BigDye® Terminator v3.1 Cycle Sequencing Kit

Protocol

Applied Biosystems

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Introduction 1

Chapter Summary

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

About the Kit

New Formulation The BigDye® Terminator v3.1 Cycle Sequencing Kit has a new formulation that delivers:

- Increased robustness
- More even peak heights
- Longer read lengths

The thermal cycling and clean up protocols for cycle sequencing have been modified to optimize results using the new formulation.

Features and Compatibilities

- The BigDye Terminator v3.1 Cycle Sequencing Kit does not require new instrument (matrix) files for the ABI PRISM® 310 Genetic Analyzer, and ABI PRISM® 377 DNA Sequencers or new spectral calibrations for theABI PRISM® 3700 DNA Analyzer, ABI PRISM® 3100 Genetic Analyzer, and ABI PRISM® 3100-Avant Genetic Analyzer.

Applied Biosystems recommends that you verify the quality of your current matrix or spectral before proceeding. If it is necessary to generate a new matrix or spectral, use the appropriate matrix and/or sequencing standard for your instrument.

  - The 310 and 377 instruments use the 310/377 BigDye® Terminator v3.1 Matrix Standards (PN 4336948) for instrument (matrix) file generation.
  - The 3700 instrument requires the 3700 BigDye® Terminator v3.1 Matrix Standard (PN 4336975) for spectral calibration.
  - The 3100 and 3100-Avant instruments require the 3100 BigDye® Terminator v3.1 Matrix Standard (PN 4336974) for spectral calibration.

- The alcohol precipitation methods are different from those recommended for earlier versions of BigDye® terminators.
- The existing mobility files can be used with their respective platforms. New mobility files are not necessary.
- The basecallers are the same.

BigDye Terminator v3.1 Cycle Sequencing Kit

The BigDye Terminator v3.1 Cycle Sequencing Kit provides the required reagent components for the sequencing reaction in a ready reaction, pre-mixed format. You need only provide your template and the template-specific primer.

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 (for example, BAC clones).

Note: This kit includes BigDye® Terminator v1.1/3.1 Sequencing Buffer (5X), which has been specifically optimized for use with the new BigDye® ready reaction mixes.

Instruments

Instrument Platforms

The BigDye Terminator v3.1 Cycle Sequencing Kit is for use with the following instruments:

- ABI PRISM® 3700 DNA Analyzer
- ABI PRISM® 3100 Genetic Analyzer
- ABI PRISM® 3100-Avant Genetic Analyzer
- ABI PRISM® 310 Genetic Analyzer
- ABI PRISM® 377 DNA Sequencer (all models*)

General instructions are given for using the kit reagents to generate samples for these instruments. For more detailed instructions, refer to the appropriate instrument user's manual or chemistry guide.

Thermal Cyclers

The protocols provided in this document were optimized using Applied Biosystems thermal cyclers, including the GeneAmp® PCR Systems 9700, 9600, 2700, and 2400.

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°/second), poor (noisy) data may result.

*Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.

Required Software

Dye/Filter Sets and Matrix Standards for the 310 and 377 Instruments

The dye/filter sets and matrix standards required for the 310 and 377 instruments are listed in the table below.

IMPORTANT! The instrument (matrix) file for the BigDye terminators v3.1 cannot be used for the BigDye terminators (original), BigDye terminators v2.0, dRhodamine terminators, or BigDye® primers (original), but can be used for BigDye terminators v3.0.

Instrument	Dye/Filter Set	Standards for Instrument (Matrix) File Generation
310 Genetic Analyzer	Filter Set E	310/377 BigDye® Terminator v3.1 Matrix Standards (PN 4336948)*
377 DNA Sequencers†	Filter Set E	

*The BigDye Terminator v3.1 Cycle Sequencing Kit does not require a new spectral calibration file if you currently have a BigDye Terminator v3.0 spectral file on your instrument.

†Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.

Dye Sets and Spectral Standards for the 3700, 3100, and 3100-Avant Instruments

IMPORTANT! Spectral calibrations for the BigDye terminators v3.1 are not compatible with the BigDye terminators (original), BigDye terminators v2.0, dRhodamine terminators, or BigDye primers (original).

Instrument	Dye Set	Standards for Spectral Calibration
3700 DNA Analyzer with Data Collection v2.0 3700 DNA Analyzer with Data Collection v1.1 and v1.1.1	H D	3700 BigDye® Terminator v3.1 Matrix Standard (PN 4336975)*
3100 Genetic Analyzer 3100-Avant Genetic Analyzer	Z	3100 BigDye® Terminator v3.1 Matrix Standard (PN 4336974)*

*The BigDye Terminator v3.1 Cycle Sequencing Kit does not require a new spectral calibration file if you currently have a BigDye® Terminator v3.0 spectral file on your instrument.

Instructions

For Generating Matrices

For the 377 and 310 instruments, refer to the product insert (included with matrix or sequence standards) for instructions on using the BigDye Matrix Standards v3.1 to generate matrices.

For Performing Spectral Calibrations

- For the 3700 instrument, refer to the product insert for instructions on using the 3700 BigDye Terminator v3.1 Matrix or Sequencing Standard to perform spectral calibration.
- For the 3100 and 3100-Avant instruments, refer to the product insert for instructions on using the BigDye Terminator v3.1 Matrix or Sequencing Standard to perform spectral calibration.

Dye Set/Primer (Mobility) Files

To analyze sequencing data generated with BigDye® chemistries v3.1, you need dye set/primer (mobility) files that were created for v3.0 chemistries. The dye set/primer (mobility) files can be downloaded from the Internet.

Dye set/primer (mobility) files can be downloaded from the Applied Biosystems website:

http://www.appliedbiosystems.com/support/software

If you do not have access to the Internet, contact Applied Biosystems Technical Support, or your local field applications specialist (call your local sales office for more information).

Reagents and Storage

Available Kits

The following kits are available:

Kit	Number of Reactions	Part Number
The BigDye Terminator v3.1 Cycle Sequencing Kit*	100	4337455
	1000	4337456
	5000	4337457
	25000	4337958

*The BigDye Terminator v3.1 Cycle Sequencing Kit does not require a new spectral calibration file if you currently have a BigDye Terminator v3.0 spectral file on your instrument.

The *BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol* (PN 4337035) is available separately and can be ordered at no charge.

Description of Reagents

A listing of the kit reagents is given below.

- Ready Reaction Mix
- pGEM®-3Zf(+) double-stranded DNA Control Template
- –21 M13 Control Primer (forward)
- BigDye Terminator v1.1/3.1 Sequencing Buffer (5X)

Storage and Use of the Kit

- Store the kit at –15 to –25 °C.

Note: The BigDye sequencing buffer can be stored at 4 °C.

- Avoid excess (that is, no more than 5–10) freeze-thaw cycles. Aliquot reagents in smaller amounts if necessary.
- Before each use of the kit, allow the frozen stocks to thaw at room temperature (do not heat).

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

- Whenever possible, keep thawed materials on ice during use. Do not leave reagents at room temperature for extended periods.

Materials Supplied by the User

Overview

In addition to the reagents supplied in this kit, other items are required.

This section lists general materials needed for:

- Cycle sequencing
- Purifying extension products

Note: Many of the items listed in this section are available from major laboratory suppliers (MLS) unless otherwise noted. Equivalent sources may be acceptable where noted.

Refer to the individual instrument protocols for the specific items needed for each instrument.

⚠ WARNING CHEMICAL HAZARD. Before handling the chemical reagents needed for cycle sequencing, read the safety warnings on the reagent bottles and in the manufacturers' Material Safety Data Sheets (MSDSs), and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Dispose of waste in accordance with all local, state/provincial, and national environmental and health regulations.

Materials for Cycle Sequencing

The table below lists the plates or tubes required for the recommended Applied Biosystems thermal cyclers (page 1-3).

Thermal Cycler	Plate or Tube	Applied Biosystems Part Number
GeneAmp® PCR System 9700	MicroAmp® 96-Well Reaction Plate	N801-0560
	384-Well Reaction Plate	4305505
	MicroAmp® Reaction Tubes, 0.2-mL	N801-0533
	MicroAmp® Caps, 12 or 8/strip	N801-0534 or N801-0535
	ABI PRISM® Optical Adhesive Cover Starter Pack or ABI PRISM® Optical Adhesive Covers	4313663 or 4311971
GeneAmp® PCR System 9600	MicroAmp® 96-Well Reaction Plate	N801-0560
	MicroAmp® Reaction Tubes, 0.2-mL	N801-0533
	MicroAmp® Caps, 12 or 8/strip	N801-0534 N801-0535
	ABI PRISM™ Optical Adhesive Cover Starter Pack or ABI PRISM® Optical Adhesive Covers	4313663 or 4311971
GeneAmp® PCR System 2400 and 2700	MicroAmp® Reaction Tubes, 0.2-mL	N801-0533
	MicroAmp® Caps, 12 or 8/strip	N801-0534 N801-0535

Materials for Purifying Extension Products

Method	Material	Supplier
Ethanol/EDTA Precipitation	Ethanol (EtOH), 200 proof, Molecular Biology grade EDTA, 125 mM Sealing tape	MLS MLS Costar 6570 Thermowell Sealing Tape
Ethanol/EDTA/ Sodium Acetate Precipitation	Ethanol (EtOH), 200 proof, Molecular Biology grade Sodium acetate (NaOAc), 3 M, pH 5.2 Sealing tape	MLS Applied Biosystems (PN 400320) Costar 6570 Thermowell Sealing Tape
Plate Column Purification **Note:** For 96-well reaction plates	96-Well columns for purification Sealing tape 2.2% SDS in deionized water	See “Recommended 96-Well Spin Plates” on page 4-14 Costar 6570 Thermowell Sealing Tape See “Plate and Spin Column Purification” on page 4-11
Spin Column Purification	Centri-Sep™ spin column, 1-mL, 32 columns, 100 columns Sealing tape 2.2% SDS in deionized water	Applied Biosystems PN 401763, PN 401762 Costar 6570 Thermowell Sealing Tape See “Plate and Spin Column Purification” on page 4-11

Other Equipment

You will also need a variable speed centrifuge with microtiter plate holders capable of reach a spin speed of at least 1400 × *g*. Applied Biosystems recommends a Beckman Allegra 6A centrifuge with a GH-3.8A rotor.

Materials for Electrophoresis

Instrument	Material	Supplier
ABI PRISM 3700 DNA Analyzer, 3100 and 3100-Avant Genetic Analyzers	Hi-Di™ Formamide, 25-mL bottle	Applied Biosystems (PN 4311320)
	3700/3730 BigDye® Terminator v3.1 Sequencing Standard	Applied Biosystems (PN 4336943)
	BigDye® Terminator v3.1 Sequencing Standard	Applied Biosystems (PN 4336935)
	3700 BigDye Terminator v3.1 Matrix Standard	Applied Biosystems (PN 4336975)
	3100 BigDye Terminator v3.1 Matrix Standard	Applied Biosystems (PN 4336974)
ABI PRISM 310 Genetic Analyzer	Template Suppression Reagent (TSR)	Applied Biosystems (PN 401674)
	EDTA	MLS
	BigDye® Terminator v3.1 Sequencing Standard	Applied Biosystems (PN 4336935)
	310/377 BigDye Terminator v3.1 Matrix Standards	Applied Biosystems (PN 4336948)
ABI PRISM 377 DNA Sequencer	Formamide	MLS
	EDTA	MLS
	25 mM EDTA with 50 mg/mL blue dextran	Applied Biosystems (PN 402055)
	BigDye® Terminator v3.1 Sequencing Standard	Applied Biosystems (PN 4336935)
	310/377 BigDye Terminator v3.1 Matrix Standards	Applied Biosystems (PN 4336948)

General Safety

Documentation User Attention Words

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

Note: Calls attention to useful information.

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

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

Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.

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

Site Preparation and Safety Guide

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

Chemical Safety

Chemical Hazard Warning

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

- Read and understand the material safety data sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.

- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (*e.g.,* fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical Waste Hazard Warning

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

- Read and understand the material safety data sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Handle chemical wastes in a fume hood.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (*e.g.,* fume hood). For additional safety guidelines, consult the MSDS.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

About MSDSs

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

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

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

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

Ordering MSDSs

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

To order documents by automated telephone service:

1.	From the U.S. or Canada, dial **1.800.487.6809**.
2.	Follow the voice instructions to order documents (for delivery by fax). **Note:** There is a limit of five documents per fax request.

To obtain documents through the Applied Biosystems Web site:

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

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

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 forward primer for use in performing control reactions.

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

An Additional Control Sold Separately The BigDye® Terminator v3.1 Sequencing Standard Kit provides an additional control to help in troubleshooting electrophoresis runs. It contains lyophilized sequencing reactions that require only resuspension and denaturation before use.

There are two v3.1 sequencing standard kits, as shown in the table below. Please use the correct sequencing standard for your instrument. Refer to the product inserts for instructions on using each sequencing standard.

Instrument	Kit	PN
ABI PRISM® 3700 DNA Analyzer	3700/3730 BigDye® Terminator v3.1 Sequencing Standard	4336943
ABI PRISM® 3100 Genetic Analyzer	BigDye® Terminator v3.1 Sequencing Standard	4336935
ABI PRISM® 3100-Avant Genetic Analyzer		
ABI PRISM® 310 Genetic Analyzer		
ABI PRISM® 377 DNA Sequencers*		

*Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.

Template Preparation Methods

Single- and Double-Stranded Templates

Refer to the *Automated DNA Sequencing Chemistry Guide* (PN 4305080) 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,* with extra phenol extraction and isopropanol precipitation if very clean DNA is desired
- Cesium chloride (CsCl) banding

Commercial Kits

Commercial kits are also available for BAC DNA preparation:

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

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, ensuring adequate signal in the sequencing reaction.

Importance of Purifying Product

For optimal results, purify the PCR product before sequencing. In general, any method that removes dNTPs and primers should work. We recommend Centricon®-100 columns (PN N930-2119). The protocol for using these columns is provided in "Purifying PCR Fragments" on page 2-4

Refer to the *Automated DNA Sequencing Chemistry Guide* (PN 4305080) for information on sequencing PCR templates.

*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.

Purifying PCR Fragments

To purify PCR fragments by ultrafiltration:

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.

DNA Quality

Poor Template Quality

Poor template quality is the most common cause of sequencing problems. The following are characteristics of poor quality templates:

- Noisy data or peaks under peaks
- No usable sequence data
- Weak signal

Always follow recommended procedures to prepare templates.

Contamination

Potential contaminants include:

- Proteins
- RNA
- Chromosomal DNA

- Excess PCR primers, dNTPs, enzyme, and buffer components (from a PCR amplification used to generate the sequencing template)
- Residual salts
- Residual organic chemicals such as phenol, chloroform, and ethanol
- Residual detergents

Determining DNA Quality

The following methods can be used to examine DNA quality:

- Agarose gel electrophoresis

  Purified DNA should run as a single band on an agarose gel.

Note: Uncut plasmid DNA can run as three bands: supercoiled, nicked, and linear.

- Spectrophotometry

  The A_{260}/A_{280} ratio should be 1.7 to 1.9. Smaller ratios usually indicate contamination by protein or organic chemicals.

Agarose gels reveal the presence of contaminating DNAs and RNAs, but not proteins. Spectrophotometry can indicate the presence of protein contamination, but not DNA and RNA contamination. Use these methods together to get the most information about your DNA before sequencing.

DNA Quantity

Quantitating DNA

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	20–50 ng
Single-stranded	25–50 ng
Double-stranded	150–300 ng
Cosmid, BAC	0.5–1.0 µg
Bacterial genomic DNA	2–3 µg

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

The template quantities stated above should work with all primers. You may be able to use even less DNA when using capillary instruments for detection. The amount of PCR product to use in sequencing also depends on the length and purity of the PCR product.

Performing Cycle Sequencing 3

Chapter Summary

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

Introduction

Overview The cycle sequencing protocols used for the BigDye® Terminator v3.1 Cycle Sequencing Kit have been modified to optimize results using the new chemistries. Applied Biosystems does not recommend the use of the protocols for the BigDye® Terminator v1.0 or v2.0 Ready Reaction Cycle Sequencing Kits.

Cycle Sequencing Single- and Double-Stranded DNA

Overview

This section describes how to prepare reactions and perform cycle sequencing on a variety of templates, including M13, plasmids, and PCR products.

Preparing the Reactions for 96-Well Reaction Plates or Microcentrifuge Tubes

The type of tube required depends on the thermal cycler that you are using. Refer to "Materials for Cycle Sequencing" on page 1-8.

To prepare the reaction mixtures:

1.	For each reaction add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix*	8.0 µL
	Template	See the table in "Template Quantity" on page 2-6.
	Primer	3.2 pmol
	Deionized water	q.s.
	Total Volume	20 µL
2.	Mix well and spin briefly.	

*See "Using BigDye Terminator v1.1/3.1 Sequencing Buffer" below.

Using BigDye Terminator v1.1/3.1 Sequencing Buffer

The BigDye® Terminator v1.1/3.1 Sequencing Buffer (5X)* is supplied at a 5X concentration. If you use it for sequencing reactions, be sure the final reaction volume is at a concentration of 1X. For example, for a half reaction in 20 µL final volume, you would use 4 µL of ready reaction premix and 2 µL of BigDye sequencing buffer as shown below.

Reagent	Concentration	Volume
Ready Reaction Premix	2.5X	4 µL
BigDye Sequencing Buffer	5X	2 µL
Primer	—	3.2 pmol
Template	—	See "Template Quantity" on page 2-6.
Water	—	to 20 µL
Final Volume	**1X**	20 µL

Note: The use of this buffer without optimization may result in deterioration of sequence quality. Applied Biosystems does not support diluted reactions or guarantee the performance of BigDye® chemistry when it is diluted.

*The BigDye Terminator v1.1/3.1 Sequencing Buffer is intended for use only with BigDye Terminator v1.1/3.l Cycle Sequencing Kits.

Preparing the Reactions for 384-Well Plates

The type of tube required depends on the thermal cycler that you are using. Refer to "Materials for Cycle Sequencing" on page 1-8.

Note: The wells in a 384-well reaction plate have a volume capacity of 35 µL. Therefore, we recommend doing a 10 µL reaction. This allows the post-reaction cleanup step to be performed in the same well.

To prepare the reaction mixtures:

1.	For each reaction add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix*	4.0 µL
	Template	See the table in "Template Quantity" on page 2-6.
	Primer	3.2 pmol
	Deionized water	q.s.
	Total Volume	10 µL
2.	Mix well and spin briefly.	
3.	Use on a GeneAmp® PCR System 9700 Dual 384-Well Sample Block Module.	

*Note: For instructions on using BigDye sequencing buffer, see "Using BigDye Terminator v1.1/3.1 Sequencing Buffer" on page 3-3.

Cycle Sequencing on the System 9700, 9600, 2700, or 2400

To sequence single- and double-stranded DNA on the GeneAmp® PCR System 9700 (in 9600 emulation mode), 9600, or 2400:

1.	Place the tubes in a thermal cycler and set to the correct volume.
2.	Perform an initial denaturation. a. Rapid thermal ramp to 96 °C b. 96 °C for 1 min
3.	Repeat the following for 25 cycles: • Rapid thermal ramp* to 96 °C • 96 °C for 10 sec • Rapid thermal ramp to 50 °C • 50 °C for 5 sec • Rapid thermal ramp to 60 °C • 60 °C for 4 min
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."

*Rapid thermal ramp is 1 °C/second.

Cycle Sequencing Large DNA Templates

Overview

This section describes how to prepare reactions and perform cycle sequencing on large DNA templates such as:

- BAC DNA
- Cosmid DNA
- Genomic DNA

Thermal Cyclers

Only the following thermal cyclers can be used with this protocol:

- GeneAmp PCR System 9600
- GeneAmp PCR System 9700 (in 9600 emulation mode)

Reoptimize this protocol for use on other thermal cyclers.

Preparing Sequencing Reactions

The type of tube required depends on the thermal cycler that you are using. Refer to "Materials for Cycle Sequencing" on page 1-8.

To prepare the sequencing reaction:

1.	For each reaction add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix*	8.0 µL
	Template	See the table in "Template Quantity" on page 2-6.
	Primer	3.2 pmol
	Deionized water	q.s.
	Total Volume	20 µL
2.	Mix well and spin briefly.	
3.	Use on a GeneAmp® PCR System 9600 or 9700 Sample Block Module.	

*Note: For instructions on using BigDye sequencing buffer, see "Using BigDye Terminator v1.1/3.1 Sequencing Buffer" on page 3-3.

Performing Cycle Sequencing

To perform cycle sequencing on BAC DNA:

1.	Place the tubes in a thermal cycler and set the volume to 20 µL.
2.	Heat the tubes at 95 °C for 5 minutes.

To perform cycle sequencing on BAC DNA: *(continued)*

3.	Repeat the following for 50 cycles:* • Rapid thermal ramp† to 95 °C • 95 °C for 30 sec • Rapid thermal ramp to 50–55 °C (depending on template) • 50–55 °C for 10 sec • Rapid thermal ramp to 60 °C • 60 °C for 4 min
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.”

*Some laboratories have found that increasing the number of cycles gives better results.
†Rapid thermal ramp is 1 °C/sec.

Purifying Extension Products 4

Chapter Summary

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

Choosing a Method of Purification

Purpose The best results are obtained when unincorporated dye terminators are completely removed prior to electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling.

Purification Methods The components of the BigDye® Terminator v3.1 Cycle Sequencing Kit have been optimized to produce excellent results under a wide variety of conditions. However, this kit may require changes to the clean-up protocols used for previous kits. To obtain clean sequencing data, Applied Biosystems recommends the following purification methods:

- Ethanol/EDTA precipitation
- Ethanol/EDTA/sodium acetate precipitation
- Plate and spin column purification

Use the method that works best for your particular application.

Note: The precipitation protocols given here have been optimized for use with the v3.1 formulation at the specified sequencing volumes (20 µL in 96-well format and 10-µL in 384-well format) and are not recommended for other versions.

IMPORTANT! To clean up sequencing reactions at volumes less than those specified, reduce each component of the precipitation protocol proportionately.

Ethanol/EDTA Precipitation

Recommended Protocol

With the BigDye terminators v3.1, the ethanol/EDTA precipitation method produces consistent signal, while minimizing unincorporated dyes. It is particularly good at getting rid of unincorporated dye-labeled terminators.

Note: While this method produces the cleanest signal, it may cause loss of small molecular weight fragments.

IMPORTANT! Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some sequencing results.

IMPORTANT! 95% ethanol is usable, but you must make sure the final ethanol concentration for precipitation remains the same (67–71%).

Precipitating in 96-Well Reaction Plates

WARNING **CHEMICAL HAZARD. EDTA**. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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

To precipitate 20-µL sequencing reactions in 96-well reaction plates:

1.	Remove the 96-well reaction plate from the thermal cycler and briefly spin.
2.	Add 5 µL of 125 mM EDTA to each well. **Note:** Make sure the EDTA reaches the bottom of the wells.
3.	Add 60 µL of 100% ethanol to each well.
4.	Seal the plate with aluminum tape and mix by inverting 4 times.
5.	Incubate at room temperature for 15 min.
6.	**If you are using ...** / **Then ...** a Beckman Allegra 6A centrifuge with a GH-3.8A rotor: set it at 4 °C and spin the plate at 1650 × *g* for 45 min. any other centrifuge: use a plate adapter and spin the plate at the maximum speed as follows: • 1400–2000 × *g* for 45 min or • 2000–3000 × *g* for 30 min **IMPORTANT!** Proceed to the next step immediately. If this is not possible, then spin the plate for an additional 2 min before performing the next step.

To precipitate 20-µL sequencing reactions in 96-well reaction plates: *(continued)*

7.	Invert the plate and spin up to 185 × *g*, then remove from the centrifuge.
8.	Add 60 µL of 70% ethanol to each well.
9.	With the centrifuge set to 4 °C, spin at 1650 × *g* for 15 min.
10.	Invert the plate and spin up to 185 × *g* for 1 min, then remove from the centrifuge. **Note:** Start timing when the rotor starts moving.
11.	To continue, resuspend the samples in injection buffer. To store, cover with aluminum foil, and store at 4 °C. **IMPORTANT!** Make sure the wells are dry. You may use a Speed-Vac for 15 min to dry the plate. **IMPORTANT!** Make sure the samples are protected from light while they are drying.

Precipitating in 384-Well Reaction Plates

WARNING **CHEMICAL HAZARD. EDTA**. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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

To precipitate 10-µL sequencing reactions in 384-well reaction plates:

1.	Remove the 384-well reaction plates from the thermal cycler. Remove the seal from each plate and briefly spin the plates.
2.	Add 2.5 µL of 125 mM EDTA to each well. **Note:** Make sure the EDTA reaches the bottom of the wells.
3.	Add 25 µL of 100% ethanol to each well.
4.	Seal the plates with aluminum tape and mix by inverting 4 times.
5.	Incubate at room temperature for 15 min.

To precipitate 10-µL sequencing reactions in 384-well reaction plates: *(continued)*

<table>
<tr><td>6.</td><td>
<table>
<tr><th>If you are using ...</th><th>Then ...</th></tr>
<tr><td>a Beckman Allegra 6A centrifuge with a GH-3.8A rotor</td><td>set it at 4 °C and spin the plate at 1650 × g for 45 min.</td></tr>
<tr><td>any other centrifuge</td><td>use a plate adapter and spin the plate at the maximum speed as follows:
• 1400–2000 × g for 45 min
or
• 2000–3000 × g for 30 min</td></tr>
</table>
IMPORTANT! Proceed to the next step immediately. If this is not possible, then spin the plate for 2 minutes more immediately before performing the next step.
</td></tr>
<tr><td>7.</td><td>Invert the plate and spin up to 185 × g, then remove from the centrifuge.</td></tr>
<tr><td>8.</td><td>Add 30 µL of 70% ethanol to each well.</td></tr>
<tr><td>9.</td><td>With the centrifuge set to 4 °C, spin at 1650 × g for 15 min.</td></tr>
<tr><td>10.</td><td>Invert the plate and spin up to 185 × g for 1 min, then remove from the centrifuge.
Note: Start timing when the rotor starts moving.</td></tr>
<tr><td>11.</td><td>To continue, resuspend the samples in injection buffer.
To store, cover with aluminum foil, and store at 4 °C.
IMPORTANT! Make sure the wells are dry. You may use a Speed-Vac for 15 min to dry the plate.
IMPORTANT! Make sure the samples are protected from light while they are drying.</td></tr>
</table>

Ethanol/EDTA/Sodium Acetate Precipitation

Note: Ethanol/EDTA/sodium acetate precipitation is recommended when good signal from base 1 is required. However, for reactions containing high concentrations of unincorporated terminators, some residual terminators may be carried through the precipitation. To completely remove excess terminators in these cases, ethanol/EDTA precipitation is recommended (see page 4-2).

Precipitating in 96-Well Reaction Plates

WARNING **CHEMICAL HAZARD. EDTA**. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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

To precipitate 20-µL sequencing reactions in 96-well reaction plates:

1.	Remove the 96-well reaction plate from the thermal cycler and briefly spin.
2.	Add 2 µL of 125 mM EDTA to each well. **Note:** Make sure the EDTA reaches the bottom of the wells.
3.	Add 2 µL of 3 M sodium acetate to each well. **Note:** Make sure the sodium acetate reaches the bottom of the wells.
4.	Add 50 µL of 100% ethanol to each well.
5.	Seal the plate with aluminum tape and mix by inverting 4 times.
6.	Incubate at room temperature for 15 min.

To precipitate 20-µL sequencing reactions in 96-well reaction plates: *(continued)*

<table>
<tr><td>7.</td><td>
<table>
<tr><th>If you are using ...</th><th>Then ...</th></tr>
<tr><td>a Beckman Allegra 6A centrifuge with a GH-3.8A rotor</td><td>set it at 4 °C and spin the plate at 1650 × g for 45 min.</td></tr>
<tr><td>any other centrifuge</td><td>use a plate adapter and spin the plate at the maximum speed as follows:
• 1400–2000 × g for 45 min
or
• 2000–3000 × g for 30 min</td></tr>
</table>
IMPORTANT! Proceed to the next step immediately. If this is not possible, then spin the plate for 2 minutes more immediately before performing the next step.
</td></tr>
<tr><td>8.</td><td>Invert the plate and spin up to 185 × g, then remove from the centrifuge.</td></tr>
<tr><td>9.</td><td>Add 70 µL of 70% ethanol to each well.</td></tr>
<tr><td>10.</td><td>With the centrifuge set to 4 °C, spin at 1650 × g for 15 min.</td></tr>
<tr><td>11.</td><td>Invert the plate and spin up to 185 × g for 1 min, then remove from the centrifuge.
Note: Start timing when the rotor starts moving.</td></tr>
<tr><td>12.</td><td>To continue, resuspend the samples in injection buffer.
To store, cover with aluminum foil, and store at 4 °C.
IMPORTANT! Make sure the wells are dry. You may use a Speed-Vac for 15 min to dry the plate.
IMPORTANT! Make sure the samples are protected from light while they are drying.</td></tr>
</table>

Precipitating in 384-Well Reaction Plates

WARNING **CHEMICAL HAZARD. EDTA**. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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

To precipitate 10-µL sequencing reactions in 384-well reaction plates:

1.	Remove the 384-well reaction plate from the thermal cycler and briefly spin.
2.	Add 1 µL of 125 mM EDTA to each well. **Note:** Make sure the EDTA reaches the bottom of the wells.
3.	Add 1 µL of 3 M sodium acetate to each well. **Note:** Make sure the sodium acetate reaches the bottom of the wells.
4.	Add 25 µL of 100% ethanol to each well.
5.	Seal the plate with aluminum tape and mix by inverting 4 times.
6.	Incubate at room temperature for 15 min.

To precipitate 10-µL sequencing reactions in 384-well reaction plates: *(continued)*

7.

If you are using ...	Then ...
a Beckman Allegra 6A centrifuge with a GH-3.8A rotor	set it at 4 °C and spin the plate at 1650 × *g* for 45 min.
any other centrifuge	use a plate adapter and spin the plate at the maximum speed as follows: • 1400–2000 × *g* for 45 min or • 2000–3000 × *g* for 30 min

IMPORTANT! Proceed to the next step immediately. If this is not possible, then spin the plate for 2 minutes more immediately before performing the next step.

8. Invert the plate and spin up to 185 × *g*, then remove from the centrifuge.

9. Add 35 µL of 70% ethanol to each well.

10. With the centrifuge set to 4 °C, spin at 1650 × *g* for 15 min.

11. Invert the plate and spin up to 185 × *g* for 1 min, then remove from the centrifuge.

    Note: Start timing when the rotor starts moving.

12. To continue, resuspend the samples in injection buffer.

    To store, cover with aluminum foil, and store at 4 °C.

    IMPORTANT! Make sure the wells are dry. You may use a Speed-Vac for 15 min to dry the plate.

    IMPORTANT! Make sure the samples are protected from light while they are drying.

Plate and Spin Column Purification

Overview

This section provides instructions for adding a sodium dodecyl sulfate (SDS)/heat treatment to the spin column and spin plate purification methods. This SDS/heat treatment effectively eliminates unincorporated dye terminators from cycle sequencing reactions.

Preparing Extension Products

Use this procedure to prepare extension products for both spin column and 96-well spin plate purification.

DANGER **CHEMICAL HAZARD. Sodium dodecyl sulfate (SDS).** Exposure causes eye, skin, and respiratory tract irritation. Exposure may cause severe allergic respiratory and skin reaction. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare extension products:

1.	Prepare 2.2% SDS in deionized water. This SDS solution is stable at room temperature.
2.	Add an appropriate amount of the 2.2% SDS solution to each sample to bring the final SDS concentration to 0.2%. For example: Add 2 µL of 2.2% SDS to each 20-µL completed cycle sequencing reaction.
3.	Seal the tubes and mix thoroughly.
4.	Heat the tubes to 98 °C for 5 min, then allow the tubes to cool to ambient temperature before proceeding to the next step. **Note:** A convenient way to perform this heating/cooling cycle is to place the tubes in a thermal cycler and set it as follows: • 98 °C for 5 min • 25 °C for 10 min

To prepare extension products: ***(continued)***

5.	Spin down the contents of the tubes briefly.
6.	Continue with spin column or 96-well plate purification.

Performing Spin Column Purification

Recommended Spin Columns

We recommend Centri-Sep™ spin columns (Applied Biosystems, PN 401763 for 32 columns and PN 401762 for 100 columns).

Optimizing Spin Column Purification

IMPORTANT! When using the BigDye terminators v3.1, hydrate the column for 2 hours (see page 4-13).

Tips for optimizing spin column purification when using individual columns:

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

Note: If samples are not properly loaded, peaks from unincorporated dye terminators can result.

- Spin the column at 325–730 × *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.

Preparing the Spin Column

To perform spin column purification:

1.	Prepare the extension products according to “Preparing Extension Products” on page 4-11.
2.	Gently tap the column to cause the gel material to settle to the bottom of the column.
3.	Remove the upper end cap and add 0.8 mL of deionized water.
4.	Replace the upper end cap and vortex or invert the column a few times to mix the water and gel material.
5.	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.
6.	Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.
7.	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.
8.	Insert the column into the wash tube provided.
9.	Spin the column in a microcentrifuge at 730 × *g* for 2 minutes to remove the interstitial fluid.
10.	Remove the column from the wash tube and insert it into a sample collection tube (for example, a 1.5-mL microcentrifuge tube).

Purifying with the Spin Column

To perform purification with the spin column

1.	Remove the extension reaction/SDS mixture from its tube and load it carefully onto the center of the gel material.
2.	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.
3.	Discard the column. The sample is in the sample collection tube.
4.	Dry the sample in a vacuum centrifuge for 10–15 minutes without heat, or until dry. Do not over-dry.

Performing 96-Well Spin Plate Purification

Recommended 96-Well Spin Plates

For large-scale procedures, you can use 96-well spin plates, such as the Gel Filtration Kit from Edge Biosystems.

Note: Other spin plate systems may be used to successfully remove unincorporated dye terminators. However, due to the large number of variables associated with using spin plate systems, you will need to optimize the performance of your system in your own laboratory.

Purifying with the 96-Well Spin Plate

To perform purification with the spin plate:

1.	Prepare the extension products according to “Preparing Extension Products” on page 4-11.
2.	Prepare the 96-well spin plate per the manufacturer’s instructions.
3.	Perform the purification per the manufacturer’s instructions. **IMPORTANT!** When using the Edge Biosystems gel filtration kit only, centrifuge at 850 × *g* for 2 min.

Selecting Sequencing Primers A

Selecting Sequencing Primers

Overview

The choice of sequencing primer sequence, method of primer synthesis, and approach to primer purification can have a significant effect on the quality of the sequencing data obtained in dye terminator cycle sequencing reactions with this kit.

These decisions are particularly important when sequencing is done on real-time detection systems where signal strength is critical. Some of the recommendations given here are based on information that is general knowledge, while others are based on practical experience gained by Applied Biosystems scientists.

Optimizing Primer Selection

The following recommendations are provided to help optimize primer selection:

- Primers should be at least 18 bases long to ensure good hybridization.
- Avoid runs of an identical nucleotide, especially guanine, where runs of four or more Gs should be avoided.
- Keep the G-C content in the range 30–80%.
- For cycle sequencing, primers with melting temperatures (T_m) above 45 °C produce better results than primers with lower T_m.
- For primers with a G-C content less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the T_m>45 °C.
- Use of primers longer than 18 bases also minimizes the chance of having a secondary hybridization site on the target DNA.
- Avoid primers that have secondary structure or that can hybridize to form dimers.
- Several computer programs for primer selection are available. They can be useful in identifying potential secondary structure problems and determining if a secondary hybridization site exists on the target DNA.

Control DNA Sequence

B

Control Sequence

Partial Sequence of pGEM-3Zf(+)

The pGEM®-3Zf(+) sequence below is 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

Sample Electrophoresis C

Some Important Reminders

- Dye set/primer (mobility) files for the BigDye® terminators v3.1 are the same for those for v3.0, but different than those for the dRhodamine terminators, BigDye terminators original, and BigDye terminators v2.0.
- If a mobility file for the wrong sequencing chemistry is used, some bases may be miscalled due to different dye labeling for the different chemistries.

Note: See "Dye Set/Primer (Mobility) Files" on page 1-6 for information on obtaining the v3.1 dye set/primer (mobility) files.

Electrophoresis on the ABI PRISM 3700 DNA Analyzer

Requirements

Run Modules

Configuration	Run Module
POP-5™ polymer, 50-cm	Seq1_1POP5DefaultModule
	Seq1_2POP5DefaultModule
POP-6™ polymer, 50-cm	Seq1_1POP6DefaultModule
	Seq1_2POP6DefaultModule

Dye Set/Primer (Mobility) Files

Polymer	Dye Set/Primer (Mobility) File
POP-5 polymer	DT3700POP5{BDv3}v1.mob
POP-6 polymer	DT3700POP6{BDv3}v1.mob

Standards

IMPORTANT! Use Dye Set H.

Dye Set	Standards
H	3700/3730 BigDye® Terminator v3.1 Sequencing Standard (PN 4336943)
H	3700 BigDye® Terminator v3.1 Matrix Standard (PN 4336975)

Note: Refer to the product insert for instructions on using the standards for this instrument.

Performing Sample Electrophoresis

For information on how to perform sample electrophoresis on the 3700 instrument, refer to the following manuals:

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

Electrophoresis on the ABI PRISM 3100 and 3100-Avant Genetic Analyzers

Requirements

Electrophoresis and data analysis of samples on the ABI PRISM® 3100 and 3100-Avant Genetic Analyzers require the following:

Run Modules

Configuration	Run Module
POP-4™ polymer, 36-cm	UltraSeq_POP4Default Module
POP-4 polymer, 80-cm	LongSeq80_POP4DefaultModule
POP-6™ polymer, 36-cm	RapidSeq36_POP6DefaultModule
POP-6 polymer, 50-cm	StdSeq50_POP6DefaultModule

Dye Set/Primer (Mobility) Files

Polymer	Dye Set/Primer (Mobility) File
POP-4™ polymer	DT3100POP4{BDv3}v1.mob
POP-6™ polymer	DT3100POP6{BDv3}v1.mob

Standards

IMPORTANT! Use Dye Set Z.

Dye Set	Standards
Z	BigDye® Terminator v3.1 Sequencing Standard (PN 4336935)
Z	3100 BigDye® Terminator v3.1 Matrix Standard (PN 4336974)

Note: Refer to the product insert for instructions on using the standards for this instrument.

Performing Sample Electrophoresis

For information on how to perform sample electrophoresis on the 3100 instrument, refer to the following manuals:

- *ABI PRISM 3100 Genetic Analyzer Sequencing Chemistry Guide* (PN 4315831)
- *ABI PRISM 3100 Genetic Analyzer User's Manual* (PN 4315834)

Electrophoresis on the ABI PRISM 310 Genetic Analyzer

Requirements

Electrophoresis and data analysis of samples on the ABI PRISM® 310 Genetic Analyzer requires the following:

Filter Set E Run Modules

Configuration	Run Module
POP-4™ polymer, 1-mL syringe, 47-cm, 50-µm i.d. capillary, Ld = 36 cm	P4StdSeq (1 mL) E
POP-4 polymer, Rapid Sequencing, 1-mL syringe, 47-cm, 50-µm i.d. capillary, Ld = 36 cm	P4RapidSeq (1 mL) E
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 Rapid (1 mL) E

Dye Set/Primer (Mobility) Files

Polymer	Dye Set/Primer (Mobility) File
POP-4 polymer	DT310POP4{BDv3}v2.mob
POP-6 polymer	DT310POP6{BDv3}v2.mob

Matrix Standards

IMPORTANT! The instrument (matrix) file for the BigDye terminators v3.1 cannot be used for the BigDye terminators (original), BigDye terminators v2.0, dRhodamine terminators, or BigDye® primers (original).

Dye/Filter Set	Standards for Instrument (Matrix) File Generation
E	310/377 BigDye® Terminator v3.1 Matrix Standards (PN 4336948)

Note: Refer to the product insert for instructions on using the standards for this instrument.

Performing Sample Electrophoresis

For information on how to perform sample electrophoresis on the 310 instrument, refer to the following manuals:

- *ABI PRISM 310 Genetic Analyzer Sequencing Chemistry Guide* (PN 4303189)
- *ABI PRISM 310 Genetic Analyzer User's Manual* (PN 4317588)

Electrophoresis on the ABI PRISM 377 DNA Sequencers

Requirements

Electrophoresis and data analysis of samples on the ABI PRISM® 377 DNA Sequencers (all models*) require the following:

Filter Set E Run Modules

Configuration*	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

*Any plate check and prerun module can be used on the ABI PRISM 377 DNA Sequencers.

Dye Set/Primer (Mobility) Files

Gel Formulation	Dye Set/Primer (Mobility) File
4.5% acrylamide (29:1) or 5% Long Ranger™ gel	DT377{BDv3}v2.mob

*Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.

Matrix Standards

IMPORTANT! The instrument (matrix) file for the BigDye terminators v3.1 cannot be used for the BigDye terminators (original), BigDye terminators v2.0, dRhodamine terminators, or BigDye primers (original), but can be used with BigDye terminators v3.0.

Dye/Filter Set	Standards for Instrument (Matrix) File Generation
E	310/377 BigDye® Terminator v3.1 Matrix Standards (PN 4336948)

Note: Refer to the product insert for instructions on using the standards for this instrument.

Using the Lane Guide Kit

If you are using the BigDye® chemistries v3.1 on the 377 instrument in conjunction with the ABI PRISM® Lane Guide™ Lane Identification Kit, refer to that kit's protocol (PN 4313804) for instructions on resuspending and loading samples.

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* (PN 4315153).

Performing Sample Electrophoresis

For information on how to perform sample electrophoresis on the 377 instrument, refer to the following manuals:

- *Automated DNA Sequencing Chemistry Guide* (PN 4305080)
- *ABI PRISM 377 DNA Sequencer User's Manual* (PN 4307164)

Troubleshooting

D

Observation	Possible Causes	Recommended Actions
No recognizable sequence	Insufficient template	• Quantitate DNA template • Increase amount of DNA in the sequencing reactions
	Inhibitory contaminant in the template	Clean up the template
	Insufficient primer	• Quantitate the primer • Increase amount of primer in the sequencing reactions
	Primer has no annealing site	Use a primer that is complementary to the template
	Poor primer design or incorrect primer sequence	Redesign the primer
	Missing reagent	Repeat the reactions, carefully following the protocol
	Old or mishandled reagents	Use fresh reagents
	Thermal cycler power failure	Repeat the reactions
	Thermal cycling conditions incorrect	• Calibrate the thermal cycler regularly • Use correct thermal cycling parameters • Use correct tube for your thermal cycler • Set ramp rate to 1 °C/sec
	Extension products lost during reaction cleanup	• Make sure you use the correct centrifugation speeds and times for precipitation and spin column procedures • Make sure ethanol concentration is correct for precipitation protocols

Observation	Possible Causes	Recommended Actions
No recognizable sequence (continued)	Extension products not resuspended	Carefully resuspend the sample pellet in loading buffer
	Lane tracking failure (377 instrument)	Check the lane tracking. If necessary, retrack and reextract the lanes
	Electrokinetic injection failure (capillary instruments)	Repeat the injections
Noisy data throughout the sequence with low signal strength	Insufficient DNA in the sequencing reactions	• Use more DNA in the sequencing reactions • Load or inject more of the resuspended sequencing reactions
	Degraded template	Prepare fresh DNA and repeat the reactions
	Old or mishandled reagents	Use fresh reagents
	Thermal cycling conditions incorrect	• Calibrate the thermal cycler regularly • Use correct thermal cycling parameters • Use correct tube for your thermal cycler • Set ramp rate to 1 °C/sec
	Lane tracking failure (377 instrument)	Check the lane tracking. If necessary, retrack and reextract the lanes
	Electrokinetic injection failure (capillary instruments)	Repeat the injections
Noisy data throughout the sequence with good signal strength	Inhibitory contaminant in the template	Clean up the template
	Multiple templates in the sequencing reaction	Examine template on an agarose gel to be sure only one template is present
	Multiple priming sites	• Make sure primer has only one priming site. • If necessary, redesign primer
	Multiple primers when sequencing PCR products	Purify your PCR template to remove excess primers

Observation	Possible Causes	Recommended Actions
Noisy data throughout the sequence with good signal strength (continued)	Primer with N-1 contamination	Use HPLC-purified primer
	High signal saturating the detector	• Use less DNA in the sequencing reactions • Load or inject less of the resuspended sequencing reactions
	Incorrect run module	Use correct run module
	Incorrect instrument (matrix) file	Use correct instrument file for terminator chemistry
Noise up to or after a specific point in the sequence	Mixed plasmid separation	Make sure you have only one template
	Multiple PCR products	Make sure you have only one template
	Primer-dimer contamination in PCR sequencing	• Optimize your PCR amplification • Make sure there is no sequence complementarity between the two PCR primers • Make sure your sequencing primer does not overlap the sequences of the PCR primers • Use a Hot Start technique such as with Amplitaq Gold polymerase
	Slippage after repeat region in template	• Try an alternate sequencing chemistry • Use an anchored primer
Poor mobility correction	Incorrect dye set/primer (mobility) file	Use correct dye set/primer file
	Incorrect Peak 1 location for data analysis	Select a new Peak 1 location
	Gel with very different separation properties than the gel matrices used to construct the dye set/primer (mobility) file	Use correct dye set/primer file for your gel type

Observation	Possible Causes	Recommended Actions
Excess dye peaks at beginning of sequence	Poor removal of unincorporated dye terminators	Use ethanol/EDTA precipitation protocol to remove unincorporated dye terminators.
		When preparing extension products for plate and spin column purification, increase the final SDS concentration to 0.4%.
		With Centri-Sep spin columns, take care to load sample on the center of the gel surface **Note:** Do not touch the gel surface with the pipet tip. **IMPORTANT!** Be sure you hydrate the column for at least 2 hours.
		Spin samples for recommended times (spinning too long precipitates more dyes with the sample)
		With microfuge tubes, aspirate the supernatant rather than decanting (decanting leaves excess ethanol on the sides of the tube)
		Select the start point for data analysis to exclude excess dye peaks
Pull-up peaks or bleedthrough	Total signal strength over 4000	• Quantitate DNA template • Decrease amount of DNA in the sequencing reactions
		Load or inject less of the resuspended sequencing reactions

Obtaining Technical Support E

Services and Support

Applied Biosystems Web Site

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

http://www.appliedbiosystems.com

and click the link for services and support.

At the services and support page, you can:

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

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

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