicon Centricon-30 column is preferable.
	Sample contains PCR inhibitor (*e.g.*, heme compounds, EDTA, or certain dyes)	Quantitate DNA and add minimum necessary volume of PCR product. Repeat amplification.
Individual alleles are missing when inheritance data is examined	Mutation in primer annealing site of one allele	Change the primer.

Troubleshooting PCR Product Detection

Topics This section offers troubleshooting suggestions for the following problem areas:

- Problems with automatic data analysis (page 11-7)
- Problems with current (page 11-8)
- Problems with signal strength and quality (page 11-10)
- Problems with peak number and position (page 11-12)
- Problems with peak quality and resolution (page 11-14)

Table 11-4 Problems with Automatic Data Analysis

Observation	Possible Causes	Recommended Actions
Data was not automatically analyzed	Sample Sheet not completed or completed incorrectly	Complete the Sample Sheet as described in your user's manual.
	Injection List not completed or completed incorrectly	Complete the Injection List as described in your user's manual.
	Analysis preferences set incorrectly in data collection program	Check the collection software preferences to make sure that Autoanalyze with GeneScan® Analysis Software is selected under the GeneScan Injection List Defaults.
	Insufficient free RAM	Restart the computer before collecting data. **Note** You should always restart the computer before collecting data.
	Conflicting extensions	Choose Extensions Manager from the Control Panels. Turn off any extensions that were not part of the original installation and restart computer.

continued on next page

Table 11-5 Problems with Current

Observation	Possible Causes	Recommended Actions
No current	Too little or no buffer in anode buffer reservoir	Replenish buffer reservoir.
	Too little or no buffer in position 1 of autosampler	Replenish buffer in position 1 of autosampler.
	Electrode bent	Replace or straighten electrode and recalibrate autosampler. **Note** If you replace the electrode be sure to clip it to the correct size.
	Capillary bent away from electrode	Tape capillary securely to heat plate to keep capillary from shifting position. Place the tape on the heat plate just above the electrode holder. Refer to the *ABI PRISM® 310 Genetic Analyzer User's Manual*.
	Unfilled capillary or bubbles in capillary	Check system for leaks. Replace capillary if necessary and rerun module.
	Major leaks in system. Polymer does not enter capillary	Check system for leaks. **Note** Filling the capillary should cause the Gel Pump value in the Status Window to increase by only 1–2 steps. If the instrument detects a syringe leak, a warning message appears on the screen.
	Pump blockage (pump is plugged with urea or crystallized buffer)	Remove and clean pump block. Refer to the *ABI PRISM 310 Genetic Analyzer User's Manual.*
	Loose valve fittings or syringe	Tighten valve fittings and syringe.
	Anode buffer valve does not open	Open buffer valve. **Note** The valve should depress easily when you push the top with your finger tip. After you release the pressure the valve should spring to the "open" position. If the valve is stuck, it should be cleaned.
	Plugged, broken, or nonconducting capillary	Replace the capillary.
	Poor quality water in buffer solutions	Remake buffer with freshly autoclaved, distilled, deionized water
	Incorrect polymer solution formulation	Make or install new polymer solution
	Corrupted firmware	Resend firmware by performing a cold boot reset.
	Syringe Pump Force too low. Capillary is not being filled completely	Call DNA Technical Support.

Table 11-5 Problems with Current *(continued)*

Observation	Possible Causes	Recommended Actions
Low current	Small bubble in capillary blocking current flow	Replenish gel in capillary.
	Small bubble in pump block	Remove bubble by repriming the pump block with polymer.
	Plugged, broken, or nonconducting capillary	Replace the capillary.
	Poor quality water in buffer solutions	Remake buffer with freshly autoclaved, distilled, deionized water.
	Old, defective, or incorrectly made buffer or polymer solution	Replace buffer or polymer solution.
Fluctuating current	Too little buffer in anodic jar	Replenish buffer jar.
	Small bubble in capillary blocking current flow	Replenish gel in capillary.
	Small bubble in pump block	Remove bubble by repriming the pump block with polymer.
	Broken or cracked capillary	Replace the capillary.
	Arcing to conductive surface on the instrument	Clean the hotplate and autosampler. Ensure that the ambient temperature is between 15 and 30 °C and the humidity is below 80%. Check for excessive condensation on the instrument.
	Position of electrode is not sufficiently below the buffer surface	Replenish buffer.
		Reposition electrode and recalibrate autosampler.
Current is normal at beginning of run and then decreases rapidly over the next several minutes	Loss of anodic buffer capacity	Replace the buffer.
Current too high	Decomposition of urea in polymer solution	Add fresh polymer solution to the syringe.
	Incorrect buffer formulation (most likely too concentrated)	Replace buffer with appropriate 1X running buffer.
	Arcing to conductive surface on the instrument	Clean the hotplate and autosampler. Ensure that the ambient temperature is between 15 and 30 °C and the humidity is below 80%. Check for excessive condensation on the instrument.

continued on next page

Table 11-6 Problems with Signal Strength and Quality

Observation	Possible Causes	Recommended Actions
No signal	No sample added	Add 1 µL PCR product to formamide/size standard mix.
	Sample not at bottom of tube	Spin sample tube in microcentrifuge.
	Air bubble at bottom of sample tube	Spin sample tube in microcentrifuge to remove air bubbles.
	Capillary misaligned with electrode	Align capillary and electrode. **Note** The capillary should be adjacent to, but not touching, the electrode. The capillary should protrude 0.5 mm past the electrode.
	Capillary bent out of sample tube	Align capillary and electrode. Recalibrate autosampler. **Note** To verify whether a bent capillary is the problem, watch the movement of the autosampler tray during run operation.
	Autosampler not calibrated correctly	Calibrate autosampler in X, Y, and Z directions. **IMPORTANT** The capillary should almost touch the Z calibration point.
	Sealed sample tube septum (*i.e.*, septum will not open to allow electrode into sample tube)	Replace septum.
	Septum not placed in the sample tube properly	
Signal too low	Insufficient sample added	Did you add a full 1 µL of PCR product to formamide/size standard mix? If no, add 1 µL PCR product to formamide/size standard mix. If yes, concentrate your PCR product before adding to formamide/size standard mix or examine the efficiency of the PCR. Check pipette calibration.
	Samples added to formamide that has degraded to formic acid and formate ions (leading to insufficient sample injected)	Use freshly deionized formamide. (See "Deionized Formamide" on page A-3 for directions.)
	Ions in sample (leading to insufficient sample injected)	Dialyze sample to remove ions.
	Sample not thoroughly mixed with formamide/size standard mixture	Mix sample into formamide/size standard mixture by pipetting up and down several times.
	Insufficient [F]dNTPs added to PCR reaction	Reamplify using more [F]dNTPs or examine the efficiency of the PCR.

Table 11-6 Problems with Signal Strength and Quality *(continued)*

Signal too high	Too much sample injected into capillary.	Decrease injection time or injection voltage.
		Dilute sample before adding to formamide/size standard mix.
		Reamplify using less [F]dNTPs.
	Unincorporated [F]dNTPs	Purify the PCR product.
High baseline	Dirty capillary window	Clean capillary window with 95% ethanol.
	Capillary moved out of position in front of laser window	Position capillary in front of laser window.
	Precipitate in polymer	Allow polymer to equilibrate to room temperature before using.
		Use fresh polymer.
	Incorrectly prepared and/or old buffer or polymer solutions	Replace buffer and polymer with fresh solutions.
	Fluorescing material in the capillary holder	Clean the capillary holder.
	Defective capillary	Replace the capillary.
	Matrix made incorrectly resulting in too much correction (also indicated by troughs under peaks)	Remake matrix. Be sure to: ♦ Remove the primer peak (or aberrant off-scale peaks) from the scan range. ♦ Pick the start and stop points on flat parts of the baseline when viewing raw data. ♦ Make the matrix using same polymer, buffer, and run conditions as sample injections.
Noisy baseline	Incorrectly prepared and/or old buffer or polymer solutions	Replace buffer and polymer with fresh solutions.
	Dirty capillary holder aperture	Clean the capillary holder.
	Defective capillary	Replace the capillary.
Spikes in baseline	Precipitate in polymer	Allow polymer to equilibrate to room temperature before adding to capillary.
		Use fresh polymer.
	Old polymer	Use fresh polymer.

continued on next page

Table 11-7 Problems with Peak Number and Position

Observation	Possible Causes	Recommended Actions
Extra peaks in additional colors displayed underneath the position of one strong peak	Too much sample injected into capillary (indicated if any peak is greater than 4000 RFU)	Decrease injection time or injection voltage.
		Dilute PCR sample before adding to formamide/size standard mix. Reamplify using less DNA.
	Incorrect matrix chosen or poor matrix	Check matrix selection on Injection List. If correct, create a new matrix.
Extra peaks when sample is known to contain DNA from a single source (If extra peaks are 1–4 nt larger or smaller than expected peak, it may be a PCR artifact. See "Problems with Extra Peaks" on page 11-5.)	Samples not fully denatured	Make sure the samples are heated at 95 °C for 5 minutes prior to loading onto autosampler.
	Unoptimized PCR	Check efficiency of the PCR. See Chapter 6, "Optimizing PCR," for detailed suggestions.
	Renaturation of denatured samples	Load samples immediately following denaturation, or store on ice until you are ready to load. **IMPORTANT** Do not store samples on ice for more than 2 hours before loading. **Note** Too much DNA also promotes renaturation, but before you add less DNA you will need to assess the signal strength and quality.
	Dust or dirt in polymer	Filter the polymer with a 0.2-µm or 0.4-µm disk filter attached to a plastic syringe.
Size standard peaks not recognized when defining size standard	Height of a size standard peak less than the Peak Amplitude Threshold for the size standard color (in Analysis Parameters) **Note** 50 RFU is the default threshold.	Rerun sample, adding the recommended amount of size standard.
	Peaks missing from size standard definition	Check GeneScan Analysis Parameters to make sure the correct scan range is defined.
	Minimum Peak Half Width is set too high (in Analysis Parameters)	Lower the value for the Minimum Peak Half Width.

Table 11-7 Problems with Peak Number and Position *(continued)*

Peak positions off throughout size range **Note** See Chapter 5 for detailed information on factors that affect sizing.	Incorrect Sample Sheet	Check Sample Sheet selection in data collection program.
	Change in size-calling method	Use consistent size-calling method.
	Incorrect internal size standard	Use correct GeneScan Internal Lane Size Standard.
	Incorrect polymer composition	Check urea concentration and polymer composition against protocol.
	Incorrect electrophoresis temperature	Check the Injection List for temperature setting. If correct on Injection List, check the Log for a recording of the actual electrophoresis temperature.
	Incorrectly defined size standard	Define size standard peak sizes separately for each incorrectly sized injection.
Inconsistent peak mobilities at beginning of run (*i.e.*, peaks come off at higher scan numbers in the first injection)	Capillary temperature not at equilibrium	Repeat the injection of the first sample. **Note** The run temperature can be set in the Manual Control window while the samples are being prepared, but we still recommend repeating the first sample.
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.
	Syringe out of polymer	Fill syringe with fresh polymer.
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, invert syringe to coat capillary walls, and discard polymer. Then fill syringe with fresh running polymer.

continued on next page

Table 11-8 Problems with Peak Resolution

Observation	Possible Causes	Recommended Actions
Poor resolution	Poor capillary performance	Replace capillary.
	Incorrectly prepared and/or old buffer or polymer solutions	Replace buffer and polymer with fresh solutions.
	Injection time too long (broad peaks)	Decrease injection time.
	Incorrectly prepared and/or degraded sample	Prepare new sample.
	Incorrect buffer formulation	Check if buffer formulation matches protocol requirements.
	Incorrect polymer composition	Check if polymer composition matches protocol requirements.
	Electrophoresis voltage too high.	Decrease electrophoresis voltage by as much as 4 kV. **Note** Increase electrophoresis time accordingly.
	Sample concentrated by evaporation leaving excess salt behind.	Do not concentrate sample by evaporation. Use an Amicon Centricon-100 column if necessary.
	Incomplete strand separation due to insufficient heat denaturation	Make sure the samples are heated at 95 °C for 5 minutes prior to loading onto autosampler.
	Too much DNA in sample	Dilute sample before adding to formamide.
	Wrong capillary used for POP-4™ runs	Verify that you are using a 47-cm, 50-µm i.d. (green mark) capillary.
	Oil in sample (from DNA Thermal Cycler 480)	Carefully pipette PCR product without oil carryover.
		Remove oil by organic extraction.
	Poor quality water	Use freshly autoclaved, distilled, deionized water.
	Syringe empty or incorrect Syringe Max Travel value	Fill syringe if necessary and recalibrate Syringe Max Travel value.
	Capillary too short	Increase capillary length. **Note** Increase electrophoresis time accordingly.

Reagent Preparation

A

5% GeneScan Polymer with 10% Glycerol

To prepare 50 g of 5% GeneScan Polymer with 10% glycerol:

Step	Action
1	To a 50-mL screw cap tube, add: ♦ 35.7 g GeneScan Polymer (7% w/w) ♦ 5 g glycerol ♦ 5 g 10X TBE (see page A-2) ♦ distilled, deionized H_2O to 50 g
2	Mix by inverting several times, then vortex on high for 30 seconds.

Note 5% GeneScan polymer with 10% glycerol lasts for 3 months at room temperature or for 100 sample injections on the instrument.

You can quickly dilute the 5% GeneScan Polymer to any percentage from 1–5% by adding the appropriate amount of dilution buffer to the 5% GeneScan Polymer. The dilution buffer is also used as the electrode buffer for SSCP applications. See "1X TBE with 10% Glycerol" on page A-2 for details.

GeneScan Polymer for Non-denaturing Applications

Use the chart below as a guide for making 5 mL of the desired concentration of GeneScan Polymer. Use 10X Genetic Analyzer Buffer with EDTA (P/N 402824).

IMPORTANT Weigh the components of the solution because of the high viscosity of the polymer. When weighing, dispense the polymer, buffer, and water into a 10-mL conical tube or similar storage container. Vortex the GeneScan Polymer solution to ensure adequate mixing of the polymer, buffer, and water. The higher the concentration of polymer, the more vortexing required.

Desired Concentration	10X Genetic Analyzer Buffer with EDTA	GeneScan Polymer	Deionized water
2%	0.5 g	1.43 g	3.07 g
3%	0.5 g	2.14 g	2.36 g
4%	0.5 g	2.86 g	1.64 g
5%	0.5 g	3.57 g	0.93 g

continued on next page

10X TBE

To make 50 mL of 10X TBE:

Step	Action
1	To a 50-mL screw cap tube, add the following: ♦ 5.4 g Tris base ♦ 2.8 g Boric acid ♦ 0.4 g Na_2EDTA ♦ Distilled, deionized H_2O to 50 mL **IMPORTANT** Be sure to use disodium EDTA to make 10X TBE stock. Some major laboratory suppliers provide monosodium EDTA.
2	Mix ingredients thoroughly by vortexing.
3	Verify that the pH reads between 8.2 and 8.3.

Note 10X TBE lasts indefinitely at 2–6 °C and for 6 months at room temperature. If a precipitate forms in the TBE buffer, discard it and prepare fresh buffer.

1X TBE with 10% Glycerol

This buffer can be used to dilute the GeneScan Polymer concentrate (see "5% GeneScan Polymer with 10% Glycerol" on page A-1). It is also used as the electrode buffer for SSCP applications.

To prepare 250 mL of 1X TBE with 10% glycerol:

Step	Action
1	To a 500-mL glass beaker, add: ♦ 25 g 10X TBE ♦ 25 g glycerol ♦ distilled, deionized H_2O to 250 g
2	Mix by stirring, then filter through a 0.2-µm cellulose nitrate filter.

Note SSCP dilution buffer lasts for 3 months at room temperature or for 100 sample injections or 48 hours on the instrument.

continued on next page

Deionized Formamide

IMPORTANT Always use deionized formamide. Over time, formamide hydrolyzes to formic acid and formate. The formate ions migrate preferentially into the capillary during electrokinetic injection causing a loss of signal intensity. Deionized formamide stock lasts for 3 months at –15 to –25 °C

Step	Action
1	Mix 50 mL of formamide and 5 g of AG501 X8 ion-exchange resin. **! WARNING ! CHEMICAL HAZARD. Formamide is a known teratogen. It can cause birth defects. Wash thoroughly after handling formamide. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves. Wash thoroughly after handling formamide.**
2	Stir for 30 minutes at room temperature.
3	Check that the pH is greater than 7.0 using pH paper. If the pH is not greater than 7.0, decant the formamide into a beaker containing another 5 g of ion-exchange resin and repeat 30-minute stirring at room temperature.
4	When the pH is greater than 7.0, allow the beads to settle to the bottom of the beaker. Remove the supernatant (formamide), taking care not to disturb the beads.
5	Dispense the deionized formamide into aliquots of 500 µL and store for up to 3 months at –15 to –25 °C.
6	Use one aliquot per set of samples. Discard any unused deionized formamide.

Creating Matrix Files B

Creating the GeneScan Matrix File

Overview

The matrix file contains the information necessary for software to correct the spectral overlap of the dyes in the virtual filter set (see Chapter 4 for more information on the dyes available). Once a matrix file has been created, it can be used for subsequent runs performed:

- With the same kit or chemistry
- On the same instrument
- Using the same:
  - run modules
  - set of dyes
  - polymer

After running the matrix standards, use their sample files to generate a matrix file using GeneScan® Analysis Software.

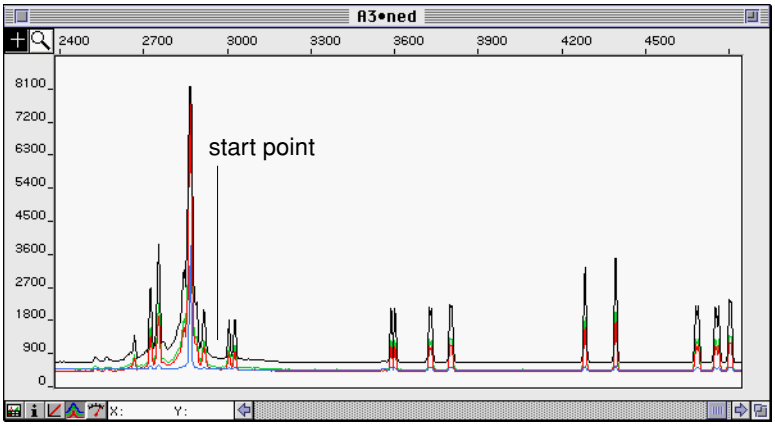
How to Verify the Raw Data

Before creating the matrix file, verify that the raw data from the standards is good.

To view the raw data in GeneScan Analysis Software:

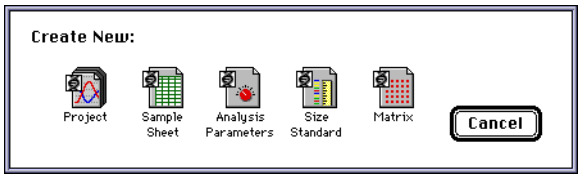
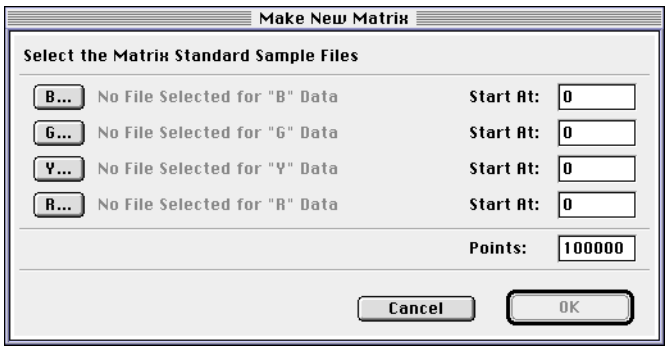
Step	Action
1	Create a new project if you did not select Autoanalyze in the GeneScan Run Defaults preferences in the data collection software: a. Choose New from the File menu. b. Select the Project icon. An untitled Analysis Control window opens. c. Choose Add Sample Files from the Project menu. d. Find and open the Run Folder for the matrix standards run. e. Select the four Sample files representing the blue, green, yellow, and red dye-labeled “runs,” and then click Add. f. Click Done after the Sample files are transferred.
2	In the Analysis Control window, select the four matrix standard Sample files by clicking on the first Sample file, holding down the mouse button, and releasing on the last Sample file.
3	Choose Raw Data from the Project menu. Electropherograms displaying raw data from the four matrix standard Sample files appear.

To verify the raw data:

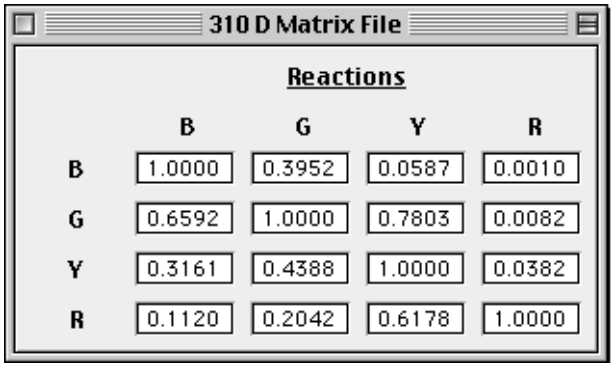
Step	Action
1	Verify data peaks are present in all four samples. Peak data should be on-scale and the dye of interest should have a value of at least 200.
2	Check for any data anomalies, such as an unstable baseline. Rerun samples that have an unstable baseline.
3	Select a starting point for the matrix data as shown below. The starting point for matrix data should be slightly beyond the point where the primer peak falls back to the baseline (approximately 2950 scans in this example). 
4	Choose a stop point such that at least three matrix standard peaks will be within the range analyzed.

How to Generate The Matrix File

To generate the matrix files:

Step	Action
1	Choose New from the File menu. 
2	Click the Matrix icon. This will open the Make New Matrix dialog box. 

To generate the matrix files: *(continued)*

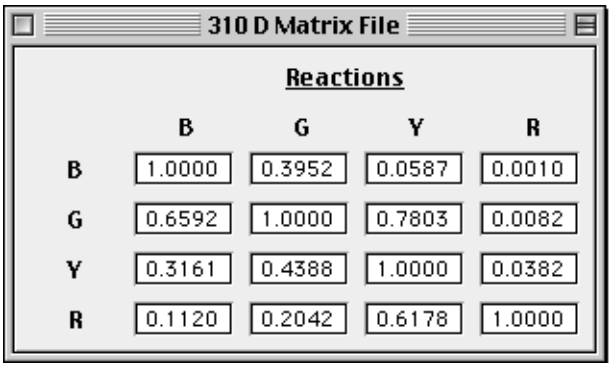
Step	Action
3	Click the B, G, Y, and R buttons to choose the standard sample files. Choose the sample file representing blue dye for B, green dye for G, etc.
4	Enter the starting point for each file. The Start At point should be after the primer peak. Define the Points value. This is the number of points after the start point to be analyzed.
5	Click OK. A successful matrix opens an untitled Matrix Values window with a 4x4 matrix of numerical values. 
6	Use the Save As… command to name and save the matrix file. Choose a name that reflects the chemistry, the virtual filter set, and the run conditions.

How to Check Matrix Quality

Check the quality of the matrix by:

- reviewing the values in the Matrix Values window
- reviewing the analyzed data of the matrix run

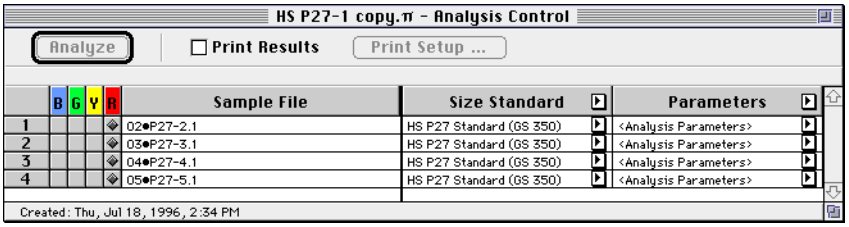
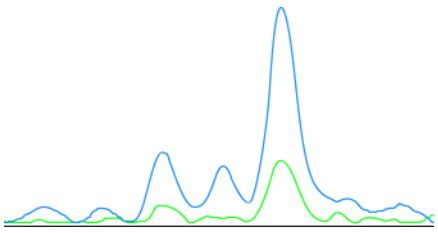
Review the matrix values in the Matrix Values window as follows:

Step	Action
1	View the Matrix Values window. 
2	The numbers on the diagonal (Blue against Blue, Green against Green, etc.) must all be 1.00. The numbers off the diagonal should be less than 1.00. **Note** In Virtual Filter C, Green under Blue (the second box from the top in the first column on the left) is sometimes slightly above 1.00. This is acceptable.

Check matrix quality as follows:

<table>
<tr><th>Step</th><th colspan="2">Action</th></tr>
<tr><td>1</td><td colspan="2">From the Project containing your matrix standard Sample files, open the Analysis Control window.

In the Analysis Control window, select the colors for each sample.

 
</td></tr>
<tr><td>2</td><td colspan="2">Select the four matrix standard Sample files.</td></tr>
<tr><td>3</td><td colspan="2">Choose Assign New Matrix in the Project menu. Select the matrix file.</td></tr>
<tr><td>4</td><td colspan="2">Select numbers 1, 2, 3, and 4 on the left side of the window to highlight the colors for each row.</td></tr>
<tr><td>5</td><td colspan="2">Use the Set Analysis Parameters dialog box in the Settings menu to set the Analysis Range.</td></tr>
<tr><td>6</td><td colspan="2">Click Analyze.</td></tr>
<tr><td>7</td><td colspan="2">Choose Results from the Windows menu and check each electropherogram by:
a. Clicking 4 in the # of Panels menu
b. Clicking 1 under Dye/Samples
c. Clicking #1 on the Sample Files side of the Results window</td></tr>
<tr><td rowspan="3">8</td><td>If…</td><td>Then…</td></tr>
<tr><td>each peak is one color with the other colors flat under it</td><td>the matrix is good.</td></tr>
<tr><td>the other colors are not flat under the peaks (as shown below)
  </td><td>the matrix is poor.</td></tr>
<tr><td rowspan="3">9</td><td>If…</td><td>Then…</td></tr>
<tr><td>the matrix is good</td><td>Save the matrix file to the ABI folder.</td></tr>
<tr><td>the matrix is poor</td><td>Redo the matrix by using different start and stop points.

If this does not improve the matrix data, run new matrix standards.</td></tr>
</table>

Preparing 5´-End Labeled Primers

C

Overview

In This Appendix

This appendix provides detailed instructions for preparing 5´-end labeled primers directly on any Applied Biosystems DNA synthesizer using any of the Applied Biosystems dye phosphoramidites.

This appendix contains the following topics:

Topic	See Page
Abbreviations and Definitions	C-1
Introduction to 5´-end Labeling	C-2
Instrument Setup	C-4
Synthesis and Purification of 5´-end Labeled Primers	C-6
Calculating Absorbance for DNA Samples	C-10

Abbreviations and Definitions

Abbreviation	Definition
O.D. (Optical Density) unit	The amount of a substance dissolved in 1.0 mL that will give an absorbance reading of 1.00 in a spectrophotometer with a 1-cm path length. The wavelength is assumed to be 260 nm unless stated otherwise.
3´	3- prime hydroxyl end of an oligonucleotide
5´	5- prime hydroxyl end of an oligonucleotide
A_x	Absorbance of a solution at wavelength x (nm) in a spectrophotometer with a 1-cm path length
kcal	kilocalories
OPC®	Oligonucleotide Purification Cartridge
q.s.	Quantity sufficient to bring solution to desired volume
TBE buffer	Tris-borate-EDTA buffer
TEAA	Triethylammonium acetate
TEMED	N,N,N´,N´-tetramethylethylenediamine
U	Enzyme unit
v/v	Volume per volume

Introduction to 5´-end Labeling

Ease of Preparation

You can prepare 5´-end labeled primers on any Applied Biosystems DNA synthesizer using one of three dye phosphoramidites: 6-FAM, HEX, or TET. Fluorescently labeled primers are as easy to make as unlabeled oligonucleotides.

Designing Fluorescently-labeled Primers

The principles of custom primer design are the same for fluorescently labeled primers as for unlabeled primers used in traditional procedures. Choose a sequence for your custom primer that binds to the desired template location with maximum stability and specificity and with minimum destabilization of internal structures.

For guidelines on choosing sequences for custom primers, see page 6-3.

continued on next page

Dye Preparation

For spectral homogeneity, all three dye phosphoramidites are prepared from single-isomer fluorescein dyes: 6-carboxyfluorescein and its tetra- and hexachlorinated analogs. The phosphoramidites are synthesized, purified, and formulated specifically for use on all Applied Biosystems nucleic acid synthesizers. The dyes and their linkers are stable under standard detritylation, coupling, capping, and oxidation conditions.

Figure C-1 shows the structures of the dye phosphoramidites.

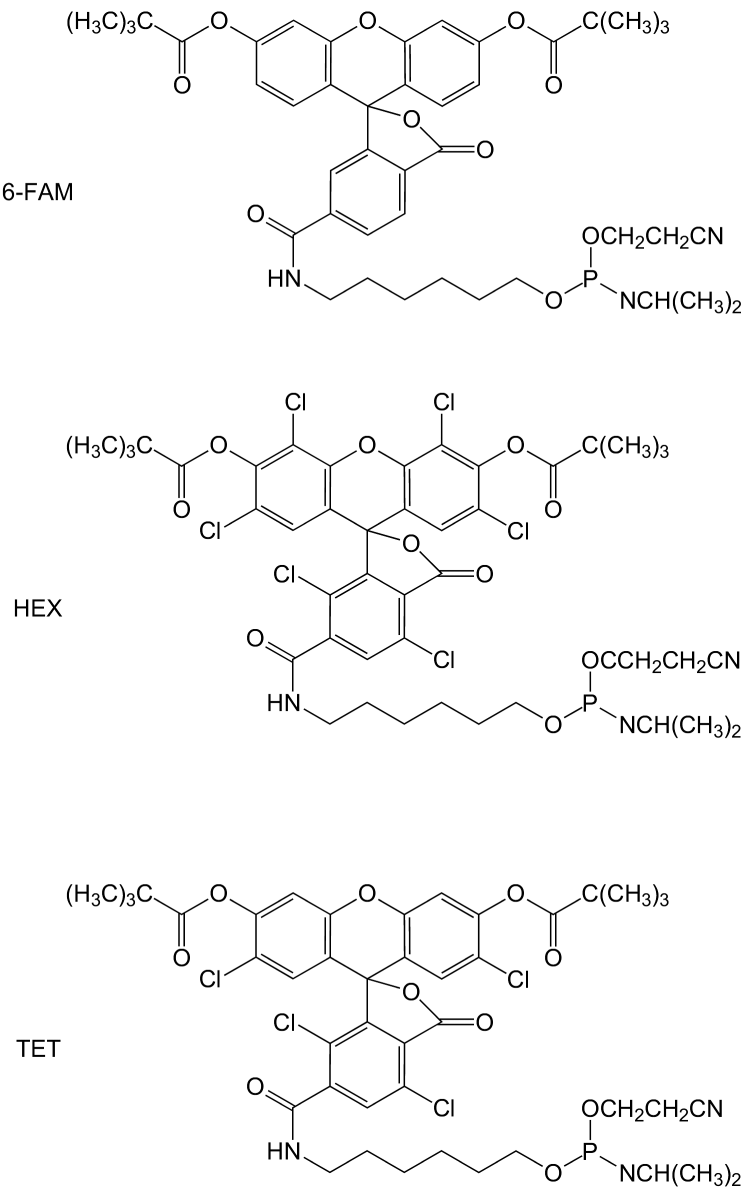


Figure C-1 Dye phosphoramidites use 6-carboxyfluorescein and its chlorinated analogs

Instrument Setup

Installing Dye on Synthesizer

To install dye-labeled phosphoramidites on the DNA/RNA synthesizer:

Step	Action
1	Open the dye-labeled phosphoramidites and desiccant at room temperature.
2	Dilute with dry acetonitrile (<50 ppm water) by the manual method with a dry syringe: ♦ For 0.2-µmol, 1-µmol, and 10-µmol scale syntheses, add 1 mL of dry acetonitrile to prepare a 0.1 M solution. ♦ For 40-nmol scale syntheses, add 2 mL to prepare a 0.05 M solution. **Note** Do not perform autodilution on the ABI 392 and ABI 394 instruments. Significant acetonitrile is lost during argon bubbling.
3	Place the diluted dye phosphoramidite at any monomer position on your DNA/RNA synthesizer; typically, position 5 or greater.

Refer to the Functions, Cycles and Procedures section in your synthesizer user's manual for more detailed directions.

Creating a Bottle Change Procedure

This program reduces the time for the delivery to waste from the dye phosphoramidite bottle to one second. The program is for the ABI 392 instrument with the dye phosphoramidite at bottle position 5. Program other instruments or bottle positions similarly.

To create a Bottle Change procedure:

392 Bottle Change Procedure

Step	Function	Number	Time
1	Begin	106	0
2	Block Flush	1	5
3	18 to Waste	64	7
4	18 to 5	74	3
5	Flush to 5	10	10
6	Interrupt	104	0
7	Flush to 5	10	5
8	Phos Prep	101	15
9	5 to Waste	54	1
10	18 to Waste	64	7
11	Block Flush	1	5
12	End	107	0

continued on next page

Creating a Begin Procedure

Reduce to one second the delivery to waste from the dye phosphoramidite bottle position. The following procedure is for the ABI 392 instrument with the dye phosphoramidite at bottle position 5. Other instruments or bottle positions are similarly programmed.

To create a Begin procedure:

392 Begin Procedure

Step	Function	Number	Time
1	Begin	106	0
2	Phos Prep	101	15
3	A to Waste	50	2
4	G to Waste	51	2
5	C to Waste	52	2
6	T to Waste	53	2
7	5 to Waste	54	1
8	Tet to Waste	58	2
9	18 to Waste	64	10
10	Block Flush	1	10
11	End	107	0

Synthesis and Purification of 5´-end Labeled Primers

Synthesizing the Primer

Step	Action
1	Enter the oligonucleotide sequence with the dye phosphoramidite (denoted by its bottle position) at the 5´ end.
2	Create a user-defined cycle with an additional 120-second wait after the coupling wait. **Note** You can use dye phosphoramidites with standard cycles and coupling times; however, an additional 120-second coupling wait significantly improves coupling efficiency.
3	Using the base-specifier field, set the dye phosphoramidite bottle position to Yes for the 120-second wait step. All other bottle positions should be set to No. ♦ For 0.2-µmol, 1-µmol, and 10-µmol scale syntheses, use 0.1 M phosphoramidites. ♦ For 40-nmol scale syntheses, use 0.05 M phosphoramidite and the 0.2-µmol scale cycle.
4	Choose Trityl-on.

Consumption

Dye phosphoramidites yield the following approximate number of couplings:

Couplings	Scale	M	Cycle
12	40 nmol	0.05	0.2 µmol
6	0.2 µmol	0.1	0.2 µmol
4	1 µmol	0.1	1.0 µmol
1	10 µmol	0.1	10 µmol

Deprotecting

Perform ammonia deprotection for 4 hours at 55 °C.

IMPORTANT Monitor the deprotection time and temperature for HEX-labeled oligonucleotides with special care. Extended treatment in concentrated aqueous ammonia will cause degradation of the dye and subsequent formation of side products. Although these impurities can be removed by OPC purification, corresponding product yields will be lower.

continued on next page

Purifying

Purify with the Oligonucleotide Purification Cartridge (OPC) following the protocol below. The selective OPC® media binds only the fluorescently labeled product. Unlabeled impurities are washed away. (For more details regarding OPC purification, see *DNA User Bulletin 59*, *New Applications for the Oligonucleotide Purification Cartridge*, March 1991.

Step	Action
1	Dry the crude, fluorescently labeled oligonucleotide and dissolve it in 1 mL of 0.1 M TEAA (P/N 400613).
2	Pass 5 mL of dry acetonitrile, followed by 5 mL of 2 M TEAA, through the OPC to waste.
3	Pass the fluorescently labeled oligonucleotide solution through the OPC at a rate of about one drop per second. Collect the eluate and pass it through a second time.
4	Pass 5 mL of 8% acetonitrile in 0.1 M TEAA (v/v), followed by 5 mL of water, through the OPC to waste.
5	Elute, drop-by-drop, the purified, dye-labeled oligonucleotide with 1 mL of 20% acetonitrile in water (v/v) (P/N 400314).
6	Store the purified fluorescently labeled oligonucleotide in the freezer, either dry or as a neutral aqueous solution. Keep it in the dark.

The capacity of an OPC cartridge allows for maximum recovery of purified product from 8–10 O.D. of crude, fluorescently labeled oligonucleotide. All of the product from a 40-nmol synthesis can be purified using a single OPC cartridge. However, a 0.2-µmol synthesis requires three or four cartridges for total product purification, depending on crude yield.

You can also purify fluorescently labeled oligonucleotides by reverse-phase HPLC, employing the same column and mobile-phase conditions as for unlabeled oligonucleotides. The greater hydrophobicity of fluorescently labeled oligonucleotides results in significantly longer elution times than those of unlabeled species.

Analyzing the Oligos

You can analyze crude or OPC-purified fluorescently labeled oligonucleotides by reverse-phase HPLC, PAGE, and capillary electrophoresis using MicroGel™ Gel-Filled Capillaries. However, the dye phosphoramidite imparts greater hydrophobicity to the oligonucleotide. The fluorescently labeled oligonucleotide will migrate approximately one base slower than on PAGE, and will be visible under long-wavelength UV light. Also, by HPLC and MicroGel CE, the fluorescently labeled oligonucleotide will elute later than it does for unlabeled sequences.

Figure C-2 on page C-8 shows a typical fluorescently labeled oligonucleotide analyzed using MicroGel capillary electrophoresis on the Applied Biosystems Model 270A-HT.

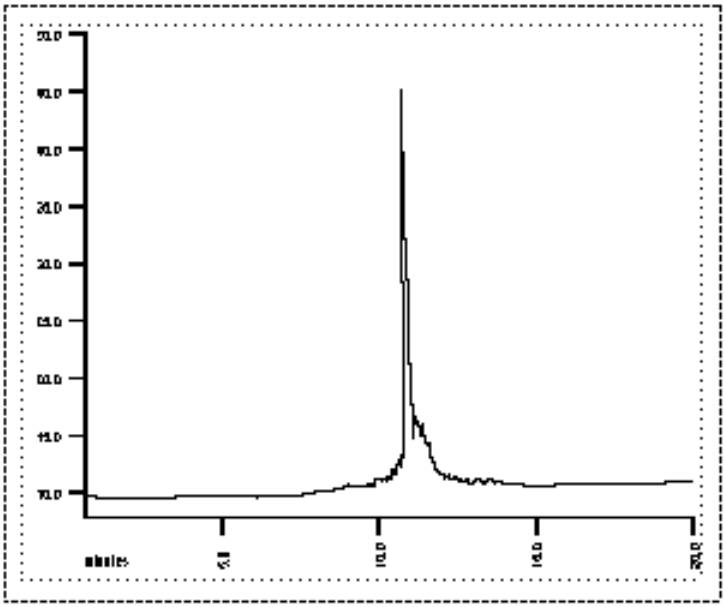


Figure C-2 MicroGel electropherogram of an OPC-purified 18-mer with the sequence: 5´-HEX-TGT AAA ACG ACG GCC AGT-3´

Evaluating Yield

The following are typical yields of a fluorescently labeled 20-mer. Base composition (purine/pyrimidine ratio) and length may affect yield.

Scale	Expected Yield		Purification
40 nmol	1–2 O.D.	33–66 µg	OPC
0.2 µmol	5–10 O.D.	165–330 µg	OPC
1 µmol	20–40 O.D.	0.66–1.32 mg	HPLC
10 µmol	100–200 O.D.	3.3–6.6 mg	HPLC

Molecular Weight and Emission Specifications

Dye + Linker	MW	Emission λ_{max} (nm)
6-FAM	491.5	494
TET	629.3	521
HEX	698.2	535

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Storing the Dyes

- Store dry dye phosphoramidite powder over desiccant in a freezer at –15 to –25 °C.

Note These products are stable for at least one year under these conditions.

- Use diluted dye phosphoramidites as soon as possible. Coupling efficiency may fall below 90% after four days on the instrument. Although OPC purification will remain effective, less fluorescently labeled oligonucleotide may be produced.

IMPORTANT As with nucleoside phosphoramidites, dye phosphoramidites in solution undergo significant loss of coupling efficiency if removed from the DNA synthesizer, regardless of care or technique.

Calculating Absorbance for DNA Samples

Introduction

Spectrophotometric absorbance readings are used to determine quantitative information about DNA samples. The method of expressing this information differs between research specialties, however, creating the potential for confusion and error. For example, the O.D. unit is the standard of DNA quantification in the field of DNA synthesis. In molecular biology, concentration values of pmol/µL or µg/µL are far more relevant. The information in this section is designed to help you correctly convert absorbance information into several different units. Not all of these conversions are for the procedures outlined in this appendix; they are provided for your convenience.

Optical Density

Definition

One Optical Density (O.D.) unit is the amount of a substance dissolved in 1.0 mL which gives an absorbance reading of 1.00 in a spectrophotometer with a 1 cm path length. The wavelength is assumed to be 260 nm unless stated otherwise.

Formula

O.D. = $A_{260} \times$ Stock Volume (mL) $\times$ Dilution Factor

Dilution Factor = Dilution Volume (mL)/Aliquot Volume (mL)

Example 1

Question: How many O.D. units are present in a 1.5 mL PD-10 column fraction?

- A 0.1 mL aliquot is brought to 1.0 mL. The absorbance at 260 nm is 0.16 in a 1-mL cuvette with a 1 cm path length.

Answer:

$$\text{O.D.} = 0.16 \times 1.5 \text{ mL} \times 1.0 \text{ mL} = \frac{2.4 \text{ O.D. units}}{0.1 \text{ mL}}$$

Example 2

Question: How many O.D. units are present in 0.5 mL of a purified dye primer?

- 0.3 mL of this solution is loaded into a 0.3-mL cuvette (1 cm path length), and the absorbance at 260 nm is 0.6.
- Since no dilution of the stock occurred, the dilution factor is 1. The sample can be recovered from the cuvette and pooled with the rest of the stock dye primer.

Answer:

$$\text{O.D.} = 0.6 \times 0.5 \text{ mL} \times 0.3 \text{ mL} = \frac{0.3 \text{ O.D. unit}}{0.3 \text{ mL}}$$

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Converting A_{260} to Concentration

A_{260} values can be converted into µg/mL using Beer´s Law:

Absorbance (260 nm) = sum of extinction coefficient contributions × cuvette pathlength × oligonucleotide concentration

The following formulas, which are derived from Beer's Law, convert A_{260} readings into pmol/µL concentrations:

- Single-stranded DNA:

  C (pmol/µL) = $A_{260}/(10 \times S)$, where S = size of DNA in kilobases

- Double-stranded DNA:

  C (pmol/µL) = $A_{260}/(13.2 \times S)$, where S = size of DNA in kilobases

- Oligonucleotides:

  C (pmol/µL) = $(A_{260} \times 100)/(1.54n_A + 0.75n_C + 1.17n_G + 0.92n_T)$,

  where n_x = number of residues of base x in the oligonucleotide

Two useful facts come from this:

- One A_{260} unit of single-stranded DNA contains 33 µg/mL.
- One A_{260} unit of double-stranded DNA contains 50 µg/mL.

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D

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Part Numbers

E

ABI PRISM DNA Fragment Analysis Kits and Reagents

GeneScan Internal Lane Size Standards

GeneScan-350, 500, and 400HD contain enough material for 800 injections. GeneScan-1000 and 2500 contain enough material for 400 injections. GeneScan-500XL contains enough material for 1600 injections. Loading buffer is included.

401735	GeneScan-350 [ROX]
401736	GeneScan-350 [TAMRA]
402985	GeneScan-400HD [ROX]
401734	GeneScan-500 [ROX]
401733	GeneScan-500 [TAMRA]
403040	GeneScan-500XL [TAMRA]
403039	GeneScan-500XL [ROX]
401098	GeneScan-1000 [ROX]
401100	GeneScan-2500 [ROX]
401545	GeneScan-2500 [TAMRA]
401144	Loading Buffer

Fluorescent dNTPs

For fluorescent labeling of DNA during PCR amplification:

401894	[F]dUTP Set: [R110], [R6G], and [TAMRA]	3, 3, and 12 nmol (3 x 30 µL)
401896	[R110]dUTP	6 nmol (2 x 30 µL)
401897	[R6G]dUTP	6 nmol (2 x 30 µL)
401895	[TAMRA]dUTP	24 nmol* (2 x 30 µL)
402793	[F]dCTP Set: [R110], [R6G], and [TAMRA]	3, 3, and 12 nmol (3 x 30 µL)
402795	[R110]dCTP	6 nmol (2 x 30 µL)
402796	[R6G]dCTP	6 nmol (2 x 30 µL)
402794	[TAMRA]dUTP	24 nmol* (2 x 30 µL)

Note [TAMRA]dNTP is supplied at a concentration four times higher than [R110]dNTP and [R6G]dNTP because it produces approximately four times less signal.

continued on next page

Fluorescent dNTP PCR Kits

Each kit listed below includes a GeneAmp® kit as specified (100 reactions) along with an [F]dNTP set that contains 30 µL each of [R110]dNTP (3 nmol), [R6G]dNTP (3 nmol), and [TAMRA]dNTP (12 nmol).

N808-0220	GeneAmp PCR Reagent Kit with AmpliTaq® DNA Polymerase with [F]dUTP Set
N808-0221	GeneAmp PCR Core Reagents with [F]dUTP Set
N808-0222	GeneAmp Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit with [F]dUTP Set
N808-0223	GeneAmp PCR Reagent Kit with AmpliTaq DNA Polymerase with [F]dCTP Set
N808-0224	GeneAmp PCR Core Reagents with [F]dCTP Set
N808-0225	GeneAmp Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit with [F]dCTP Set

Fluorescent Phosphoramidites

For direct 5' end labeling on an automated DNA synthesizer:

401527	[6-FAM] Phosphoramidite	85 mg
401533	[TET] Phosphoramidite	100 mg
401526	[HEX] Phosphoramidite	105 mg

Fluorescent NHS-Esters

For post-synthesis labeling of primers containing a 5' Aminolink 2:

400981	[TAMRA] NHS-Ester	5 mg/60 µL in DMSO
400980	[ROX] NHS-Ester	5 mg/60 µL in DMSO
400808	Aminolink 2	0.25 g

Matrix Standard Sets

401114	Dye Primer Matrix Standards Kit (Filter Set A) for NHS-ester labeling Contains one tube each of 5-FAM-, JOE-, TAMRA-, and ROX-labeled DNA
402792	[F]dNTP Matrix Standards Contains one tube each of R110-, R6G-, TAMRA-, and ROX-labeled DNA
401546	Fluorescent Amidite Matrix Standards Kit (Filter Set C) for fluorescent phosphoramidite labeling Contains one tube each of 6-FAM-, TET-, HEX-, TAMRA- and ROX-labeled DNA
402996	NED Matrix Standard Used in combination with the 5-FAM, JOE and ROX dyes in the Dye Primer Matrix Standards Kit or the 6-FAM, HEX, and ROX dyes in the Fluorescent Amidite Matrix Standards Kit

continued on next page

Fluorescent Genotyping Demonstration Kits A and B

402246	Kit A-PCR Reagents Contains six fluorescent labeled PCR primer pairs labeled with [HEX], [TET] & [FAM], two control DNAs (CEPH 1347-02 and 1347-10), and a ready made mix of PCR reagents containing AmpliTaq Gold™ DNA Polymerase, GeneAmp PCR Buffer II, dNTPs, and magnesium chloride Also includes GeneScan-350 Internal Lane Size Standard and loading buffer
402247	Kit B-Amplified PCR Products Contains four tubes of pooled (combined) PCR products. To generate the products each DNA sample (CEPH 1347-01, 1347-02, 1347-10, 1347-15) has been amplified with the same six fluorescent-labeled PCR primer pairs in kit A. All of the PCR products from one tube can be detected in one gel lane.

continued on next page

ABI PRISM Linkage Mapping Set Version 2

50-Rxn Kits	300-Rxn Kits	Panel	Chromosome
403089	403118	Complete Set	1–22, X
403090	403119	1	1
403091	403120	2	1
403092	403121	3	2
403093	403122	4	2
403094	403123	5	3,4
403095	403124	6	3,4
403096	403125	7	3,4
403097	403126	8	5,6
403998	403127	9	5,6
403099	403128	10	5,6
403100	403129	11	7,8
403101	403130	12	7,8
403102	403131	13	9,10,11
403103	403132	14	9,10,11
403104	403133	15	9,10,11
403105	403134	16	9,10,11
403106	403135	17	12,13
403107	403136	18	12,13
403108	403137	19	12,13
403109	403138	20	14
403110	403139	21	15,16
403111	403140	22	15,16
403112	403141	23	17,18
403113	403142	24	17,18
403114	403143	25	19,20,21,22
403115	403144	26	19,20,21,22
403116	403145	27	19,20,21,22
403117	403146	28	X

450096	Individual Primer Pairs from the ABI PRISM™ Linkage Mapping Set Version 2 Must be ordered through Applied Biosystems Custom Oligonucleotide Synthesis Service (specify locus name)	3000 pmol
403061	True Allele™ PCR Premix	18 mL, enough for 2000 rxns
403062	Control DNA CEPH 1347-02	180 µL, enough for 150 rxns

ABI PRISM 310 Genetic Analyzer

Autosampler Tray Kits

402867	48-Tube Sample Tray Kit Includes: 48-Tube Sample Trays (2), 0.5-mL Tube Septa (500), 0.5-mL Sample Tubes (500). (Individual Part Numbers: One 48-Tube Sample Tray, P/N 005572; 0.5-mL Tube Septa, P/N 401956; 0.5-mL Sample Tubes, P/N 401957)
402868	96-Tube Sample Tray Kit Includes: 96-Tube Septa Clips (4), 0.2-mL Tube Septa Strips (24 strips, 480 septa), 0.2-mL Sample Tubes (1000), MicroAmp Tray and Retainer (10 sets), MicroAmp Base (10). (Individual Part Numbers: Septa Clips, P/N 402866; 0.2-mL Tube Septa Strips, P/N 402059; 0.2-mL MicroAmp Tubes, P/N N801-0580; MicroAmp Tray and Retainer, P/N 403081; MicroAmp Base, P/N N801-0531; 96-Well Tray Adapter, P/N 4305051)

Polymers and Consumables

Polymers and Consumables for the ABI PRISM 310 Genetic Analyzer

402838	Performance Optimized Polymer 4 (POP-4™) 500 sample runs	5 mL
401885	GeneScan Polymer For native (non-denaturing) applications	50 mL
402818	GeneScan Polymer w/TSR Includes two 4-mL vials of template suppression reagent	50 mL
402837	Performance Optimized Polymer 6 (POP-6™) Generally used for sequencing No template suppression reagent included 200 sample run	3 mL
402844	Performance Optimized Polymer 6 (POP-6) w/TSR Includes two 4-mL vials of template suppression reagent 200 sample runs	3 mL
403076	POP-6 w/TSR for Shared Instruments Includes eight 4-mL vials of template suppression reagent 200 sample runs	3 mL
402824	10X Genetic Analyzer Buffer with EDTA Used with POP-4, POP-6 and GeneScan Polymer	25 mL
402839	310 Capillaries, 47-cm x 50-µm (internally uncoated) Used with POP-4 500 sample runs (100 runs/capillary)	5/pkg
402840	310 Capillaries, 61-cm x 50-µm (internally uncoated) Used with POP-6 for long read sequencing 200 sample runs (100 runs/capillary)	2/pkg
401957	Genetic Analyzer Sample Tubes (0.5-mL)	500/pkg

Polymers and Consumables for the ABI PRISM 310 Genetic Analyzer *(continued)*

401956	Genetic Analyzer Septa for 0.5-mL Sample Tubes For 48-Tube Tray	500/pkg
402059	Genetic Analyzer Septa Strips (0.2-mL tube) For 96-Tube Tray	485/pkg (20 strips)
402866	Genetic Analyzer Retainer Clips (96-Tube Tray Septa Clips)	4/pkg
N801-0580	MicroAmp 0.2-mL Sample Tubes	1000/pkg
403081	MicroAmp Tray and Retainer	10 sets
N801-0531	MicroAmp Base	10/pkg
4305051	96-Well Tray Adapter	1 each
401958	Genetic Analyzer Capillary Cutters	2 each
401955	Genetic Analyzer Buffer Vials (4.0-mL) Includes cap adapters	50/pkg
005914	Platinum cathode electrode	1 each
604418	1.0-mL Glass Syringe Used for GeneScan and Sequencing Applications Contains syringe O-rings and ferrule	1 each
604042	GeneScan Glass Syringe (2.5-mL) Contains syringe O-rings and ferrule	1 each
603803	DNA Sequencing Glass Syringe (250-µL) Contains syringe O-rings and ferrule	1 each
221102	Syringe O-rings O-ring inside of glass syringe assembly	1 each
005401	Syringe ferrule Ferrule inside of glass syringe assembly	1 each
005404	Capillary Fitting Screw fitting used to hold the capillary in the pump block	1 each
005572	0.5-mL Sample Tray Holds 48 0.5-mL sample tubes	1 each
603796	Waste vial Vial attaches to the gel pump block, collects waste generated during gel pump priming with Sequence Polymer	1 each
005402	Anode buffer jar Buffer jar attaches to gel pump block, holds the anode buffer	1 each
604076	Valve, waste vial Gel pump block manual valve, the waste vial attaches to the fitting on this valve	1 each
604075	Valve, plastic syringe, Luer Gel pump block manual valve, the DNA sequencing polymer plastic syringe attaches to the fitting on this valve	1 each

Polymers and Consumables for the ABI PRISM 310 Genetic Analyzer *(continued)*

310021	Thermal Tape For affixing the capillary to the heat plate	1 each

Chemical Installation Kits

These kits are shipped with new instruments for the purpose of installation and training.

402089	ABI PRISM® 310 Basic Install Kit Included with purchase of ABI PRISM 310 Genetic Analyzer Includes: 310 Genetic Analyzer Buffer with EDTA, 310 Leak Test Capillary, Sensitivity Standard, Genetic Analyzer Buffer Vials, Buffer Vial Septa, Genetic Analyzer Capillary Cutters, 5-cc Syringe
401822	ABI PRISM 310 GeneScan Install Kit Included with purchase of the GeneScan Analysis module (672-30) Includes: POP-4, 47-cm x 50-µm i.d. Capillaries, Microsatellite Demo Kit B, GeneScan-500 [TAMRA] Internal Lane Size Standard, formamide, Amberlite MB-1A, Fluorescent Amidite Matrix Standards, Dye Primer Matrix Standards
402090	ABI PRISM 310 DNA Sequencing and GeneScan Install Kit Included when both the DNA Sequencing and GeneScan Modules (677-30 & 672-30) are purchased Includes: POP-6, TSR, Taq FS Terminator Sequence Standard, 61-cm x 50-µm i.d. Capillaries, POP-4, 47-cm x 50-µm i.d. Capillaries, Microsatellite Demo Kit B, GeneScan-500 [TAMRA] Internal Lane Size Standard, formamide, Amberlite MB-1A, Dye Terminator Matrix Standards, Fluorescent Amidite Matrix Standards, Dye Primer Matrix Standards
401820	ABI PRISM 310 DNA Sequencing Install Kit Included with purchase of the DNA sequencing Analysis module (677-30) Includes: POP-6, TSR, Taq FS Terminator Sequence Standard, 61-cm x 50-µm i.d. Capillaries

Reference Materials

903565	*ABI PRISM 310 Genetic Analyzer User's Manual*
904435	*GeneScan® Analysis Software User's Manual*
4303032	ABI PRISM 310 Training CD

Updates

Part numbers are subject to change. Consult the Applied Biosystems World Wide Web Site (www.appliedbiosystems.com/techsupport) for updated information.

Index

Symbols

[F]dNTPs
- advantages of using 2-5
- dye chemical form 4-2
- literature references D-1

Numerics

10X TBE, preparing reagent solution A-2

1X TBE with 10% glycerol, preparing reagent solution A-2

3´ A addition 6-18 to 6-20
- enzymatic treatment 6-19 to 6-20
- modifying Mg^{2+} 6-19
- modifying thermal cycling conditions 6-19
- reverse-primer tailing 6-19
- why incomplete 3´ A addition causes problems 6-18

5´-end labeled primers
- advantages of using 2-5
- determining relative quantities 3-10
- literature references D-2
- preparing
  - abbreviations and definitions C-1
  - calculating absorbance for DNA samples C-10 to C-11
  - instrument setup C-4 to C-5
    - creating a bottle change procedure C-4
    - installing dye on the synthesizer C-4
  - introduction to 5´-end labeled primers C-2 to C-3
  - synthesis and purification C-6 to C-9
    - analyzing the oligos C-7 to C-8
    - consumption C-6
    - deprotecting C-6
    - evaluating yield C-8
    - molecular weight and emission specifications C-8
    - purifying C-7
    - storing the dyes C-9
    - synthesizing the primer C-6

A

ABI PRISM 310
- analyzing data 3-2 to 3-7
  - sample files, analyzing 3-2
  - size standard peak assignments, verifying 3-5 to 3-7
- data collection, how it works 4-5
- electrokinetic injection parameters, optimizing 2-9 to 2-12
- evaluating data quality 3-8 to 3-9
  - bad data example 3-9
  - good data example 3-8
- matrix, evaluating quality 3-11 to 3-14
  - purpose of matrix 3-11
  - recognizing problems 3-11 to 3-14
  - solving matrix problems 3-14
  - when to remake matrix 3-11
- nucleic acids, quantitating 3-10
- *See Also* microsatellite marker analysis

ABI PRISM dyes
- available dyes 4-2
- dye spectra, understanding 4-3 to 4-4
- dye/filter sets, choosing 4-5 to 4-7
  - chemical forms, table of 4-6
  - reagents and primer dye sets, table of 4-7
  - recommended combinations, table of 4-6

ABI PRISM Linkage Mapping Set, primer dye set 4-7

ABI PRISM multicolor fluorescence technology, increasing throughput, multiplexing 2-2 to 2-3

AFLP
- bacterial and fungal genomes analyzed 10-10
- for more information 10-4
- literature references D-9
- microbial fingerprinting 10-1
  - preselective amplification 10-5
  - primer selection 10-9
  - selective amplification 10-6
- plant genomes analyzed 10-12 to 10-17
- plant mapping 10-2
  - preselective amplification 10-7
  - primer selection 10-11
  - selective amplification 10-8
- primer dye sets 4-7
- simplifying complex patterns 10-7
- template preparation and adaptor ligation 10-5
- testing new genomes 10-9
- what is AFLP 10-1

allele binning. *See* Genotyper

Amicon Centricon-100 Microconcentrator, using to desalt DNA 2-3

AmpFℓSTR kits 9-22
- discrimination power 9-25
- primer dye sets 4-7
- reliability 9-24
- table of loci 9-21

AmpliTaq DNA Polymerase
- description of 6-8
- LD, description of 6-8
- Stoeffel Fragment, description of 6-8

AmpliTaq Gold DNA Polymerase
- 3´ A addition 6-18 to 6-20
  - enzymatic treatment 6-19 to 6-20
  - modifying Mg^{2+} 6-19
  - modifying thermal cycling conditions 6-19
  - reverse-primer tailing 6-19
  - why incomplete 3´ A addition causes problems 6-18
- description of 6-8
- enzyme concentration 6-7

AmpliWax PCR Gem-mediated Hot Start, performing 6-14

analyzing data
- evaluating data quality 3-8 to 3-9
  - bad data example 3-9
  - good data example 3-8
- matrix, evaluating quality 3-11 to 3-14
  - purpose of matrix 3-11
  - recognizing problems 3-11 to 3-14

solving matrix problems 3-14
when to remake matrix 3-11
nucleic acids, quantitating 3-10
sample files, analyzing 3-2
size standard peak assignments, verifying 3-5 to 3-7
animal paternity 9-17 to 9-18
allele frequency 9-18
DNA-based tests, advantages 9-17
genotyping 9-18
improved breeding 9-17
StockMarks Kits 9-17
anomalous size standard peaks, how affects size-calling 5-6
applications
AFLP
bacterial and fungal genomes analyzed 10-10
for more information 10-4
literature references D-9
microbial fingerprinting 10-1
preselective amplification 10-5
primer selection 10-9
selective amplification 10-6
plant genomes analyzed 10-12 to 10-17
plant mapping 10-2
preselective amplification 10-7
primer selection 10-11
selective amplification 10-8
simplifying complex patterns 10-7
template preparation and adaptor ligation 10-5
testing new genomes 10-9
what is AFLP 10-1
animal paternity 9-17 to 9-18
allele frequency 9-18
DNA-based tests, advantages 9-17
genotyping 9-18
improved breeding 9-17
StockMarks Kits 9-17
human identification 9-19 to 9-25
advantages of STRs 9-19
AmpFℓ STR kits 9-22
AmpFℓ STR loci, table of 9-21
automated sizing and genotyping 9-20
for more information 9-25
high throughput, about 9-20
using allelic ladders 9-23 to 9-24
using STRs 9-19
Linkage Mapping Set 9-2 to 9-4
loss of heterozygosity, screening for 9-5 to 9-12
advantages 9-5
analyzing LOH data 9-11 to 9-12
example 9-12
limitations 9-5
what is LOH 9-5
replication error, screening for 9-13 to 9-15
advantages 9-13
examples 9-14
limitations 9-13
what is RER 9-13
single-strand conformation polymorphism (SSCP) 7-2 to 7-18
about SSCP 7-2
analyzing the data 7-12 to 7-13
creating matrix file 7-12
setting analysis parameters 7-12
materials required 7-3
optimizing run conditions 7-14 to 7-16
PCR amplification, labeling, and controls 7-4 to 7-6
preparing for a run 7-8 to 7-11
creating the module 7-9
preparing and loading samples 7-8 to 7-9
starting the run 7-10
prerun checklist 7-7
troubleshooting 7-17
Applied Biosystems
Custom Oligonucleotide Synthesis Service, help 2-7
artifact peaks, result of too much signal 2-8

B

Beer's Law, converting A_{260} to concentration C-11
buffers
preparation A-2

C

capillary, bad capillary, how effects size-calling 5-5
cattle, StockMarks 9-18
CEPH 1347-02
part number 2-16
See Also DNA
co-electrophoresis
guidelines 2-2
contamination, avoiding 6-16 to 6-17
conventions used 1-2
cross-platform size, comparing sizes 5-3 to 5-4
precision results, table of 5-4
Custom Oligonucleotide Synthesis Service, help 2-7
custom primers.*See* primers, designing custom primers

D

data
analyzing 3-2 to 3-7
sample files 3-2
size-standard peak assignments, verifying 3-5 to 3-7
evaluating data quality 3-8 to 3-9
bad data example 3-9
good data example 3-8
data collection, how it works 4-5
deionized formamide, preparing reagent solution A-3
design factors 2-2 to 2-16
control DNA, using 2-16
electrokinetic injection parameters, optimizing 2-9 to 2-12
electrophoresis conditions, optimizing 2-13 to 2-15
fluorescent labeling methods, choosing 2-5 to 2-7
samples, determining loading concentrations 2-8
working with multiple colors 2-2 to 2-4
ensuring signal intensity 2-3 to 2-4
guidelines 2-2
increasing throughput, multiplexing 2-2 to 2-3
diluting PCR amplification products 6-23
DNA
control DNA, using 2-16
control, for microsatellite marker analysis 8-5
salt concentration, decreasing 2-3
dNTP concentration, when determining reagent concentration 6-6
Documents-on-Demand 1-4
dyes
accounting for differences in signal strengths 2-3
available dyes 4-2
chemical forms, table of 4-2

dye spectra, understanding 4-3 to 4-4
wavelengths, table of 4-4
dye/filter sets, choosing 4-5 to 4-7
chemical forms, table of 4-6
reagents and primer dye sets, table of 4-7
recommended combinations, table of 4-6
dye/filter sets, emission spectra 4-8 to 4-10
Virtual Filter Set A 4-8
Virtual Filter Set C 4-9
Virtual Filter Set D 4-9
Virtual Filter Set F 4-10

E

electrokinetic injection parameters
optimizing 2-9 to 2-12
electrophoresis
how effects size-calling 5-6
optimizing conditions 2-13 to 2-15
e-mail address, technical support 1-3
emission spectrum, definition of 4-3
enzyme
choosing 6-8 to 6-9
enzyme choice table 6-9
PCR enzyme overview 6-8 to 6-9
concentration, determining reagent concentration 6-7
evaluating data quality 3-8 to 3-9
bad data example 3-9
good data example 3-8
excitation efficiency, definition of 4-3
excitation spectrum, definition of 4-3
excitation wavelength, table of 4-4
experiment design factors. *See* GeneScan, fragment analysis, design factors

F

fluorescent dNTPs
See [F]dNTPs
fluorescent labeling 2-2 to 2-4
available dyes 4-2
control DNA, using 2-16
dye spectra, understanding 4-3 to 4-4
wavelengths, table of 4-4
dye/filter sets, choosing 4-5 to 4-7
chemical forms, table of 4-6
reagents and primer dye set, table of 4-7
recommended combinations, table of 4-6
dye/filter sets, emission spectra 4-8 to 4-10
Virtual Filter Set A 4-8
Virtual Filter Set C 4-9
Virtual Filter Set D 4-9
Virtual Filter Set F 4-10
electrokinetic injection parameters, optimizing 2-9 to 2-12
electrophoresis conditions, optimizing 2-13 to 2-15
ensuring signal intensity 2-3 to 2-4
guidelines 2-2
increasing throughput, multiplexing 2-2 to 2-3
methods, choosing 2-5 to 2-7
samples, determining loading concentrations 2-8
fragment migration rates, from modifying run voltage 2-14

G

GeneScan
analyzing microsatellite markers 8-10 to 8-11
creating new matrix file 8-10
setting analysis parameters 8-11
control DNA, using 2-16
design factors 2-2 to 2-16
electrokinetic injection parameters, optimizing 2-9 to 2-12
electrophoresis conditions, optimizing 2-13 to 2-15
ensuring signal intensity 2-3 to 2-4
fluorescent labeling methods, choosing 2-5 to 2-7
increasing throughput, multiplexing 2-2 to 2-3
samples, determining loading concentrations 2-8
working with multiple colors 2-2 to 2-4
guidelines 2-2
matrix file, creating B-1 to B-4
checking matrix quality B-3 to B-4
generating the matrix file B-2 to B-3
how to verify raw data B-1 to B-2
size standards 5-2 to 5-17
accuracy versus precision 5-3
comparing sizes across platforms 5-3 to 5-4
GeneScan Internal Lane Size Standards 5-7 to 5-17
GeneScan-1000 5-14 to 5-15
GeneScan-2500 5-16 to 5-17
GeneScan-350 5-8 to 5-9
GeneScan-400HD 5-10 to 5-11
GeneScan-500 5-12 to 5-13
process overview 5-2 to 5-3
troubleshooting 5-5 to 5-6
possible problems 5-5 to 5-6
preventing sizing problems 5-5
GeneScan Polymer with 10% glycerol, preparing reagent solution A-1
GeneScan-1000 size standard
anomalous size standard peaks, 262 and 692-bp fragment peaks 5-6
denaturing electropherogram 5-15
fragment lengths, table of 5-14
non-denaturing electropherogram 5-15
preparation of 5-14
special uses 5-14
what to use it for 5-14
GeneScan-2500 size standard
anomalous size standard peaks, 508-bp fragment peak 5-6
denaturing electropherogram 5-17
fragment lengths, table of 5-16
non-denaturing electropherogram 5-17
preparation of 5-16
special uses 5-16
what to use it for 5-16
GeneScan-350 size standard
anomalous size standard peak, 250-bp fragment peak 5-6
denaturing electropherogram 5-9
fragment lengths, table of 5-8
non-denaturing electropherogram 5-9
preparation of 5-8
what to use it for 5-8
GeneScan-400HD size standard
denaturing electropherogram 5-11
fragment lengths, table of 5-10
non-denaturing electropherogram 5-11
preparation of 5-10
special uses 5-10

what to use it for 5-10
GeneScan-500 size standard
anomalous size standard peaks, 250-bp fragment peak 5-6
denaturing electropherogram 5-13
fragment lengths, table of 5-12
non-denaturing electropherogram 5-13
preparation of 5-12
special uses 5-12
what to use it for 5-12
Genotyper
analyzing microsatellite markers 8-12 to 8-24
allele binning methods 8-12
allele binning, definition 8-12
benefits of allele binning 8-12
creating defined set of category members 8-21 to 8-24
generating category member automatically 8-18 to 8-21
using the Histogram window 8-13 to 8-17
using the Plot window 8-17 to 8-18
StockMarks example 9-18
global sizing methods 5-2
guidelines
co-electrophoresis 2-2
control DNA, using 2-16
for performing experiments 2-2
temperature control parameters, modifying 6-15

H

help
Custom Oligonucleotide Synthesis Service 2-7
e-mail address 1-5
Documents-on-Demand 1-4
Internet address 1-3
regional offices 1-5 to 1-7
technical support 1-3 to 1-7
horse, StockMarks 9-18
Hot Start technique 6-13 to 6-14
AmpliWax PCR Gem-mediated Hot Start, performing 6-14
Hot Start procedure 6-13
human identification 9-19 to 9-25
advantages of STRs 9-19
AmpFℓSTR kits 9-22
discrimination power 9-25
reliability 9-24
AmpFℓSTR loci, table of 9-21
automated sizing and genotyping 9-20
for more information 9-25
high throughput, about 9-20
using allelic ladders 9-23 to 9-24
using STRs 9-19

I

Important, user attention word 1-2
injection time, modifying 2-9 to 2-11
injection voltage, modifying 2-11
Internet address
Custom Oligonucleotide Synthesis Service 2-7
manual updates 1-2
technical support 1-3

L

Linkage Mapping Set 9-2 to 9-4
advantages 9-2
collecting and analyzing LMS V2 microsatellite data 9-3
for more information 9-3
primer dye set 4-7
troubleshooting 9-4
what is LMS 9-2
local sizing methods 5-2
LOH. *See* loss of heterozygosity, screening for
loss of heterozygosity, screening for 9-5 to 9-12
advantages 9-5
analyzing LOH data 9-11 to 9-12
example 9-12
limitations 9-5
literature references D-7
troubleshooting 9-16
what is LOH 9-5

M

magnesium ion concentration
determining reagent concentration 6-6
matrix file
creating B-1 to B-4
checking matrix quality B-3 to B-4
generating the matrix file B-2 to B-3
how to verify raw data B-1 to B-2
evaluating quality of 3-11 to 3-14
purpose of matrix 3-11
recognizing problems 3-11 to 3-14
solving matrix problems 3-14
when to remake matrix 3-11
matrix problems, how effects size-calling 5-6
maximum emission wavelength, table of 4-4
microsatellite marker analysis
about 8-2
analyzing the data-using GeneScan 8-10 to 8-11
creating new matrix file 8-10
setting analysis parameters 8-11
analyzing the data-using Genotyper 8-12 to 8-24
allele binning methods 8-12
allele binning, definition 8-12
benefits of allele binning 8-12
creating defined set of category members 8-21 to 8-24
generating category members automatically 8-18 to 8-21
using the Histogram window 8-13 to 8-17
using the Plot window 8-17 to 8-18
applications
animal paternity 9-17 to 9-18
applications
loss of heterozygosity, screening for 9-5 to 9-12
human identification 9-19 to 9-25
Linkage Mapping Set 9-2 to 9-4
replication error, screening for 9-13 to 9-15
materials required 8-3
PCR amplification, labeling, and controls 8-4 to 8-5
preparing for a run 8-7 to 8-9
preparing and loading samples 8-7 to 8-8
starting the run 8-8 to 8-9
prerun checklist 8-6
troubleshooting 8-25 to 8-28
common problems 8-25
LOH and RER screening 9-16
stutter, aiding in allele calling 8-28
stutter, example 8-25 to 8-27
multiplexing PCR
increasing throughput 2-2 to 2-3
optimizing 6-10 to 6-11
PCR, how to 2-3

N

NHS-esters, dye chemical form 4-2
Note, user attention word 1-2

O

off-scale data, how effects size-calling 5-6
oligonucleotide sequence
  analysis of C-7
  deprotection C-6
  purification of C-7
  typical synthesis yields C-8

P

part numbers E-1 to E-8
PCR
  and microsatellite marker analysis 8-4 to 8-5
  and single-strand conformation polymorphism (SSCP) analysis 7-4 to 7-6
  co-electrophoresis, guidelines 2-2
  multiplexing, how to 2-3
  optimizing
    3´ A addition 6-18 to 6-20
    contamination, avoiding 6-16 to 6-17
    designing custom primers 6-3 to 6-5
    enzyme, choosing the right enzyme 6-8 to 6-9
    Hot Start technique 6-13 to 6-14
    literature references D-1
    multiplexing PCR 6-10 to 6-11
    reaction volumes and tube types, choosing 6-2
    reagent concentrations, determining 6-6 to 6-7
    RNA templates, using 6-12
    stutter products 6-21 to 6-22
    temperature control parameters, modifying 6-15
  products, guidelines for working with 2-2
  troubleshooting
    amplification 11-1 to 11-6
    product detection 11-7 to 11-14
PCR product carryover, avoiding contamination from 6-16
Performance Optimized Polymer 4 (POP-4)
  analyzing the data-using GeneScan 8-10 to 8-11
    creating new matrix file 8-10
    setting analysis parameters 8-11
  analyzing the data-using Genotyper 8-12 to 8-24
    allele binning methods 8-12
    allele binning, definition 8-12
    benefits of allele binning 8-12
    creating defined set of category members 8-21 to 8-24
    generating category members automatically 8-18 to 8-21
    using the Histogram window 8-13 to 8-17
    using the Plot window 8-17 to 8-18
  introduction to microsatellite analysis 8-2
  materials required 8-3
  PCR amplification, labeling, and controls 8-4 to 8-5
  preparing for a run 8-7 to 8-9
    preparing and loading samples 8-7 to 8-8
    starting the run 8-8 to 8-9
  prerun checklist 8-6
  troubleshooting 8-25 to 8-28
    common problems 8-25
    stutter, aiding in allele calling 8-28
    stutter, example 8-25 to 8-27
phosphoramidite reagents, dye chemical form 4-2
post-PCR end-labeling, advantages of using 2-6
precision, in size calling 5-3
primer dye kits, table of 4-7
primers
  5´-end labeled primers, preparing
    abbreviations and definitions C-1
    calculating absorbance for DNA samples C-10 to C-11
    instrument setup C-4 to C-5
    introduction to 5´-end labeled primers C-2 to C-3
    synthesis and purification C-6 to C-9
  designing custom primers 6-3 to 6-5
    definition 6-3
    ensuring successful amplification 6-3
    maximizing specificity 6-4 to 6-5
    maximizing stability 6-3 to 6-4
    melting temperature 6-3
    post-amplification manipulation 6-5

Q

QuantiBlot kit
  quantitating human DNA 9-8, 11-1, 11-3
quantitation 3-10
  choosing fluorescent labeling methods 2-5
  converting A_{260} to concentration C-11
  importance in interpreting stutter patterns 6-22
  importance in LOH-RER assays 9-8
  troubleshooting weak signals 11-3
quantum yield, definition of 4-3

R

reaction volumes, choosing 6-2
  when using small amounts of template 6-2
reagents
  determining concentrations 6-6 to 6-7
    dNTP concentration 6-6
    enzyme concentration 6-7
    magnesium ion concentration 6-6
    template concentration 6-7
  preparation A-1 to A-3
    10X TBE A-2
    1X TBE with 10% glycerol A-2
    5% GeneScan Polymer with 10% glycerol A-1
    deionized formamide A-3
references
  5´-end labeled primers D-2
  loss of heterozygosity, screening for D-7
  optimizing PCR D-1
  post-labeling with [F]dNTPs D-1
  replication error, screening for D-7
  short tandem repeat markers D-4 to D-7
  single-strand conformation polymorphism (SSCP) D-2 to D-4
replication error, screening for 9-13 to 9-15
  advantages 9-13
  examples 9-14

limitations 9-13
literature references D-7
troubleshooting 9-16
what is RER 9-13
RER.*See* replication error, screening for
resolution
definition of 2-9
modifying run voltage, effect of 2-14
RNA templates
using to optimize PCR 6-12
run time, determining required run time 2-13
run voltage
modifying, effects of 2-14 to 2-15

S

salt concentration
decreasing for adequate signal intensity 2-3
samples, loading concentrations, determining 2-8
short tandem repeat markers
human identification 9-19 to 9-25
advantages of STRs 9-19
AmpFℓSTR kits 9-22
AmpFℓSTR loci, table of 9-21
automated sizing and genotyping 9-20
for more information 9-25
high throughput, about 9-20
using allelic ladders 9-23 to 9-24
using STRs 9-19
literature references D-4 to D-7
short tandem repeat markers.*See* microsatellite marker analysis
signal intensity, adjusting. *See* samples, loading concentrations, determining
signals, problems, how to proceed 2-8
single-strand conformation polymorphism (SSCP) 7-2 to 7-18
about SSCP 7-2
analyzing the data 7-12 to 7-13
creating matrix file 7-12
setting analysis parameters 7-12
literature references D-2 to D-4
materials required 7-3
optimizing run conditions 7-14 to 7-16
PCR amplification, labeling, and controls 7-4 to 7-6
preparing for a run 7-8 to 7-11
creating the module 7-9
preparing and loading samples 7-8 to 7-9
starting the run 7-10
prerun checklist 7-7
troubleshooting 7-17
size calling.*See* size standard
size standard
accuracy versus precision 5-3
comparing sizes from across platforms 5-3 to 5-4
precision results, table of 5-4
defining 3-4
dye chemical form 4-2
GeneScan Internal Lane Size Standards 5-7 to 5-17
GeneScan-1000 5-14 to 5-15
GeneScan-2500 5-16 to 5-17
GeneScan-350 5-8 to 5-9
GeneScan-400HD 5-10 to 5-11
GeneScan-500 5-12 to 5-13
peak assignments, verifying 3-5 to 3-7
process overview 5-2 to 5-3
troubleshooting 5-5 to 5-6
possible problems 5-5 to 5-6
preventing sizing problems 5-5
SSCP.*See* single-strand conformation polymorphism (SSCP)
StockMarks 9-17 to 9-18
allele frequency 9-18
DNA-based tests, advantages 9-17
genotyping 9-18
improved breeding 9-17
kits 9-17
primer dye set 4-7
storing dyes C-9
STR (short tandem repeat) markers.*See* microsatellite marker analysis
stutter products 6-21 to 6-22
definition 6-21
evaluating data with stutter 6-22
preparing PCR products for analysis 6-23
stutter facts 6-21

T

technical support 1-3 to 1-7
e-mail address 1-5
Documents-on-Demand 1-4
Internet address 1-3
regional offices 1-5 to 1-7
temperature control parameters, modifying 6-15
template concentration, determining reagent concentration 6-7
troubleshooting
Linkage Mapping Set (LMS V2) 9-4
LOH and RER screening 9-16
microsatellite analysis 8-25 to 8-28
common problems 8-25
stutter, aiding in allele calling 8-28
stutter, example 8-25 to 8-27
PCR amplification 11-1 to 11-6
problems with extra peaks 11-5 to 11-6
problems with missing peaks 11-6
problems with poor amplification 11-1 to 11-4
PCR product detection 11-7 to 11-14
problems with automatic data analysis 11-7
problems with current 11-8 to 11-9
problems with peak number and position 11-12 to 11-13
problems with peak resolution 11-14
problems with signal strength and quality 11-10 to 11-11
single-strand conformation polymorphism (SSCP) 7-17
size standard problems 5-5 to 5-6
possible problems 5-5 to 5-6
preventing sizing problems 5-5
Tth DNA Polymerase, recombinant (r*Tth*) 6-9
tube types, choosing 6-2
when using small amounts of template 6-2

U

UlTma DNA Polymerase, description of 6-9
updates, using the Internet to access 1-2
user attention words 1-2

V

Virtual Filter Set A
representative emission spectra 4-8

Virtual Filter Set C
making matrix files for 3-11
matrix problems resulting from using 4-9
Virtual Filter Set D
representative emission spectra 4-9
Virtual Filter Set F
representative emission spectra 4-10
virtual filter sets, available sets 4-5
See Also individual virtual filter set
voltage, modifying injection voltage 2-11

W

Warning, user attention word 1-2
wavelengths, table of emission and excitation 4-4

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