ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kits

Original and Version 2.0

Protocol

Applied Biosystems

Printed in the U.S.A.

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1 Introduction

Chapter Summary

In This Chapter The following topics are covered in this chapter:

Two Kits Available

Protocol for Two Kits

This protocol describes how to use the following kits:

- ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit
- ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0

IMPORTANT The protocol is identical for both of the kits.

Comparing the Two Kits

The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 contains the same components as the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (original kit). However, the ratio of dideoxy to deoxy terminators has been changed in the v2.0 kit. The new formulation distributes more signal to the longer DNA fragments. Reactions generated with the v2.0 kit show higher signal in longer fragments relative to shorter fragments.

Recommended Use of Version 2 Kit

The v2.0 kit can be used in place of the original kit on all of the sequencing platforms (see “Instrument Platforms” on page 1-7), but is primarily recommended for use with the ABI PRISM 3700 DNA Analyzer and the ABI PRISM 377 DNA Sequencer with 48-cm well-to-read. The v2.0 kit is very effective when following the extended read protocol described in *Achieving Longer High Accuracy Reads on the 377 Sequencer* (P/N 4315153).

BigDye Terminator Ready Reaction Kits

Both the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 provide AmpliTaq® DNA Polymerase, FS, BigDye terminators, and all the required components for the sequencing reaction.

In the Ready Reaction format, the dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, r*Tth* pyrophosphatase (a component in AmpliTaq DNA Polymerase, FS), magnesium chloride, and buffer are premixed into a single tube of Ready Reaction Mix and are ready to use. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on polymerase chain reaction (PCR) fragments, and on large templates, *e.g.*, BAC clones.

The dNTP mix includes dITP in place of dGTP to minimize band compressions. The dNTP mix also uses dUTP in place of dTTP. dUTP improves the incorporation of the T terminator and results in a better T pattern.

Cycle Sequencing with AmpliTaq DNA Polymerase, FS

Both kit formulations contain the sequencing enzyme AmpliTaq DNA Polymerase, FS. This enzyme is a variant of *Thermus aquaticus* DNA polymerase that contains a point mutation in the active site. This results in less discrimination against dideoxynucleotides.

This enzyme also has a second mutation in the amino terminal domain that virtually eliminates the 5´→3´ nuclease activity of AmpliTaq DNA Polymerase. The enzyme has been formulated with a thermally stable inorganic pyrophosphatase to eliminate problems associated with pyrophosphorolysis.

Cycle sequencing protocols that rely on the use of AmpliTaq DNA Polymerase, FS offer the following advantages over traditional sequencing methods:

- Less hands-on operation
- No alkaline denaturation step required for double-stranded DNA
- Same protocol for both single- and double-stranded templates
- Less starting template needed
- More reproducible results

BigDye Terminators

Applied Biosystems has developed a set of dye terminators labeled with novel, high-sensitivity dyes. The dye structures contain a fluorescein donor dye, *e.g.*, 6-carboxyfluorescein (6-FAM), linked to a dichlororhodamine (dRhodamine) acceptor dye. The excitation maximum of each dye label is that of the fluorescein donor, and the emission spectrum is that of the dRhodamine acceptor. See “Dye Spectra” on page 1-6.

The donor dye is optimized to absorb the excitation energy of the argon ion laser in the Applied Biosystems DNA sequencing instruments. The linker affords extremely efficient energy transfer (quantum efficiency nearly 1.0, *i.e.*, 100%) between the donor and acceptor dyes. The BigDye™ terminators are 2–3 times brighter than the rhodamine dye terminators when incorporated into cycle sequencing products.

The BigDye terminators are labeled with the following dRhodamine acceptor dyes:

Terminator	Acceptor Dye	Color of Raw Data on ABI PRISM 3700 or 310 Electropherograms	Color of Raw Data on ABI PRISM 377 or 373 Gel Image
A	dR6G	Green	Green
C	dROX	Red	Red
G	dR110	Blue	Blue
T	dTAMRA	Black	Yellow

Comparing Peak Height Patterns

Data generated with dRhodamine dye terminators or BigDye terminators gives more even peak-height patterns than data generated with rhodamine dye terminators. In particular, the weak G after A pattern characteristic of the rhodamine dye terminators is greatly reduced (Figure 1-1 through Figure 1-4 on page 1-5).

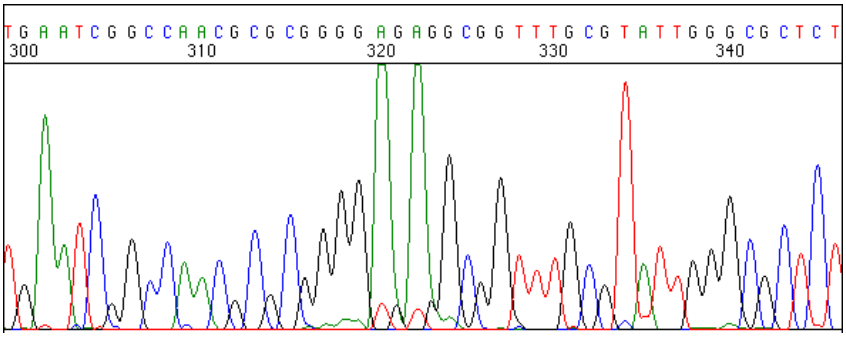


Figure 1-1 Region of pGEM®-3Zf(+) sequenced with rhodamine dye terminators

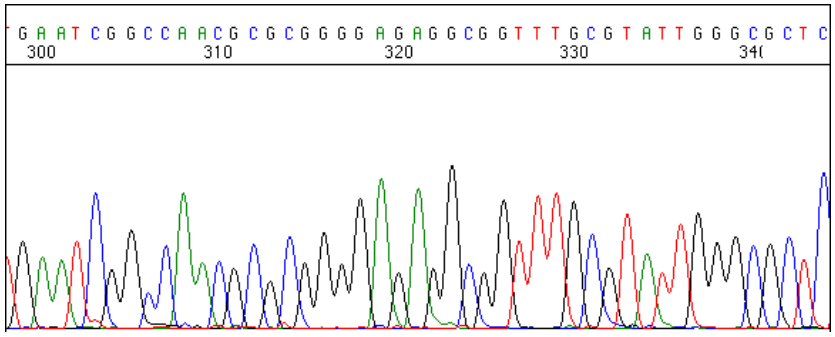


Figure 1-2 Region of pGEM-3Zf(+) sequenced with dRhodamine terminators

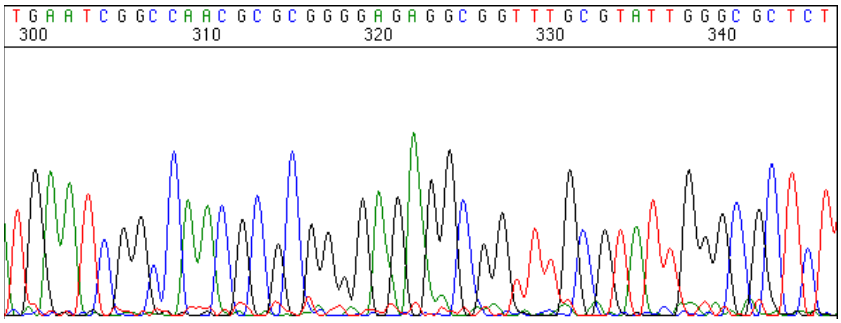


Figure 1-3 Region of pGEM-3Zf(+) sequenced with BigDye terminators

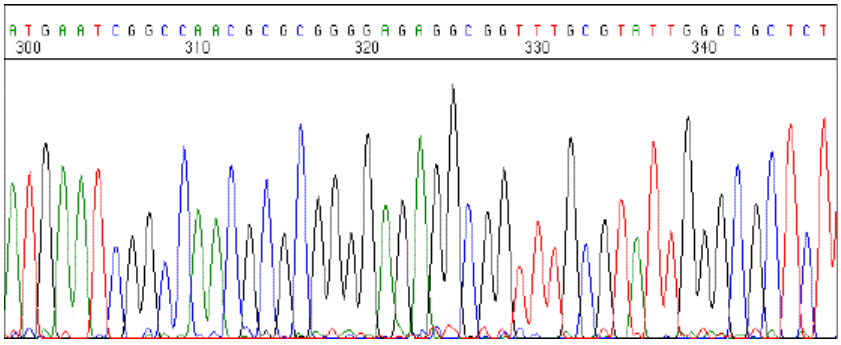
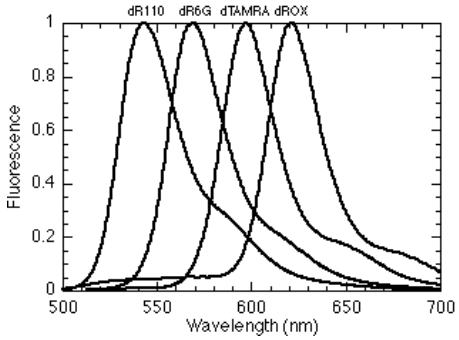


Figure 1-4 Region of pGEM-3Zf(+) sequenced with BigDye terminators v2.0

Dye Spectra

The normalized emission spectra of the dRhodamine dyes in the BigDye terminators are shown below.



Instrument Platforms and Required Software

Instrument Platforms

The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits are for use with the following instruments:

- ABI PRISM 3700 DNA Analyzer
- ABI PRISM 310 Genetic Analyzer
- ABI PRISM 377 DNA Sequencers
  - ABI PRISM 377
  - ABI PRISM 377-18
  - ABI PRISM 377 with XL Upgrade
  - ABI PRISM 377 with 96-Lane Upgrade
- ABI PRISM 373 DNA Sequencers with BigDye Filter Wheel
  - ABI PRISM 373
  - ABI PRISM 373 with XL Upgrade

  These kits are designed for use with ABI PRISM 373 DNA Sequencers and ABI PRISM 373 DNA Sequencers with XL Upgrade on which the ABI PRISM BigDye Filter Wheel has been installed. Refer to the *ABI PRISM BigDye Filter Wheel User Bulletin* (P/N 4304367) for more information.

Thermal Cyclers

This protocol has been optimized for all Applied Biosystems thermal cyclers, including:

- GeneAmp PCR Systems 9700, 9600, and 2400
- ABI PRISM 877 Integrated Thermal Cycler
- CATALYST 800 Molecular Biology LabStation
- DNA Thermal Cycler 480
- DNA Thermal Cycler (TC1)

If you use a thermal cycler not manufactured by Applied Biosystems, you may need to optimize thermal cycling conditions. Ramping time is very important. If the thermal ramping time is too fast (>1°/sec), poor (noisy) data may result.

Run Modules and Dye Set/Primer (Mobility) Files

You must use Filter Set E run modules and dye set/primer (mobility) files on *all* instrument platforms *except* the ABI PRISM 373 DNA Sequencer. Use Filter Set A on ABI PRISM 373 DNA Sequencers with the ABI PRISM BigDye Filter Wheel.

IMPORTANT Users of the ABI PRISM 3700 DNA Analyzer refer to the *ABI PRISM 3700 DNA Analyzer User's Manual* (P/N 4306152) for information on run modules and dye set/primer (mobility) files.

- Run modules and dye set/primer (mobility) files are included in the current versions of data collection software.
- The run modules and dye set/primer (mobility) files can be downloaded from the Internet:

  www.appliedbiosystems.com/techsupport
- If you do not have access to the Internet, you can get the files from Applied Biosystems Technical Support, or from your local field applications specialist (call your local sales office for more information).

Run Modules

- Run modules are the same as for the dRhodamine terminators and BigDye primers.

Dye Set/Primer (Mobility) Files

- You must install new dye set/primer (mobility) files for the BigDye terminators (original and version 2 kits).
- Dye set/primer file names for the dRhodamine terminators are similar to those for the BigDye terminators. Their respective mobility files can be mistaken for each other easily.
- If a mobility file for the wrong sequencing chemistry is used, some bases will be miscalled.

  This is because in the dRhodamine chemistry C is labeled with dTAMRA and T is labeled with dROX, whereas in the BigDye terminator chemistry C is labeled with dROX BigDye and T is labeled with dTAMRA BigDye.

  In addition there are differences in the mobility shifts of dRhodamine terminators and BigDye Terminators.

Instrument (Matrix) File Required

Data analysis requires a Filter Set E instrument (matrix) file:

IMPORTANT Users of the ABI PRISM 3700 DNA Analyzer refer to the *ABI PRISM 3700 DNA Analyzer User's Manual* (P/N 4306152) for information on instrument (matrix) files.

For the ABI PRISM 310, 377, and 373 with BigDye Filter Wheel:

- Instrument (matrix) files are the same for dRhodamine terminator chemistry and Big Dye terminator chemistries (original and version 2).
- Instrument (matrix) files are made using the ABI PRISM dRhodamine matrix standards (P/N 403047). Refer to the *Automated DNA Sequencing Chemistry Guide* (P/N 4305080; www.appliedbiosystems.com/techsupport) for information on creating instrument files.

Reagents and Storage

Available Kits

The following kits are available:

Kit	Number of Reactions	Part Number
ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit	100	4303149
	1000	4303150
	5000	4303151
ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0	100	4314414
	1000	4314415
	5000	4314416
	25,000	4314849

Description of Reagents

A description of the kit components is listed below.

- Terminator Ready Reaction Mix:
  - A-Dye Terminator labeled with dichloro[R6G]
  - C-Dye Terminator labeled with dichloro[ROX]
  - G-Dye Terminator labeled with dichloro[R110]
  - T-Dye Terminator labeled with dichloro[TAMRA]
  - Deoxynucleoside triphosphates (dATP, dCTP, dITP, dUTP)
  - AmpliTaq DNA Polymerase, FS
  - $MgCl_2$
  - Tris-HCl buffer, pH 9.0
- pGEM®-3Zf(+) double-stranded DNA Control Template, 0.2 µg/µL
- –21 M13 Control Primer (forward), 0.8 pmol/µL

Storage and Use of the Kits

Store the kits at –15 to –25 °C. Before each use of either kit, allow the frozen stocks to thaw at room temperature (do not heat). Whenever possible, thawed materials should be kept on ice during use.

IMPORTANT Mix each stock thoroughly and then centrifuge briefly to collect all the liquid at the bottom of each tube.

Materials Supplied by the User

Overview

IMPORTANT This section describes materials that are required for sample preparation. Refer to the instrument's user manual for materials that are required for the operation of the instrument.

Materials for Cycle Sequencing

ABI PRISM 3700 DNA Analyzer	
Material	**Supplier**
GeneAmp PCR Systems 9700 or 9600	Applied Biosystems
Thermal cycling tubes, see page 1-14	Applied Biosystems
ABI PRISM 310 Genetic Analyzer	
Thermal Cycler, see page 1-7.	Applied Biosystems
Thermal cycling tubes, see page 1-14	Applied Biosystems
ABI PRISM 377 or 373 with BigDye Filter Wheel	
Thermal Cycler, see 1-7.	Applied Biosystems
Thermal cycling tubes, see page 1-14	Applied Biosystems

Materials for Purifying Extension Products

ABI PRISM 3700 DNA Analyzer	
Material	**Supplier**
Choose one of the following:	
♦ 384-Well Plate Columns for Purification	See page 4-2.
♦ 96-Well Plate Columns for Purification	See page 4-2.
♦ Ethanol (EtOH), non-denatured, 95%	MLS
♦ Isopropanol, 100% anhydrous	MLS
Aluminum foil tape, adhesive-backed	3M (Scotch Tape P/N 439)[a]
ABI PRISM 310 Genetic Analyzer	
Choose one of the following:	
♦ Spin column, Centri-Sep™, 1-mL 32 columns, 100 columns	Applied Biosystems P/N 401763, P/N 401762
♦ Ethanol (EtOH), non-denatured, 95%	MLS
♦ Isopropanol, 100% anhydrous	MLS
♦ 75% Isopropanol	MLS
♦ Ethanol, non-denatured, and 95% Sodium acetate (NaOAc), 3 M, pH 4.6	MLS, and Applied Biosystems (P/N 400320)
Aluminum foil tape, adhesive-backed	3M (Scotch Tape P/N 439)[a]
ABI PRISM 377 or 373 with BigDye Filter Wheel	
Choose one of the following:	
♦ 96-Well Plate Columns for Purification	See page 4-2.
♦ Spin column, Centri-Sep™, 1-mL 32 columns, 100 columns	Applied Biosystems P/N 401763, P/N 401762
♦ Ethanol (EtOH), non-denatured, 95%	MLS
♦ Isopropanol, 100% anhydrous	MLS
♦ 75% Isopropanol	MLS
♦ Ethanol, non-denatured, and 95% Sodium acetate (NaOAc), 3 M, pH 4.6	MLS, and Applied Biosystems (P/N 400320)
Aluminum foil tape, adhesive-backed	3M (Scotch Tape P/N 439)[a]

a. Contact 3M in the USA at (800) 364-3577 for your local 3M representative. Use of other tapes may result in leakage or contamination of the sample.

Materials for Electrophoresis

ABI PRISM 3700 DNA Analyzer	
Material	**Supplier**
Choose one of the following: ♦ Deionized water ♦ 2-Pyrrolidinone ♦ Hi-Di™ Formamide, 25-mL bottle	 MLS MLS Applied Biosystems (P/N 4311320)
Matrix Standard Set DS-01, dROX, dTAMRA, dR6G, dR110	Applied Biosystems (P/N 4305609)
ABI PRISM 310 Genetic Analyzer	
Formamide	MLS
EDTA	MLS
ABI PRISM dRhodamine Matrix Standards Kit	Applied Biosystems (P/N 403047)
ABI PRISM 377 or 373 with BigDye Filter Wheel	
Formamide	MLS
EDTA	MLS
25 mM EDTA with 50 mg/mL blue dextran	Applied Biosystems (P/N 402055)
ABI PRISM dRhodamine Matrix Standards Kit	Applied Biosystems (P/N 403047)

Thermal Cycling Tubes Required

The table below shows several thermal cyclers, along with the appropriate plates and tubes for each.

Thermal Cycler	Plate or Tube	Applied Biosystems Part Number
GeneAmp PCR System 9700	MicroAmp 384-Well Reaction Plate	4305505
	MicroAmp 96-Well Reaction Plate	N801-0560
	MicroAmp Reaction Tubes, 0.2-µL	N801-0533
GeneAmp PCR System 9600	MicroAmp 96-Well Reaction Plate	N801-0560
	MicroAmp Reaction Tubes, 0.2-µL	N801-0533
	MicroAmp Caps, 12 or 8/strip	N801-0534 N801-0535
GeneAmp PCR System 2400	MicroAmp Reaction Tubes, 0.2-µL	N801-0533
	MicroAmp Caps, 12 or 8/strip	N801-0534 N801-0535
DNA Thermal Cycler 480[a]	GeneAmp Thin-Walled Reaction Tubes, 0.5-mL	N801-0537
	GeneAmp Thin-Walled Reaction Tubes with Flat Cap	N801-0737
DNA Thermal Cycler (TC1)[a]	GeneAmp Thin-Walled Reaction Tubes, 0.5-mL	N801-0537

a. These thermal cyclers require mineral oil that can be obtained from Applied Biosystems (P/N 0186-2302)

Safety

Documentation User Attention Words

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

Note This word is used to call attention to information.

IMPORTANT This word calls attention to information that is necessary for correct use of the kit or instrument.

CAUTION This word informs the user that damage to the instrument could occur if the user does not comply with the information. It also indicates a potentially hazardous situation that could result in minor or moderate injury to the user.

! WARNING ! This word informs the user that serious physical injury or illness to the user or other persons could occur if these required precautions are not taken.

! DANGER ! Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury.

Ordering MSDSs

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

To order MSDSs...	Then...
Over the Internet	Use www.appliedbiosystems.com/techsupport a. Select **MSDS Search** button b. Enter keywords (or partial words), or a part number, or the MSDSs' Documents on Demand index number c. Select **Search** d. Select the Adobe® Acrobat symbol to view, print, or download the document, or check the box of the desired document and delivery method (fax or e-mail)
By automated telephone service from any country	Use "Documents on Demand" on page B-4.
By telephone in the United States	Dial 1-800-327-3002, then press **1**

<table>
<tr><th>To order MSDSs...</th><th>Then...</th></tr>
<tr><td>By telephone from Canada</td><td>
<table>
<tr><th>If you want ordering instructions in...</th><th>Then dial 1-800-668-6913 and...</th></tr>
<tr><td>English</td><td>Press 1, then 2, then 1 again</td></tr>
<tr><td>French</td><td>Press 2, then 2, then 1</td></tr>
</table>
</td></tr>
<tr><td>By telephone from any other country</td><td>See the back cover of this protocol booklet.</td></tr>
</table>

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

Chemical Hazard Warning

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

- **Read and understand the material safety data sheets (MSDSs) provided by the chemical's manufacturer before you store, handle, or work with any chemicals or hazardous materials.**
- **Minimize contact with and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.*, safety glasses, gloves, or clothing). Consult the listing in the MSDS.**
- **Do not leave chemical containers open. Use only with adequate ventilation.**
- **Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.**
- **Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.**

Preparing the Templates 2

Chapter Summary

In This Chapter The following topics are covered in this chapter:

Control DNA Templates

Using Control DNA

Include a control DNA template as one of the templates in a set of sequencing reactions. The results from the control can help determine whether failed reactions are the result of poor template quality or sequencing reaction failure.

Control DNA Sequence

We recommend M13mp18 as a single-stranded control and pGEM®-3Zf(+) as a double-stranded control. All Applied Biosystems DNA sequencing kits provide pGEM control DNA. All dye terminator cycle sequencing kits include a –21 M13 control primer.

The partial sequence of pGEM-3Zf(+) from the –21 M13 forward primer, followed by the ensuing 1000 bases is shown in Appendix A, “Control DNA Sequence.”

An Additional Control

The BigDye Terminator Cycle Sequencing Standard (P/N 4304154) provides an additional control to help in troubleshooting electrophoresis runs. This standard contains lyophilized sequencing reactions that require only resuspension and denaturation before use.

Template Preparation Methods

Single- and Double-Stranded Templates

Refer to the *Automated DNA Sequencing Chemistry Guide* (P/N 4305080, www.appliedbiosystems.com/techsupport) for information on preparing single- and double-stranded templates.

BAC DNA Templates

With larger DNA targets such as bacterial artificial chromosomes (BACs), the quality of DNA template is important to the success of the sequencing reaction. Two methods have given good sequencing results:

- Alkaline lysis[1]
- Cesium chloride (CsCl) banding

Internet Addresses for BAC DNA Protocols

For other BAC DNA preparation protocols, refer to the following Internet addresses:

- Centre National de Séquençage (CNS, or Génoscope): http://www.cns.fr/externe/arabidopsis/protoBAC.html
- University of Oklahoma Advanced Center for Genome Technology: http://www.genome.ou.edu/DblAcetateProcV3.html
- Washington Univ School of Medicine Genome Sequencing Center: http://genome.wustl.edu/gsc/Protocols/BAC.shtml

Commercial Kits

Commercial kits are also available for BAC DNA preparation:

- QIAGEN-tip 100 (QIAGEN: P/N 10043, 25 reactions; 10045, 100 reactions)
- QIAGEN-tip 500 (QIAGEN: P/N 10063, 25 reactions; 10065, 100 reactions)

1. Marra, M., Weinstock, L.A., and Mardis, E.R. 1996. End sequence determination from large insert cloning using energy transfer fluorescent primers. *Genomic Methods* 6: 1118–1122.

PCR Templates

Cycle sequencing provides the most reproducible results for sequencing PCR templates. Although PCR fragments can be difficult to denature with traditional sequencing methods, cycle sequencing provides several chances to denature and extend the template, which ensures adequate signal in the sequencing reaction.

Importance of Purifying Product

For optimum results, purify the PCR product before sequencing. In general, any method that removes dNTPs and primers should work. We recommend Centricon-100 columns (P/N N930-2119). The protocol for using these columns is provided in "Purifying PCR Fragments."

Purifying PCR Fragments

To purify PCR fragments by ultrafiltration:

Step	Action
1	Assemble the Centricon-100 column according to the manufacturer's recommendations.
2	Load 2 mL deionized water onto the column.
3	Add the entire sample to the column.
4	Spin the column at 3000 × *g* in a fixed-angle centrifuge for 10 minutes. **Note** The manufacturer recommends a maximum speed of 1000 × *g*, but 3000 × *g* has worked well in Applied Biosystems laboratories. If you are following the manufacturer's guidelines, increase the time to compensate.
5	Remove the waste receptacle and attach the collection vial.
6	Invert the column and spin it at 270 × *g* for 2 minutes to collect the sample. This should yield approximately 40–60 µL of sample.
7	Add deionized water to bring the purified PCR fragments to the original volume.

Use of the Primer Island Transposition Kit

Overview

BigDye terminators are also suitable for sequencing plasmid templates generated using the Primer Island Transposition Kit (P/N 402984). This kit uses transposons to insert primer binding sites into cloned DNA.

About Transposons

Transposons are mobile genetic elements, regions of DNA capable of inserting themselves (or copies of themselves) into the genome. Transposons encode the proteins that facilitate their insertion into the target DNA.

Inserting Artificial Transposons

This property of transposons can be exploited to place unique primer binding sites randomly throughout any large segment of DNA. These primer sites may be used subsequently as templates for PCR and/or sequencing reactions. Transposon insertion is an alternative to subcloning or primer walking when sequencing a large cloned DNA region.[2,3]

The Primer Island Transposition Kit provides reagents for generating artificial transposon insertions into target DNA *in vitro*. The artificial transposon contains the PI(+) and PI(–) priming sites. The Primer Island reagents are combined with a target DNA of choice and used to transform *Escherichia coli*.

Technique

To identify the *E. coli* carrying the transposon, the transformed bacteria are plated on Luria-Bertani (LB) agar plates containing carbenicillin and trimethoprim antibiotics. Each carbenicillin- and trimethoprim-resistant colony has integrated a copy of the transposon into the target DNA.

Follow the *Primer Island Transposition Kit Protocol* (P/N 402920) for transposon insertion and template preparation.

2. Devine, S.E., and Boeke, J.D. 1994. Efficient integration of artificial transposons into plasmid targets *in vitro*: a useful tool for DNA mapping, sequencing, and functional analysis. *Nucleic Acids Res.* 22: 3765–3772.

3. Devine, S.E., Chissoe, S.L., Eby, Y., Wilson, R.K., and Boeke, J.D. 1997. A transposon-based strategy for sequencing repetitive DNA in eukaryotic genomes. *Genome Res.* 7: 551–563.

Template and Primer Quantities

Overview

If possible, quantitate the amount of purified DNA by measuring the absorbance at 260 nm or by some other method.

Template Quantity

The table below shows the amount of template to use in a cycle sequencing reaction.

Template	Quantity
PCR product:	
100–200 bp	1–3 ng
200–500 bp	3–10 ng
500–1000 bp	5–20 ng
1000–2000 bp	10–40 ng
>2000 bp	40–100 ng
Single-stranded	50–100 ng
Double-stranded	200–500 ng
Cosmid, BAC	0.5–1.0 µg
Bacterial genomic DNA	2–3 µg

Note In general, higher DNA quantities give higher signal intensities.

Note The template quantities stated above should work with all primers. you may be able to use even less DNA, especially when sequencing with the –21 M13 primer. The amount of PCR product to use in sequencing will also depend on the length and purity of the PCR product.

Template Volume

Cycle-sequencing reactions are made up in a final volume of 20 µL. The volume includes 8 µL for DNA template and 4 µL for primer. If your DNA is not concentrated enough and you need to add more than 8 µL of DNA template, then you can compensate for the additional volume by using a more concentrated solution of primer.

For example, if your concentration of primers is increased from 0.8 pmol/µL to 3.2 pmol/µL, then the volume of primers can be reduced from 4 µL to 1 µL. Because less volume is used for the primers, more volume can then be added for the template. In this example, the volume of DNA template could be increased from 8 µL to 11 µL.

Performing Cycle Sequencing 3

Chapter Summary

In This Chapter The following topics are covered in this chapter:

Sequencing Plasmids and PCR Products

Overview

This section describes how to prepare reactions and perform cycle sequencing on plasmids and PCR Products.

Sequencing Plasmids on the 3700

IMPORTANT If you are sequencing plasmids and PCR products on the ABI PRISM 3700 DNA Analyzer, refer to the *ABI PRISM 3700 DNA Analyzer Sequencing Chemistry Guide* (P/N 4309125) for information about reaction set up and cycle sequencing.

Instruments

The following thermal cyclers can be used with this protocol:

- GeneAmp PCR Systems 9700, 9600, and 2400
- ABI PRISM 877 Integrated Thermal Cycler
- CATALYST 800 Molecular Biology LabStation
- DNA Thermal Cycler 480
- DNA Thermal Cycler (TC1)

Preparing the Reactions

The type of tube required depends on the thermal cycler that you are using. Refer to “Thermal Cycling Tubes Required” on page 1-14.

To prepare the reaction mixtures:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>For each reaction add the following reagents to a separate tube:
<table>
<tr><th>Reagent</th><th>Quantity</th></tr>
<tr><td>Terminator Ready Reaction Mix</td><td>8.0 µL</td></tr>
<tr><td>Template
Single-stranded DNA
Double-stranded DNA
PCR product DNA</td><td>
50–100 ng
200–500 ng
See table in “Template Quantity” on page 2-6.</td></tr>
<tr><td>Primer</td><td>3.2 pmol</td></tr>
<tr><td>Deionized water</td><td>q.s.</td></tr>
<tr><td>Total Volume</td><td>20 µL</td></tr>
</table></td></tr>
<tr><td>2</td><td>Mix well and spin briefly.</td></tr>
</table>

To prepare the reaction mixtures: *(continued)*

Step	Action
3	If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480, overlay reaction mixture with 40 µL of light mineral oil.

Cycle Sequencing on the GeneAmp 9700, 9600, or 2400

To sequence DNA on the GeneAmp PCR System 9700, 9600, or 2400:

Step	Action
1	Place the tubes in a thermal cycler, and set the volume to 20 µL.
2	Repeat the following for 25 cycles: ♦ Rapid thermal ramp[a] to 96 °C ♦ 96 °C for 10 seconds. ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 5 seconds. ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 4 minutes.
3	Rapid thermal ramp to 4 °C and hold until ready to purify.
4	Spin down the contents of the tubes in a microcentrifuge.
5	Proceed to Chapter 4, “Purifying Extension Products.”

a. Rapid thermal ramp is 1 °C/sec.

Cycle Sequencing on the TC1 or DNA Thermal Cycler 480

To sequence DNA on the TC1 or DNA Thermal Cycler 480:

Step	Action
1	Place the tubes in a thermal cycler, and set the volume to 20 µL.
2	Repeat the following for 25 cycles: ♦ Rapid thermal ramp[a] to 96 °C ♦ 96 °C for 30 seconds. ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 15 seconds. ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 4 minutes.
3	Rapid thermal ramp to 4 °C and hold until ready to purify.
4	Spin down the contents of the tubes in a microcentrifuge.

To sequence DNA on the TC1 or DNA Thermal Cycler 480:

Step	Action
5	Proceed to Chapter 4, “Purifying Extension Products.”

a. Rapid thermal ramp is 1 °C/sec.

Sequencing BAC DNA

Thermal Cyclers

The following thermal cyclers can be used with this protocol:

- GeneAmp PCR Systems 9600 or 9700 (in 9600 emulation mode)
- ABI PRISM 877 Integrated Thermal Cycler
- CATALYST 800 Molecular Biology LabStation

This protocol needs to be reoptimized for use on other thermal cyclers.

Sequencing BAC DNA on the 3700

IMPORTANT If you are sequencing BAC DNA on the ABI PRISM 3700 DNA Analyzer, refer to the *ABI PRISM 3700 DNA Analyzer Sequencing Chemistry Guide* (P/N 4309125) for information about reaction set up and cycle sequencing.

Preparing Sequencing Reactions

The type of tube required depends on the thermal cycler that you are using. Refer to “Thermal Cycling Tubes Required” on page 1-14.

To prepare the sequencing reaction:

Step	Action
1	For each reaction, add the following reagents to a separate tube:

Reagent	Quantity
Terminator Ready Reaction Mix	16 µL
DNA Template	0.5–1.0 µg
Primer	5–10 pmol
Deionized water	*q.s.*
Total Volume	40 µL

Step	Action
2	Mix well and spin briefly.

Performing Cycle Sequencing

To perform cycle sequencing on BAC DNA:

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 30 µL.
2	Heat the tubes at 95 °C for 5 minutes.
3	Repeat the following for 30 cycles:[a] ♦ Rapid thermal ramp[b] to 95 °C ♦ 95 °C for 30 seconds. ♦ Rapid thermal ramp to 50–55 °C (depending on template) ♦ 50–55 °C for 10 seconds. ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 4 minutes.
4	Rapid thermal ramp to 4 °C and hold until ready to purify.
5	Spin down the contents of the tubes in a microcentrifuge.
6	Proceed to Chapter 4, “Purifying Extension Products.”

a. Some laboratories have found that increasing the number of cycles gives better results.

b. Rapid thermal ramp is 1 °C/sec.

Sequencing Bacterial Genomic DNA

Thermal Cyclers

The following thermal cyclers can be used with this protocol.This protocol needs to be reoptimized for use on other thermal cyclers.

- GeneAmp PCR Systems 9600 or 9700 (in 9600 emulation mode)
- ABI PRISM 877 Integrated Thermal Cycler
- CATALYST 800 Molecular Biology LabStation

Sequencing Bacterial Genomic DNA on the 3700

IMPORTANT If you are sequencing bacterial genomic DNA on the ABI PRISM 3700 DNA Analyzer, refer to the *ABI PRISM 3700 DNA Analyzer Sequencing Chemistry Guide* (P/N 4309125) for information about reaction set up and cycle sequencing.

Preparing Sequencing Reactions

The type of tube required depends on the thermal cycler that you are using. Refer to “Thermal Cycling Tubes Required” on page 1-14.

To prepare the sequencing reactions for bacterial genomic DNA:

Step	Action
1	For each reaction, add the following reagents to a separate tube: **Reagent** / **Quantity** Terminator Ready Reaction Mix / 16 µL DNA Template[a] / 2–3 µg Primer / 6–13 pmol Deionized water / *q.s.* Total Volume / 40 µL a. Shearing the DNA by passing it seven times through a 21-gauge, 1-inch long needle can improve signals.
2	Mix well and spin briefly.

Cycle Sequencing

To perform cycle sequencing:

Step	Action
1	Place the tubes in a thermal cycler, and set the volume to 40 µL.
2	Heat the tubes at 95 °C for 5 minutes.
3	Repeat the following for 45 cycles: ♦ Rapid thermal ramp[a] to 95 °C ♦ 95 °C for 30 seconds. ♦ Rapid thermal ramp to 50–55 °C (depending on template) ♦ 55 °C for 20 seconds. ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 4 minutes.
4	Rapid thermal ramp to 4 °C and hold until ready to purify.
5	Spin down the contents of the tubes in a microcentrifuge.
6	Proceed to Chapter 4, “Purifying Extension Products.”

a. Rapid thermal ramp is 1 °C/sec.

Sequencing on the CATALYST 800

Overview

Templates that have been prepared as described in chapter 2 should be suitable for use on the CATALYST 800 Molecular Biology LabStation. Follow the protocols in the Turbo Appendix of the *CATALYST 800 Molecular Biology LabStation User's Manual* (P/N 903939) to set up your reactions.

Options for Sequencing

Terminator sequencing has two options:

- A reaction premix containing the sequencing primer or premixing template with primer in the sample tube
- A reaction cocktail (lacking primers), water, and primer from one tube combined with template from another tube

Manual Ethanol Precipitation Required

Ethanol precipitation is not available for Terminator Sequencing protocols on the CATALYST 800 Molecular Biology LabStation. Ethanol precipitation or spin-column purification must be performed manually. See Chapter 4, "Purifying Extension Products."

Sequencing on the ABI PRISM 877 ITC

Predefined Temperature Profiles

Predefined temperature profiles are provided for the following on the ABI PRISM 877 Integrated Thermal Cycler:

- Terminator Sequencing uses a reaction premix containing the sequencing primer, or requires premixing template with primer in the sample tube.
- Terminator Automix Sequencing combines reaction cocktail (lacking primers), water, primer from one tube, and template from another tube.

The profile is chosen on the Chemistry page of the Sequencing Notebook and can be edited to make custom profiles. Refer to Chapter 4, “Using the ABI PRISM 877 Software,” in the ABI PRISM 877 *Integrated Thermal Cycler User’s Manual* (P/N 904414).

Ethanol Precipitation

Ethanol precipitation can be chosen for dye terminator sequencing. The proportions of ethanol and precipitation additive are set for default reaction volumes. These volumes can be changed, especially if the reaction volume is modified. After the program is completed, proceed to Chapter 4, “Purifying Extension Products.”

On extended runs (*e.g.*, overnight), we recommend withholding addition of ethanol until plate processing can be completed. This delay can be programmed on the Chemistry page of the Sequencing Notebook.

Purifying Extension Products

4

Chapter Summary

In This Chapter The following topics are covered in this chapter:

Choosing a Method of Purification

Purpose

Unincorporated dye terminators must be completely removed before the samples can be analyzed by electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with base calling.

Spin Column vs. Precipitation

Use the method that works best for your particular application.

- Precipitation methods are cheaper and faster, but they remove less of the unincorporated dye-labeled terminators that can obscure data at the beginning of the sequence.
- The plate column and spin column procedures remove more terminators, but are more costly and take time to perform.

Plate and Spin Column Purification

Overview

This section describes the recommended plate and spin columns for purifying extension products.

Recommended 384-Well Plate Columns

For large-scale procedures, you can use the following commercially available 384-well reaction plate:

- ArrayIt (Telechem, P/N DTC-384-100)
- 384 System I (Edge Biosystems, P/N 95674)

Refer to the manufacturer's instructions for procedures.

Recommended 96-Well Plate Columns

For large-scale procedures, you can use the following commercially available 96-well purification plates:

- 96-Well Spin Columns, Gel Filtration Kit (Edge Biosystems, P/N 94880)
- ArrayIt (Telechem, P/N DTC-96-100)
- Centri-Sep™ 96 plate (Princeton Separations, P/N CS-961)
- Multiscreen 96-Well Filter Plates (Millipore, P/N MADYEKIT1)

- Quantum Prep SEQueaky Kleen 96-well Terminator Removal Kit (Bio-Rad 732-6260)

Refer to the manufacturer's instructions procedures.

Recommended Spin Columns

We recommend Centri-Sep™ spin columns (Princeton Separations P/N CS-901).

Optimizing Spin Column Purification

IMPORTANT For the BigDye terminators, hydrate the column for 2 hours.

Tips for optimizing spin column purification:

- Use one column for each sample.
- Do not process more columns than you can handle conveniently at one time.
- Load the sample in the center of the column bed. Make sure that the sample does not touch the sides of the column and that the pipet tip does not touch the gel surface.

  If samples are not properly loaded, peaks from unincorporated dye terminators can result.
- Spin the column at 325–730 $\times$ *g* for best results. Use the following formula to calculate the best speed for your centrifuge:

  $g = 11.18 \times r \times (\text{rpm}/1000)^2$

  where:

  g = relative centrifugal force

  r = radius of the rotor in cm

  rpm = revolutions per minute
- Do not spin for more than 2 minutes.
- Perform the entire procedure without interruption to ensure optimal results. Do not allow the column to dry out.

Performing Spin Column Purification

To perform spin column purification:

Step	Action
1	Gently tap the column to cause the gel material to settle to the bottom of the column.
2	Remove the upper end cap and add 0.8 mL of deionized water.
3	Replace the upper end cap and vortex or invert the column a few times to mix the water and gel material.
4	Allow the gel to hydrate at room temperature for at least 2 hours. **Note** Hydrated columns can be stored for a few days at 2–6 °C. Longer storage in water is not recommended. Allow columns stored at 2–6 °C to warm to room temperature before use.
5	Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.
6	Remove the upper end cap first, then remove the bottom cap. Allow the column to drain completely by gravity. **Note** If flow does not begin immediately, apply gentle pressure to the column with a pipette bulb.
7	Insert the column into the wash tube provided.
8	Spin the column in a microcentrifuge at 730 × *g* for 2 minutes to remove the interstitial fluid.
9	Remove the column from the wash tube, and insert it into a sample collection tube (*e.g.*, a 1.5-mL microcentrifuge tube).
10	Remove the extension reaction mixture from its tube, and load it carefully onto the center of the gel material. **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 1 on page 4-7.
11	Spin the column in a microcentrifuge at 730 × *g* for 2 minutes. **Note** If using a centrifuge with a fixed-angle rotor, place the column in the same orientation as it was in for the first spin. This is important because the surface of the gel will be at an angle in the column after the first spin.
12	Discard the column. The sample is in the sample collection tube.
13	Dry the sample in a vacuum centrifuge for 10–15 minutes, or until dry. Do not overdry.

Isopropanol Precipitation

Precipitating in 384-Well Plates

IMPORTANT If you are precipitating in 384-well plates, refer to the *ABI PRISM 3700 DNA Analyzer Sequencing Chemistry Guide* (P/N 4309125) for the procedure.

Precipitating in 96-Well Plates

Note This procedure does not use salt.

To precipitate in 96-Well MicroAmp Reaction Plates:

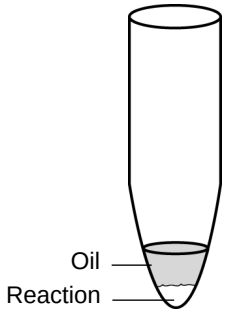
Step	Action
1	Remove the MicroAmp Tray from the thermal cycler. Remove the caps from each tube.
2	Add **one** of the following: ♦ 80 µL of 75% isopropanol ♦ 20 µL of deionized water and 60 µL of 100% isopropanol The final isopropanol concentration should be 60 ± 5%. **! WARNING ! CHEMICAL HAZARD. Isopropyl alcohol can be harmful if inhaled, ingested, or absorbed through the skin. It can cause CNS depression, and be irritating to the eyes, skin, and mucous membranes. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.**
3	Seal the tubes with strip caps or by applying a piece of 3M Scotch Tape 439 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
4	Invert the tray a few times to mix.
5	Leave the tray at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times shorter than 15 minutes will result in the loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.

To precipitate in 96-Well MicroAmp Reaction Plates: *(continued)*

Step	Action
6	Place the tray in a table-top centrifuge with tube-tray adaptor and spin it at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: ♦ 1400–2000 × *g*: 45 minutes ♦ 2000–3000 × *g*: 30 minutes **Note** A MicroAmp tube in a MicroAmp Tray can withstand 3000 × *g* for 30 minutes. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
7	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the tray onto a paper towel folded to the size of the tray.
8	If you are performing this procedure for electrophoresis on the 3700 DNA Analyzer: a. Rinse the pellet by adding 150 µL of 70% isopropanol to each well. b. Seal the plate with adhesive tape. c. Invert the plate a few times.
9	Place the inverted tray with the towel into the table-top centrifuge and spin at 700 × *g* for 1 minute.
10	Remove the tray and discard the paper towel. **Note** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.

Precipitating in Microcentrifuge Tubes

To precipitate in microcentrifuge tubes:

Step	Action
1	Pipet the entire contents of each extension reaction into a 1.5-mL microcentrifuge tube. **To remove reactions run on the TC1 or DNA Thermal Cycler 480:** Place the pipette tip into the bottom of the reaction and carefully remove the reaction from the oil.  **IMPORTANT** Transfer as little oil as possible.
2	Add **one** of the following: ♦ 80 µL of 75% isopropanol ♦ 20 µL of deionized water and 60 µL of 100% isopropanol The final isopropanol concentration should be 60 ± 5%. **! WARNING ! CHEMICAL HAZARD. Isopropyl alcohol can be harmful if inhaled, ingested, or absorbed through the skin. It can cause CNS depression, and be irritating to the eyes, skin, and mucous membranes. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.**
3	Close the tubes and vortex briefly.
4	Leave the tubes at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times shorter than 15 minutes will result in the loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.
5	Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 minutes at maximum speed. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.

To precipitate in microcentrifuge tubes: *(continued)*

Step	Action
6	Carefully aspirate the supernatants with a separate pipette tip for each sample and discard. Pellets may or may not be visible. **IMPORTANT** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.
7	Add 250 µL of 75% isopropanol to the tubes, and vortex them briefly.
8	Place the tubes in the microcentrifuge in the same orientation as in step 5, and spin for 5 minutes at maximum speed.
9	Aspirate the supernatants carefully, as in step 6.
10	Dry the samples in a vacuum centrifuge for 10–15 minutes or to dryness. (Alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90 °C for 1 minute.)

Ethanol Precipitation

Unincorporated Terminators

With ethanol precipitation, traces of unincorporated terminators may be seen at the beginning of the sequence data (up to base 40), but this is usually minimal. Some loss in the recovery of the smallest fragments may also be observed.

Precipitating in 384-Well Plates

IMPORTANT If you are precipitating in 384-well plates, refer to the *ABI PRISM 3700 DNA Analyzer Sequencing Chemistry Guide* (P/N 4309125) for the procedure.

Precipitating in 96-Well Plates

IMPORTANT Where 95% ethanol is recommended in precipitation protocols, purchase non-denatured ethanol at this concentration rather than absolute (100%) ethanol. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

To precipitate in 96-well MicroAmp plates:

Step	Action
1	Remove the MicroAmp plate from the thermal cycler. Remove the caps from each tube.
2	Add the following: ♦ 16 µL of deionized water ♦ 64 µL of non-denatured 95% ethanol The final ethanol concentration should be 60 ± 3%. **! WARNING ! CHEMICAL HAZARD. Ethanol is a flammable chemical and is irritating to the skin, eyes, respiratory system. It can cause nerve and liver damage, CNS depression, nausea, vomiting, and headache. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.**
3	Seal the tubes with strip caps or by applying a piece of 3M Scotch Tape 439 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
4	Invert the tray a few times to mix.

To precipitate in 96-well MicroAmp plates: *(continued)*

Step	Action
5	Leave the tray at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times shorter than 15 minutes will result in the loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.
6	Place the tray in a tabletop centrifuge with tube-tray adaptor and spin it at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: ♦ 1400–2000 × *g*: 45 minutes ♦ 2000–3000 × *g*: 30 minutes **Note** A MicroAmp tube in a MicroAmp Tray can withstand 3000 × *g* for 30 minutes. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
7	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the tray onto a paper towel folded to the size of the tray.
8	If you are performing this procedure for electrophoresis on the 3700 DNA Analyzer: a. Rinse the pellet by adding 150 µL of 70% ethanol to each well. b. Seal the plate with adhesive tape. c. Invert the plate a few times.
9	Place the inverted tray with the towel into the tabletop centrifuge, and spin at 700 × *g* for 1 minute.
10	Remove the tray and discard the paper towel. **Note** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.

Precipitating in Microcentrifuge Tubes

To precipitate in microcentrifuge tubes:

Step	Action
1	Pipet the entire contents of each extension reaction into a 1.5-mL microcentrifuge tube. **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 1 on page 4-7.
2	Add the following: ♦ 16 µL of deionized water ♦ 64 µL of non-denatured 95% ethanol The final ethanol concentration should be 60 ± 3%. **! WARNING ! CHEMICAL HAZARD. Ethanol is a flammable chemical and is irritating to the skin, eyes, respiratory system. It can cause nerve and liver damage, CNS depression, nausea, vomiting, and headache. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.**
3	Close the tubes and vortex briefly.
4	Leave the tubes at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times shorter than 15 minutes will result in the loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.
5	Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 minutes at maximum speed. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
6	Carefully aspirate the supernatants with a separate pipette tip for each sample and discard. Pellets may or may not be visible. **IMPORTANT** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.
7	Add 250 µL of 70% ethanol to the tubes and vortex them briefly.

To precipitate in microcentrifuge tubes: *(continued)*

Step	Action
8	Place the tubes in the microcentrifuge in the same orientation as in step 5 and spin for 10 minutes at maximum speed.
9	Aspirate the supernatants carefully as in step 6.
10	Dry the samples in a vacuum centrifuge for 10–15 minutes or to dryness. (Alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90 °C for 1 minute.)

Ethanol/Sodium Acetate Precipitation

Procedure Not for 3700 DNA Analyzer

IMPORTANT This procedure is not recommended for use on the ABI PRISM 3700 DNA Analyzer.

Precipitating in 96-Well Plates

IMPORTANT Use non-denatured 95% ethanol rather than absolute (100%) ethanol. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

To precipitate in 96-well MicroAmp trays:

Step	Action
1	Remove the MicroAmp Tray from the thermal cycler. Remove the caps from each tube.
2	Add the following: ♦ 2.0 µL of 3 M sodium acetate (NaOAc), pH 4.6 ♦ 50 µL of 95% ethanol (EtOH) The final ethanol concentration should be 65%. **! WARNING ! CHEMICAL HAZARD. Ethanol is a flammable chemical and is irritating to the skin, eyes, respiratory system. It can cause nerve and liver damage, CNS depression, nausea, vomiting, and headache. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.**
3	Seal the tubes with strip caps or by applying a piece of 3M Scotch Tape 425-3 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
4	Invert the tray a few times to mix.
5	Leave the tray at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times shorter than 15 minutes will result in the loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.

To precipitate in 96-well MicroAmp trays: *(continued)*

Step	Action
6	Place the tray in a tabletop centrifuge with tube-tray adaptor and spin it at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: ♦ 1400–2000 × *g*: 45 minutes ♦ 2000–3000 × *g*: 30 minutes **Note** A MicroAmp tube in a MicroAmp Tray can withstand 3000 × *g* for 30 minutes. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
7	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the tray onto a paper towel folded to the size of the tray.
8	Place the inverted tray with the towel into the table-top centrifuge and spin at 700 × *g* for 1 minute.
9	Add 150 µL of 70% ethanol to each pellet.
10	Cap or seal the tubes, then invert the tray a few times to mix.
11	Spin the tray for 10 minutes at maximum speed.
12	Repeat steps 7 and 8.
13	Remove the tray and discard the paper towel. **Note** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.

Precipitating Microcentrifuge Tubes

To precipitate in microcentrifuge tubes:

Step	Action
1	For each sequencing reaction, prepare a 1.5-mL microcentrifuge tube containing the following: ♦ 2.0 µL of 3 M sodium acetate (NaOAc), pH 4.6 ♦ 50 µL of 95% ethanol (EtOH) **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 1 on page 4-7. **! WARNING ! CHEMICAL HAZARD. Ethanol is a flammable chemical and is irritating to the skin, eyes, respiratory system. It can cause nerve and liver damage, CNS depression, nausea, vomiting, and headache. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.**
2	Pipet the entire contents of each extension reaction into a tube of sodium acetate/ethanol mixture. Mix thoroughly.
3	Vortex the tubes and leave at room temperature for 15 minutes to precipitate the extension products. Precipitation times shorter than 15 minutes will result in the loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.
4	Spin the tubes in a microcentrifuge for 20 min at maximum speed.
5	Carefully aspirate the supernatant with a pipette tip and discard. **IMPORTANT** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.
6	Rinse the pellet with 250 µL of 70% ethanol.
7	Vortex briefly.
8	Spin for 5 minutes in a microcentrifuge at maximum speed. Again, carefully aspirate the supernatant and discard.
9	Dry the pellet in a vacuum centrifuge for 10–15 minutes, or until dry. Do not over-dry. (Alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90 °C for 1 minute.)

Sample Electrophoresis 5

Chapter Summary

In This Chapter The following topics are covered in this chapter:

Electrophoresis on the ABI PRISM 3700 DNA Analyzer

Overview

For information on how to perform sample electrophoresis on the ABI PRISM 3700 DNA Analyzer, refer to the following manuals:

- *ABI PRISM 3700 DNA Analyzer Sequencing Chemistry Guide* (P/N 4309125)
- *ABI PRISM 3700 DNA Analyzer User's Manual* (P/N 4306152)

Electrophoresis on the ABI PRISM 310 Genetic Analyzer

Requirements

Electrophoresis and data analysis of samples requires the following:

Filter Set E Run Modules

Configuration	Run Module
POP-6™ polymer, 1-mL syringe, 61-cm, 50-µm i.d. capillary	Seq POP6 (1 mL) E
POP-6 polymer, Rapid Sequencing, 1-mL syringe, 47-cm, 50-µm i.d. capillary	Seq POP6 (1 mL) Rapid E

Dye Set/Primer (Mobility) Files

Instrument	Dye Set/Primer File
ABI PRISM® 310, POP-6 polymer	DT POP6{BD Set-Any Primer}
ABI PRISM 310, POP-6 polymer, Rapid Sequencing	DT POP6{BD Set-Any Primer}

Filter Set E Instrument (Matrix) File

Data analysis requires Filter Set E instrument (matrix) file made from the ABI PRISM dRhodamine matrix standards (P/N 4305080). See the *Automated DNA Sequencing Chemistry Guide* (P/N 4305080, www.appliedbiosystems.com/techsupport) for more information.

Resuspending the Samples

To resuspend the samples:

Step	Action
1	Resuspend each sample pellet in 12–25 µL of Template Suppression reagent (TSR, supplied with the polymer).
2	Vortex and spin the samples.
3	Heat the samples at 95 °C for 2 minutes, then chill on ice.
4	Vortex and spin the samples again. Place on ice until ready to use.
5	Refer to the ABI PRISM 310 *Genetic Analyzer User's Manual* (P/N 903565) for guidelines on loading the samples.

Note Although freezing is not recommended on a routine basis, you can keep samples prepared in TSR frozen for several weeks before running on the ABI PRISM 310 Genetic Analyzer with no detectable loss in resolution or base calling.

Electrophoresis on the ABI PRISM 377 Sequencers

Requirements

Electrophoresis and data analysis of samples require the following:

Filter Set E Run Modules

Configuration[a]	Run Module
36-cm wtr, 1200 scans/hr, any comb	Seq Run 36E-1200
36-cm wtr, 2400 scans/hr, any comb	Seq Run 36E-2400
48-cm wtr, 1200 scans/hr, any comb	Seq Run 48E-1200

a. Any plate check and prerun modules can be used with the ABI PRISM 377 DNA Sequencer.

Dye Set/Primer (Mobility) File: DT {BD Set Any-Primer}

The dye set/primer file can be used with 5 and 5.5% Long Ranger gels and 4 and 4.25% polyacrylamide gels (19:1, acrylamide:bis).

Filter Set E Instrument (Matrix) File

Data analysis requires Filter Set E instrument (matrix) file made from the ABI PRISM dRhodamine matrix standards (P/N 4305080). See the *Automated DNA Sequencing Chemistry Guide* (P/N 4305080; www.appliedbiosystems.com/techsupport) for more information.

Using the Lane Guide Kit

To resuspend and load samples using the ABI PRISM Lane Guide Lane Identification Kit, refer to the kit's protocol (P/N 4313804).

Using Long-Read Gel and Buffer Formulations

For longer sequencing read lengths follow the gel and buffer formulations described in the user bulletin entitled *Achieving Longer High Accuracy Reads on the 377 Sequencer* (P/N 4315153).

Resuspending and Loading the Samples

Note You can use any plate check and prerun modules.

To resuspend and load the samples:

Step	Action
1	Prepare a loading buffer by combining the following in a 5:1 ratio (5 parts deionized formamide to 1 part EDTA with blue dextran): ♦ Deionized formamide ♦ 25 mM EDTA (pH 8.0) with blue dextran (50 mg/mL) **! WARNING ! CHEMICAL HAZARD. Formamide is a known teratogen (*i.e.,* it can cause birth defects). Wash thoroughly after handling formamide. Wear appropriate protective eyewear, clothing, and gloves. Obtain a copy of the MSDS from the manufacturer.**
2	Resuspend each sample pellet in loading buffer as follows:

Template	Volume (µL): 18- or 36-well	Volume (µL): 48-, 64-, or 96-well
PCR product, plasmid, M13	6–8	4–6
BAC, large DNA	2	1.5

Step	Action
3	Vortex and spin the samples.
4	Heat the samples at 95 °C for 2 minutes to denature. Place on ice until ready to load.
5	Load each sample into a separate lane of the gel as follows:

Template	Volume (µL): 18- or 36-well	Volume (µL): 48-, 64-, or 96-well
PCR product, plasmid, M13	0.75–1.5	0.5–1.0
BAC, large DNA	2	48-well: 1.5 64-well: 1.5 96-well: 1.0–1.5

Note If a weak signal is obtained on the ABI PRISM 377 with XL Upgrade, rerun the samples using a CCD gain of 4. Refer to the ABI PRISM 377 *DNA Sequencer XL Upgrade User's Manual* (P/N 904412) for more information.

Electrophoresis on the ABI PRISM 373 with BigDye Filter Wheel

Requirements

Electrophoresis

Collect BigDye terminator data with Filter Set A on the ABI PRISM 373 sequencer with BigDye Filter Wheel.

Data Analysis

Data analysis requires a Filter Set A instrument (matrix) file made from the ABI PRISM dRhodamine matrix standards (P/N 4305080) and BigDye terminator mobility file.

Resuspending and Loading the Samples

To resuspend and load the samples:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare a loading buffer by combining the following in a 5:1 ratio (5 parts deionized formamide to 1 part EDTA with blue dextran):
♦ Deionized formamide
♦ 25 mM EDTA (pH 8.0) with blue dextran (50 mg/mL)
! WARNING ! CHEMICAL HAZARD. Formamide is a known teratogen (i.e., it can cause birth defects). Wash thoroughly after handling formamide. Wear appropriate protective eyewear, clothing, and gloves. Obtain a copy of the MSDS from the manufacturer.</td></tr>
<tr><td>2</td><td>Resuspend each sample pellet in loading buffer as follows:
<table><tr><th></th><th colspan="4">Volume (µL)</th></tr><tr><th>Template</th><th>18 or 24 well</th><th>32 or 36 well</th><th>48-well</th><th>64-well</th></tr><tr><td>PCR product, plasmid, M13</td><td>3–4</td><td>3–4</td><td>4</td><td>4</td></tr><tr><td>BAC, large DNA</td><td>3</td><td>3</td><td>2</td><td>2</td></tr></table></td></tr>
<tr><td>3</td><td>Vortex and spin the samples.</td></tr>
<tr><td>4</td><td>Heat the samples at 95 °C for 2 minutes to denature. Place on ice until ready to load.</td></tr>
</table>

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

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>5</td><td>Load each sample into a separate lane of the gel as follows:
<table>
<tr><th></th><th colspan="4">Volume (µL)</th></tr>
<tr><th>Template</th><th>18 or 24 well</th><th>32 or 36 well</th><th>48-well</th><th>64-well</th></tr>
<tr><td>PCR product, plasmid, M13</td><td>3–4</td><td>3–4</td><td>2–4</td><td>2–4</td></tr>
<tr><td>BAC, large DNA</td><td>3</td><td>3</td><td>2</td><td>2</td></tr>
</table>
</td></tr>
</table>

Control DNA Sequence

A

Control Sequence

Partial Sequence of pGEM-3Zf(+)

The pGEM-3Zf(+) sequence below is the the sequence of the –21 M13 forward primer, followed by the ensuing 1000 bases.

TGTAAAACGACGGCCAGT (–21 M13 primer)

GAATTGTAAT	ACGACTCACT	ATAGGGCGAA	TTCGAGCTCG	40
GTACCCGGGG	ATCCTCTAGA	GTCGACCTGC	AGGCATGCAA	80
GCTTGAGTAT	TCTATAGTGT	CACCTAAATA	GCTTGGCGTA	120
ATCATGGTCA	TAGCTGTTTC	CTGTGTGAAA	TTGTTATCCG	160
CTCACAATTC	CACACAACAT	ACGAGCCGGA	AGCATAAAGT	200
GTAAAGCCTG	GGGTGCCTAA	TGAGTGAGCT	AACTCACATT	240
AATTGCGTTG	CGCTCACTGC	CCGCTTTCCA	GTCGGGAAAC	280
CTGTCGTGCC	AGCTGCATTA	ATGAATCGGC	CAACGCGCGG	320
GGAGAGGCGG	TTTGCGTATT	GGGCGCTCTT	CCGCTTCCTC	360
GCTCACTGAC	TCGCTGCGCT	CGGTCGTTCG	GCTGCGGCGA	400
GCGGTATCAG	CTCACTCAAA	GGCGGTAATA	CGGTTATCCA	440
CAGAATCAGG	GGATAACGCA	GGAAAGAACA	TGTGAGCAAA	480
AGGCCAGCAA	AAGGCCAGGA	ACCGTAAAAA	GGCCGCGTTG	520
CTGGCGTTTT	TCCATAGGCT	CCGCCCCCCT	GACGAGCATC	560
ACAAAAATCG	ACGCTCAAGT	CAGAGGTGGC	GAAACCCGAC	600
AGGACTATAA	AGATACCAGG	CGTTTCCCCC	TGGAAGCTCC	640
CTCGTGCGCT	CTCCTGTTCC	GACCCTGCCG	CTTACCGGAT	680
ACCTGTCCGC	CTTTCTCCCT	TCGGGAAGCG	TGGCGCTTTC	720
TCATAGCTCA	CGCTGTAGGT	ATCTCAGTTC	GGTGTAGGTC	760
GTTCGCTCCA	AGCTGGGCTG	TGTGCACGAA	CCCCCCGTTC	800
AGCCCGACCG	CTGCGCCTTA	TCCGGTAACT	ATCGTCTTGA	840
GTCCAACCCG	GTAAGACACG	ACTTATCGCC	ACTGGCAGCA	880
GCCACTGGTA	ACAGGATTAG	CAGAGCGAGG	TATGTAGGCG	920
GTGCTACAGA	GTTCTTGAAG	TGGTGGCCTA	ACTACGGCTA	960
CACTAGAAGG	ACAGTATTTG	GTATCTGCGC	TCTGCTGAAG	1000

Technical Support B

To Reach Us on the Web

Applied Biosystems web site address is:

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We strongly encourage you to visit our web site for answers to frequently asked questions, and to learn more about our products. You can also order technical documents and/or an index of available documents and have them faxed or e-mailed to you through our site (see the "Documents on Demand" section below).

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

Product	Hours
Chemiluminescence	9:00 a.m. to 5:00 p.m. Eastern Time
LC/MS	9:00 a.m. to 5:00 p.m. Pacific Time
All Other Products	5:30 a.m. to 5:00 p.m. Pacific Time

See the "Regional Offices Sales and Service" section below for how to contact local service representatives outside of the United States and Canada.

To Reach Us by Telephone or Fax in North America

Call Technical Support at 1-800-831-6844, and select the appropriate option (below) for support on the product of your choice at any time during the call. (To open a service call for other support needs, or in case of an emergency, press **1** after dialing 1-800-831-6844.)

For Support On This Product	Dial 1-800-831-6844, and...
ABI PRISM® 3700 DNA Analyzer	Press **8** / FAX 650-638-5981

For Support On This Product	Dial 1-800-831-6844, and...
ABI PRISM® 3100 Genetic Analyzer	**Press** **26** / **FAX** 650-638-5891
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Fluorescent Fragment Analysis (includes GeneScan® applications)	**Press** **23** / **FAX** 650-638-5891
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BioInformatics (includes BioLIMS™, BioMerge™, and SQL GT™ applications)	**Press** **25** / **FAX** 505-982-7690
PCR and Sequence Detection	**Press** **5**, or call 1-800-762-4001, and press 1 for PCR, or 2 for Sequence Detection / **FAX** 240-453-4613
FMAT	**Telephone** 1-800-899-5858, and press 1, then press 6 / **FAX** 508-383-7855
Peptide and Organic Synthesis	**Press** **31** / **FAX** 650-638-5981
Protein Sequencing	**Press** **32** / **FAX** 650-638-5981

<table>
<tr><th>For Support On This Product</th><th>Dial 1-800-831-6844, and...</th></tr>
<tr><td>Chemiluminescence</td><td>
<table>
<tr><th>Telephone</th><th>FAX</th></tr>
<tr><td>1-800-542-2369 (U.S. only), or

1-781-271-0045 (Tropix)</td><td>781-275-8581 (Tropix)

9:00 a.m. to 5:00 p.m. ET</td></tr>
</table>
</td></tr>
<tr><td>LC/MS</td><td>
<table>
<tr><th>Telephone</th><th>FAX</th></tr>
<tr><td>1-800-952-4716</td><td>650-638-6223

9:00 a.m. to 5:00 p.m. PT</td></tr>
</table>
</td></tr>
</table>

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by phone from outside the United States or Canada	a. Dial your international access code, then 1-858-712-0317, from a touch-tone phone. Have your complete fax number and country code ready (011 precedes the country code). b. Press **1** to order an index of available documents and have it faxed to you. Each document in the index has an ID number. (Use this as your order number in step “d” below.) c. Call 1-858-712-0317 from a touch-tone phone a second time. d. Press **2** to order up to five documents and have them faxed to you.

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France (Paris) Tel: 33 (0)1 69 59 85 85 Fax: 33 (0)1 69 59 85 00	Portugal (Lisboa) Tel: 351 (0)22 605 33 14 Fax: 351 (0)22 605 33 15
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Switzerland (Rotkreuz) Tel: 41 (0)41 799 7777 Fax: 41 (0)41 790 0676	South East Europe (Zagreb, Croatia) Tel: 385 1 34 91 927 Fax: 385 1 34 91 840
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ABI PRISM® DNA Sequencing Kits and Related Products

To order ABI PRISM DNA Sequencing Kits, please contact Applied Biosystems at one of the regional sales offices listed on the back of this protocol. All reagents are quality-controlled in stable formulations. All the kits listed below include protocols. Protocols can also be ordered separately.

dRhodamine Terminator Cycle Sequencing Kits with AmpliTaq® DNA Polymerase, FS

P/N	Kit	Reactions
403044	Ready Reaction	100
403045	Ready Reaction	1000
4303143	Ready Reaction	5000
403041	Protocol	–

BigDye™ Primer Cycle Sequencing Ready Reaction Kits with AmpliTaq DNA Polymerase, FS

P/N	Primer	Reactions
403051	–21 M13	100
403049	–21 M13	5000
403052	M13 Reverse	100
403050	M13 Reverse	5000
403057	Protocol	–

BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq DNA Polymerase, FS

P/N	Kit	Reactions
4303149	Ready Reaction	100
4303150	Ready Reaction	1000
4303151	Ready Reaction	5000
4303237	Protocol	–

BigDye™ Terminator Cycle Sequencing Ready Reaction Kits v2.0 with AmpliTaq DNA Polymerase, FS

P/N	Kit	Reactions
4314414	Ready Reaction	100
4314415	Ready Reaction	1000
4314416	Ready Reaction	5000
4314849	Ready Reaction	25,000
4303237	Protocol	–

ABI PRISM® Lane Guide™ Lane Identification Kits for use on the 377 Sequencer

P/N	Kit	Reactions
4313682	Lane Guide	200
4313677	Lane Guide	1000
4313679	Lane Guide	5000
4313804	Protocol	–

ABI PRISM Matrix Standards

P/N	Kit	Instrument
4305609	Matrix Standard Set	3700
403047	dRhodamine Matrix Standards	310
403047	dRhodamine Matrix Standards	377/373

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