ABI PRISM® BigDye® Primer Cycle Sequencing Ready Reaction Kit

With AmpliTaq® DNA Polymerase, FS

Protocol

Applied Biosystems

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Contents

Introduction

Cycle Sequencing with AmpliTaq DNA Polymerase, FS

This kit formulation contains 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, which leads to a much more even peak intensity pattern.

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 Primers

Applied Biosystems has developed a set of dye primers labeled with novel, high-sensitivity dyes. The new 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 (Figure 1 on page 5).

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. Hence, the BigDye® primers are 2–3 times brighter than standard dye primers when incorporated into cycle sequencing products.

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

Primer	Acceptor Dye	Color of Raw Data on ABI PRISM 310 Electropherogram	Color of Raw Data on ABI PRISM 377 Gel Image
A	dR6G	green	green
C	dR110	blue	blue
G	dTAMRA	black	yellow
T	dROX	red	red

The new BigDye primers also have narrower emission spectra than the standard dye primers, giving less spectral overlap and therefore less noise. The brighter signal and decreased noise provide an overall four- to fivefold improvement in sensitivity, giving added flexibility in sequencing applications:

- For your convenience, the A, C, G, and T reactions are carried out in a 1:1:1:1 ratio.
- The nucleotide/dideoxynucleotide mixes have been optimized to give longer, more accurate reads.
- Large templates can be sequenced more readily. One such application is BAC end sequencing.
- Reactions using half the amount of Ready Reaction Premix can be run on some templates, such as PCR products and plasmids (see “Preparing the Reactions” on page 14).
- In some cases, reactions can be loaded onto the sequencing instrument without ethanol precipitation (see “Loading Options” on page 23).

Dye Spectra

The normalized emission spectra of the dRhodamine dyes in the BigDye primers are shown in Figure 1.

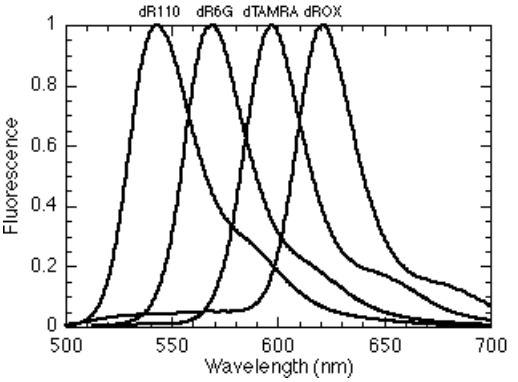


Figure 1 Emission spectra of dRhodamine dyes

BigDye Primer Ready Reaction Kit

The ABI PRISM® BigDye® Primer Cycle Sequencing Ready Reaction Kits combine AmpliTaq DNA Polymerase, FS, the new BigDye primers, and all the required components for the sequencing reaction.

The kits contain nucleotide mixes that have been specifically optimized for AmpliTaq DNA Polymerase, FS. The deoxy- and dideoxynucleotide ratios in the nucleotide mixes have been formulated to give a good signal balance above 700 bases. These formulations also contain 7-deaza-dGTP in place of dGTP to minimize band compressions.

In the Ready Reaction format, the dye-labeled primers, deoxynucleoside triphosphates, dideoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, r*Tth* pyrophosphatase, magnesium chloride, and buffer are premixed into A, C, G, and T Ready Reaction cocktails to eliminate time-consuming reagent preparation. 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.*, the ends of BAC clones.

Instrument Platforms

The protocols provided in this document were optimized using GeneAmp® PCR Instrument Systems thermal cyclers. Reactions can also be carried out on the CATALYST™ 800 Molecular Biology LabStation or the ABI PRISM® 877 Integrated Thermal Cycler.

The ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kit is for use with the ABI PRISM 310 Genetic Analyzer and ABI PRISM 377 DNA Sequencer (all models[1]). For more detailed instructions, refer to the appropriate instrument user's manual.

This kit can also be used with ABI™ 373 DNA Sequencers on which the new ABI PRISM® BigDye® Filter Wheel has been installed.[2] Refer to the *ABI PRISM BigDye Filter Wheel User Bulletin* (P/N 4304367) for more information.

IMPORTANT This kit is not designed for use with ABI 373 DNA Sequencers and ABI 373 DNA Sequencers with XL Upgrade that do not have the ABI PRISM BigDye Filter Wheel.

IMPORTANT You must install new dye set/primer (mobility) files for the BigDye primers. Run modules and instrument (matrix) files are the same as for the dRhodamine terminators and BigDye terminators.

IMPORTANT The run modules and dye set/primer files will need to be installed and the instrument files made if you have not previously used the dRhodamine-based sequencing chemistries. See "Sample Electrophoresis" on page 26 and the *Automated DNA Sequencing Chemistry Guide* (P/N 4305080, www.appliedbiosystems.com/techsupport) for more information.

Filter Set E

You must use run modules and dye set/primer (mobility) files for virtual Filter Set E when sequencing with the ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kit on the ABI PRISM 310 Genetic Analyzer or ABI PRISM 377 DNA Sequencer.

Use Filter Set A on ABI 373 DNA Sequencers with the ABI PRISM BigDye Filter Wheel.

1. 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.
2. Includes the ABI 373 and ABI 373 with XL Upgrade instruments.

Materials

Kit Reagents

This kit contains sufficient reagents to sequence either 100 or 1000 templates. All kits are stringently tested prior to shipment to ensure optimum performance. The reagents in the kit are listed below.

- A Mix: dR6G BigDye primer, ddATP, dATP, dCTP, 7-deaza-dGTP, dTTP, Tris-HCl (pH 9.0 at 25 °C), $MgCl_2$, and AmpliTaq DNA Polymerase, FS with thermostable pyrophosphatase
- C Mix: dR110 BigDye primer, ddCTP, dATP, dCTP, 7-deaza-dGTP, dTTP, Tris-HCl (pH 9.0 at 25 °C), $MgCl_2$, and AmpliTaq DNA Polymerase, FS with thermostable pyrophosphatase
- G Mix: dTAMRA primer, ddGTP, dATP, dCTP, 7-deaza-dGTP, dTTP, Tris-HCl (pH 9.0 at 25 °C), $MgCl_2$, and AmpliTaq DNA Polymerase, FS with thermostable pyrophosphatase
- T Mix: dROX BigDye primer, ddTTP, dATP, dCTP, 7-deaza-dGTP, dTTP, Tris-HCl (pH 9.0 at 25 °C), $MgCl_2$, and AmpliTaq DNA Polymerase, FS with thermostable pyrophosphatase
- pGEM®-3Zf(+) Control DNA template, 6 µg at 0.2 µg/µL

Storage and Use of the Kit

The ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kit should be stored at –15 to –25 °C.

Before each use of the kit, allow the frozen stocks to thaw at room temperature (do not heat). Mix each stock thoroughly and then centrifuge briefly to collect all the liquid at the bottom of each tube. Whenever possible, thawed materials should be kept on ice during use.

IMPORTANT Do not leave reagents at room temperature for extended periods of time.

Reagents and Equipment Supplied by the User

In addition to the reagents supplied in this kit, other items may be required depending on which instrument is used. Refer to the individual instrument protocols for the specific items needed. Many of the items listed are available from major laboratory suppliers (MLS) unless otherwise noted. Equivalent sources may be acceptable where noted.

! WARNING ! CHEMICAL HAZARD. Before handling the chemical reagents needed for BigDye primer cycle sequencing, read the safety warnings on the reagent bottles and in the manufacturers' Material Safety Data Sheets (MSDS). Always wear protective equipment (lab coat, safety glasses, and chemical-resistant gloves) when handling chemicals. Dispose of waste in accordance with all local, state, and federal health and environmental regulations and laws.

Table 1. Reagents Supplied by the User

Reagent	Source
ABI PRISM dRhodamine Matrix Standards Kit	Applied Biosystems (P/N 403047)
Performance Optimized Polymer 6 (POP-6™) with TSR for the ABI PRISM 310 Genetic Analyzer	Applied Biosystems (P/N 402844)
ABI PRISM 310 Genetic Analyzer Buffer with EDTA (for use with POP-6™ polymer)	Applied Biosystems (P/N 402824)
Deionized formamide	Major laboratory suppliers (MLS)
Deionized water	MLS
5 mM EDTA (ethylenediaminetetraacetate) for Express Load Option	MLS
25 mM EDTA with 50 mg/mL blue dextran, pH 8.0	Applied Biosystems (P/N 402055)
Ethanol (EtOH), non-denatured, 95% and 70%	MLS
Mineral oil, for the DNA Thermal Cycler (TC1) and the DNA Thermal Cycler 480	Applied Biosystems (P/N 0186-2302)
1X Sequencing Buffer (80 mM Tris-HCl, 2 mM $MgCl_2$, pH 9.0)	MLS
Sodium acetate (NaOAc), 3 M, pH 4.6	Applied Biosystems (P/N 400320)
TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)	MLS

Table 2. Equipment Supplied by the User

Item	Source
ABI PRISM 310 Genetic Analyzer, ABI PRISM 377 DNA Sequencer, or ABI PRISM 377 DNA Sequencer with XL Upgrade	Applied Biosystems
ABI PRISM 310 Capillaries, 61 cm × 50 µm i.d. (for long-read sequencing with POP-6)	Applied Biosystems (P/N 402840)
ABI PRISM 310 Capillaries, 47 cm × 50 µm i.d. (for rapid sequencing with POP-6)	Applied Biosystems (P/N 402839)
ABI PRISM Plasmid Miniprep Kit	Applied Biosystems:
♦ 100 purifications	P/N 402790
♦ 500 purifications	P/N 402791
Adhesive-backed aluminum foil tape	3M (Scotch Tape P/N 425-3)
Centricon-100 Micro-Concentrator columns	Applied Biosystems (P/N N930-2119)
Microcentrifuge, variable speed, capable of reaching 14,000 × *g*	MLS
Table top centrifuge, variable speed, capable of reaching 2000 × *g*, with 96-tube tray adaptor	MLS
Thermal cycler	Applied Biosystems
Vacuum centrifuge	Savant Speedvac (P/N DNA100) or equivalent
Vortexer	MLS

Template Preparation

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.

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.

Sequencing Standard

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 only require resuspension and denaturation before use.

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], with extra phenol extraction and isopropanol precipitation if very clean DNA is desired
- Cesium chloride (CsCl) banding

Commercial kits are also available for BAC DNA preparation:

- ProPrep BAC (LigoChem, http://www.ligochem.com)
  - Individual reactions: P/N PLK-100, 100 reactions; PLK-1000, 1000 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.

  - 96-well plates: P/N PLF-1000, 1 plate; PLF-1000, 10 plates; PLF-2500, 25 plates)

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

For other BAC DNA preparation protocols, refer to the following Web sites:

- Centre National de Séquençage (CNS, or Génoscope): http://www.cns.fr/externe/arabidopsis/protoBAC.html
- The Institute for Genome Research (TIGR): http://www.tigr.org/softlab/TPFBACmultiprep.052397.html
- University of Oklahoma Advanced Center for Genome Technology (ACGT): http://www.genome.ou.edu/DblAcetateProcV3.html
- Washington University School of Medicine Genome Sequencing Center: http://genome.wustl.edu/gsc/manual/protocols/BAC.html

PCR Templates

Cycle sequencing has been found to provide the most reproducible results when 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.

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” on page 12.

Refer to the *Automated DNA Sequencing Chemistry Guide* (P/N 4305080, www.appliedbiosystems.com/techsupport) for more information on sequencing PCR templates.

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.

Direct PCR Sequencing

PCR protocols that limit amounts of primers and dNTPs allow the product of the reaction to be used for sequencing with no purification. This is usually carried out by setting up the PCR amplification with 5–10 pmol of primers and 20–40 µM dNTPs, so that most of the primers and dNTPs are exhausted during amplification.

If the yield of the desired PCR product is high and the product is specific, *i.e.*, it produces a single band when analyzed by agarose gel electrophoresis, the sample can be diluted before sequencing and will give good results. The dilution ratio depends on the concentration of your PCR product and needs to be determined empirically (start with 1:2 and 1:10 dilutions with deionized water).

When you limit concentrations of primers and dNTPs and dilute the PCR products, the PCR parameters have to be robust. Direct PCR sequencing is most useful in applications where the same target is being amplified and sequenced repeatedly and PCR conditions have been optimized.

DNA Quantity

For most purified PCR products, using 1 µL for each reaction (A, C, G, and T) will work. If possible, quantitate the amount of purified DNA by measuring the absorbance at 260 nm or by some other method. If you do not quantitate the amount of DNA beforehand, you run the risk of using too much DNA, which can affect the final sequencing result.

The recommended amounts are shown in Table 3.

Table 3. Recommended DNA Quantities

Template	Quantity
PCR product:	
100–200 bp	2–5 ng
200–500 bp	5–10 ng
500–1000 bp	10–20 ng
1000–2000 bp	20–50 ng
>2000 bp	50–150 ng
single-stranded	150–400 ng
double-stranded	200–800 ng
cosmid, BAC	0.5–1.0 µg

The ranges given in the table above should work for 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.

Cycle Sequencing

Overview

These protocols have been optimized for all Applied Biosystems thermal cyclers, including the DNA Thermal Cycler (TC1), the DNA Thermal Cycler 480, the CATALYST 800 Molecular Biology LabStation, the ABI PRISM 877 Integrated Thermal Cycler, and the GeneAmp PCR Systems 9600, 2400, and 9700 in 9600 emulation mode. The protocols contained in this section should work for all seven instruments.

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 °C/sec), poor (noisy) data may result.

Preparing the Reactions

The flexibility of the BigDye primers allows three options for cycle sequencing:

Reaction Type	Template	Cycle
1X	♦ PCR product ♦ plasmid ♦ M13	standard
0.5X	♦ PCR product ♦ plasmid ♦ M13	standard
High-sensitivity (2X)	♦ BACs ♦ extra long PCR products ♦ other large DNA	modified

IMPORTANT Prepare separate tubes for each of the four reactions (A, C, G, and T).

1X Reactions

Step	Action				
1	Aliquot the following reagents into four PCR tubes:				
	Reagent	**A** (µL)	**C** (µL)	**G** (µL)	**T** (µL)
	Ready Reaction Premix	4	4	4	4
	DNA Template (see Table 3 on page 13 for quantity)	1	1	1	1
	Total Volume	5	5	5	5
2	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**				
	a. Add 20 µL of light mineral oil. b. Spin to layer the oil over the aqueous reaction.				

0.5X Reactions

Dilute 5X Sequencing Buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0—P/N 4305605, 600 reactions; 4305603, 5400 reactions) with four parts deionized water to 1X for use in this procedure.

Step	Action				
1	Dilute each Ready Reaction Premix (A, C, G, T) 1:1 with 1X Sequencing Buffer in a separate tube (*e.g.*, 2 µL of A Mix and 2 µL of 1X Sequencing Buffer).				
2	Aliquot the following reagents into four PCR tubes for each DNA template:				
	Reagent	**A** (µL)	**C** (µL)	**G** (µL)	**T** (µL)
	Diluted Ready Reaction Premix	4	4	4	4
	DNA Template (see Table 3 on page 13 for quantity)	1	1	1	1
	Total Volume	5	5	5	5
3	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**				
	a. Add 20 µL of light mineral oil. b. Spin to layer the oil over the aqueous reaction.				

High-sensitivity (2X) Reactions

Aliquot the following reagents into four PCR tubes:

Reagent	A (µL)	C (µL)	G (µL)	T (µL)
Ready Reaction Premix	8	8	8	8
DNA Template (see Table 3 on page 13 for quantity)	2	2	2	2
Total Volume	10	10	10	10

Note Use the GeneAmp PCR System 9600 or 9700 for cycle sequencing high-sensitivity reactions. The use of other thermal cyclers is not supported.

If the majority of your applications require high sensitivity, please contact your local Field Applications Specialist or Technical Support for more options.

Cycle Sequencing on the GeneAmp 9600, 2400, or 9700

This protocol is recommended for use with the GeneAmp PCR Systems 9600 and 2400, and 9700 in 9600 emulation mode. The total time required for the cycling in this protocol is approximately 1 hour and 15 minutes.

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 5 µL.
2	Repeat the following for 15 cycles: ♦ Rapid thermal ramp[a] to 96 °C ♦ 96 °C for 10 sec. ♦ Rapid thermal ramp to 55 °C ♦ 55 °C for 5 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
3	Repeat the following for 15 cycles: ♦ Rapid thermal ramp to 96 °C ♦ 96 °C for 10 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
4	Rapid thermal ramp to 4 °C and hold until ready to precipitate.

a. Rapid thermal ramp is 1 °C/sec.

Cycle Sequencing BAC DNA on the GeneAmp 9600 and 9700

This protocol is recommended for use with the GeneAmp PCR System 9600 and 9700 in 9600 emulation mode. The total time required for the cycling in this protocol is approximately 1 hour and 45 minutes.

Step	Action
1	Place the tubes in a thermal cycler, set the volume at 10 µL, and begin thermal cycling with the following parameters: ♦ Rapid thermal ramp[a] to 95 °C ♦ 95 °C for 5 min.
2	Repeat the following for 20 cycles: ♦ Rapid thermal ramp to 95 °C ♦ 95 °C for 30 sec. ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 15 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
3	Repeat the following for 15 cycles: ♦ Rapid thermal ramp to 95 °C ♦ 95 °C for 30 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
4	Rapid thermal ramp to 4 °C and hold until ready to precipitate.

a. Rapid thermal ramp is 1 °C/sec.

Cycle Sequencing on the TC1 or DNA Thermal Cycler 480

This protocol is recommended for use with the DNA Thermal Cycler (TC1) and the DNA Thermal Cycler 480. The total time required for the cycling in this protocol is approximately 1 hour and 45 minutes.

Step	Action
1	Place the tubes in a thermal cycler preheated to 95 °C.
2	Repeat the following for 15 cycles: ♦ Rapid thermal ramp[a] to 95 °C ♦ 95 °C for 30 sec. ♦ Rapid thermal ramp to 55 °C ♦ 55 °C for 30 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
3	Repeat the following for 15 cycles: ♦ Rapid thermal ramp to 95 °C ♦ 95 °C for 30 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
4	Rapid thermal ramp to 4 °C and hold until ready to precipitate.

a. Rapid thermal ramp is 1 °C/sec.

Cycle Sequencing on the CATALYST 800

Templates that have been prepared as described in this chapter should be suitable for use on the CATALYST 800 Molecular Biology LabStation using LabStation 3.0 protocols. 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.

Predefined temperature profiles are provided for the following:

- Double-Stranded Forward (Universal) Primer
- Double-Stranded Reverse (Universal) Primer
- Single-Stranded Forward Primer
- Quick Cycle

These are chosen during the pre-run dialogue, and can be edited to make custom profiles.

IMPORTANT Load only the reagents that you plan to use. Do not store kit reagents on the worksurface.

On extended runs (*e.g.*, overnight), we recommend withholding the addition of ethanol until you are present to complete the processing of the plate. This delay can be programmed during the prerun dialogue, in the Sample Pickup Time screen.

Directions for precipitating and centrifuging samples in MicroAmp® 9600 Trays are found in the Turbo Appendix of the *CATALYST 800 Molecular Biology LabStation User's Manual*.

After thermal cycling, remove the collection tubes containing the extension products. Follow the "Ethanol Precipitation" procedure on page 23, beginning at step 3.

Cycle Sequencing on the ABI PRISM 877 ITC

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

- Double-Stranded Forward (Universal) Primer
- Double-Stranded Reverse (Universal) Primer
- Single-Stranded Forward Primer
- Quick Cycle

These are 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* for instructions on editing temperature profiles.

IMPORTANT Load only the reagents that you plan to use. Do not store kit reagents on the worksurface.

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

Directions for precipitating and centrifuging samples in 96-well MicroAmp Trays are found in Chapter 3, "Preparing Chemistries," of the ABI PRISM 877 *Integrated Thermal Cycler User's Manual*.

Optimizing Cycle Parameters

The standard cycling conditions used in this manual have been optimized for the –21 M13 forward primer and work well for most primers. If you obtain low signal with the M13 Reverse primer, you can improve your sequencing data by altering the cycling conditions as follows:

- Standard conditions use 55 °C as the annealing temperature in the three-step cycle. Lower the annealing temperature to 45–48 °C.
- The number of three-step versus two-step cycles can also affect signal strength. Increase the number of three-step cycles to 20 instead of the standard 15 (increasing the total number of cycles to 35).

Preparing the Sample for Loading

Loading Options

There are two options for loading samples:

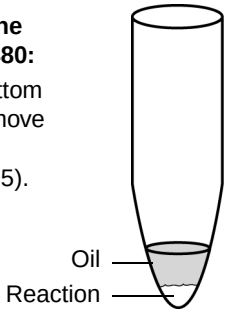
- Ethanol precipitation—samples are concentrated
- Express Load—samples are loaded directly onto a gel without being precipitated

See “Express Load Option for 36-Well Gels” on page 30.

IMPORTANT The Express Load option cannot be used on the ABI PRISM 310 Genetic Analyzer.

Ethanol Precipitation

Method 1

Step	Action
1	Add 53 µL of 95% ethanol (100 µL if sequencing BAC DNA or other high-sensitivity reactions) to a clean microcentrifuge tube. **Note** The use of sodium acetate is not necessary for precipitation.
2	Pipet the extension reactions from the bottom of each of the four tubes into the ethanol mixture. Mix thoroughly. **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 (see “Procedural Options” on page 25).  **IMPORTANT** Transfer as little oil as possible.
3	Place the tube on wet ice or leave it at room temperature for 10–15 minutes to precipitate the extension products.
4	Spin the tube in a microcentrifuge for 10–20 minutes at maximum speed. Carefully aspirate or decant the supernatant and discard. At this point, a pellet may or may not be visible.

Method 1 *(continued)*

Step	Action
5	**Optional:** Rinse the pellet with 250 µL of 70% ethanol and spin for 5 minutes in a microcentrifuge. Again, carefully aspirate or decant the supernatant and discard. This may remove some of the salts from the pellet, but doing so is often not necessary. **Note** If you use sodium acetate, you must rinse the pellet. This will reduce the carryover of salt.
6	Dry the pellet in a vacuum centrifuge for 1–3 minutes, or until dry. Do not overdry.

Method 2

Step	Action
1	Add 53 µL of 95% ethanol (100 µL if sequencing BAC DNA or other high-sensitivity reactions) to the “A” reaction tube. **Note** This method will not work if the TC1 or DNA Thermal Cycler 480 was used for thermal cycling.
2	Transfer the contents of the “A” reaction tube into the “C” reaction tube.
3	Pipet that mixture into the “G” reaction tube and so on until the contents of all four reaction tubes have been transferred into a single 1.5-mL microcentrifuge tube.
4	Spin the tube in a microcentrifuge for 10–20 minutes at maximum speed. Carefully aspirate the supernatant and discard. At this point, a pellet may or may not be visible.
5	**Optional:** Rinse the pellet with 250 µL of 70% ethanol and spin for 5 minutes in a microcentrifuge. Again, carefully aspirate the supernatant and discard. This may remove some of the salts from the pellet, but doing so is often not necessary. **Note** If you use sodium acetate, you must rinse the pellet. This will reduce the carryover of salt.
6	Dry the pellet in a vacuum centrifuge for 1–3 minutes, or until dry. Do not overdry.

Procedural Options

Removing Reactions From Beneath Mineral Oil

Removing the four reactions from under the oil can be difficult because of their small (5 µL) volumes. If this is a problem, try one of the following options:

- After cycling, add 5 µL of deionized water to each reaction for a total volume of 10 µL.

  If this is done, increase the amount of 95% ethanol used in step 1 of "Ethanol Precipitation" on page 23 to 110 µL.

- Double the amount of Ready Reaction Premix and DNA template for each reaction (A, C, G, and T) to yield several loadings' worth (*i.e.*, a 2X reaction).

Sample Electrophoresis

Overview

Electrophoresis of samples sequenced with the BigDye terminators requires run modules and dye set/primer (mobility) files for Filter Set E. These files are included in the current versions of data collection software for the ABI PRISM 310 and ABI PRISM 377 instruments.

The files can also be obtained from the Applied Biosystems site on the World Wide Web:

- www.appliedbiosystems.com/techsupport

Data analysis requires a Filter Set E instrument (matrix) file made from 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.

If you do not have access to the World Wide Web, 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

Use the appropriate run module for your run parameters on your instrument as shown below.

Instrument	Configuration	Run Module
ABI PRISM 310	Performance Optimized Polymer 6 (POP-6), 1-mL syringe	Seq POP6 (1 mL) E
	POP-6, 1-mL syringe, Rapid Sequencing	Seq POP6 Rapid (1 mL) E
ABI PRISM 377[a]	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. Use any plate check and prerun module on the ABI PRISM 377 DNA Sequencer.

Dye Set/Primer Files

Use the correct dye set/primer (mobility) file for your instrument as shown below.

Instrument	Dye Set/Primer File
ABI PRISM 310, POP-6 polymer, –21 M13 primers	DP POP6 {BD Set-21M13}
ABI PRISM 310, POP-6 polymer, M13 Reverse primers	DP POP6 {BD Set-M13 Reverse}
ABI PRISM 377[a]	DP5%LR{BD M13 FWD & REV}

a. 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).

Electrophoresis on the ABI PRISM 310

Electrophoresis on the ABI PRISM 310 Genetic Analyzer requires the appropriate run module (see page 27), dye set/primer (mobility) file (see page 27), and matrix file.

To run the samples on the ABI PRISM 310 Genetic Analyzer:

Step	Action
1	Resuspend each sample pellet in 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 to denature, then chill on ice.
4	Vortex and spin the samples again. Place on ice until ready to use. **Note** Occasionally, you may want to prepare only a portion of a sequencing reaction for analysis on the ABI PRISM 310 Genetic Analyzer and reserve the rest for analysis later or elsewhere (refer to the ABI PRISM 310 *Genetic Analyzer User's Manual* for more information).
5	Refer to the ABI PRISM 310 *Genetic Analyzer User's Manual* for guidelines on loading the samples into the autosampler tray.

Note Although 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.

Note Occasionally, you may want to prepare only a portion of a sequencing reaction for analysis on the ABI PRISM 310 instrument and reserve the rest for analysis later or elsewhere. Refer to the ABI PRISM 310 *Genetic Analyzer User's Manual* for more information.

Electrophoresis on the ABI PRISM 377

Electrophoresis on the ABI PRISM 377 DNA Sequencer (all models[1]) requires the appropriate run module (see page 27), dye set/primer (mobility) file (see page 27), and instrument (matrix) file. You can use any plate check and prerun modules.

Running ethanol-precipitated samples

Step	Action			
1	Prepare a loading buffer by combining the following in a 5:1 ratio: ♦ deionized formamide ♦ 25 mM EDTA (pH 8.0) with blue dextran (50 mg/mL) **! WARNING ! CHEMICAL HAZARD Formamide is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling.**			
2	Resuspend each sample pellet in loading buffer as follows:			
	Template	**Reaction**	**Volume (µL), 36-well**	**Volume (µL), 48- or 64-well**
	PCR product, plasmid, M13	1X	6	4
		0.5X	2–4	1–2
	BAC, large DNA	2X	2–4	1–2
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	**Reaction**	**Volume (µL), 36-well**	**Volume (µL), 48- or 64-well**
	PCR product, plasmid, M13	1X	1–1.5	1
		0.5X	2	1
	BAC, large DNA	2X	2	1

1. 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.

Express Load Option for 36-Well Gels

Step	Action
1	Combine the four reactions (A, C, G, T) with 5 µL of 5 mM EDTA (25 µL total volume).
2	Vortex briefly, then spin in a microcentrifuge.
3	Prepare a loading buffer by combining the following in a 5:1 ratio: ♦ deionized formamide ♦ 25 mM EDTA (pH 8.0) with blue dextran (50 mg/mL) **! WARNING ! CHEMICAL HAZARD Formamide is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling.**
4	Combine 4 µL of each 25-µL reaction/EDTA mixture with 4 µL of loading buffer. Store the remaining reaction/EDTA mixture at –15 to –25 °C. This mixture can be concentrated by ethanol precipitation if the Express Load procedure does not yield enough signal.
5	Heat the samples at 98 °C for 5 minutes with the lids open to denature and concentrate. Place on ice until ready to load.
6	Load 2.5 µL of each sample into a separate lane of the gel.

Setting the Data Analysis Range

The unincorporated dye primer peak at the beginning of the electropherogram (and the full-length PCR peak at the end of the electropherogram if sequencing PCR products) must be excluded from analysis for data to be normalized correctly.

Analysis of ABI PRISM 310 data is especially sensitive to the location of the Peak 1 Location and Analysis Start Point. The software should pick the these correctly in most cases. A miscalled Peak 1 Location and/or Analysis Start Point can result in spacing anomalies visible in the analyzed data.

Refer to page 6-15 of the *Automated DNA Sequencing Chemistry Guide* (P/N 4305080, www.appliedbiosystems.com/techsupport) for more information on setting the data analysis range.

Appendix A. Control Sequences

Partial Sequence of pGEM-3Zf(+) from the –21 M13 Forward Primer

The sequence of the –21 M13 forward primer, followed by the ensuing 1000 bases, is shown here.

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

Partial Sequence of pGEM-3Zf(+) from the M13 Reverse Primer

The sequence of the M13 reverse primer, followed by the ensuing 1000 bases, is shown here.

CAGGAAACAGCTATGACC (M13 reverse primer)

ATGATTACGC	CAAGCTATTT	AGGTGACACT	ATAGAATACT	40
CAAGCTTGCA	TGCCTGCAGG	TCGACTCTAG	AGGATCCCCG	80
GGTACCGAGC	TCGAATTCGC	CCTATAGTGA	GTCGTATTAC	120
AATTCACTGG	CCGTCGTTTT	ACAACGTCGT	GACTGGGAAA	160
ACCCTGGCGT	TACCCAACTT	AATCGCCTTG	CAGCACATCC	200
CCCTTTCGCC	AGCTGGCGTA	ATAGCGAAGA	GGCCCGCACC	240
GATCGCCCTT	CCCAACAGTT	GCGCAGCCTG	AATGGCGAAT	280
GGACGCGCCC	TGTAGCGGCG	CATTAAGCGC	GGCGGGTGTG	320
GTGGTTACGC	GCAGCGTGAC	CGCTACACTT	GCCAGCGCCC	360
TAGCGCCCGC	TCCTTTCGCT	TTCTTCCCTT	CCTTTCTCGC	400
CACGTTCGCC	GGCTTTCCCC	GTCAAGCTCT	AAATCGGGGG	440
CTCCCTTTAG	GGTTCCGATT	TAGTGCTTTA	CGGCACCTCG	480
ACCCCAAAAA	ACTTGATTAG	GGTGATGGTT	CACGTAGTGG	520
GCCATCGCCC	TGATAGACGG	TTTTTCGCCC	TTTGACGTTG	560
GAGTCCACGT	TCTTTAATAG	TGGACTCTTG	TTCCAAACTG	600
GAACAACACT	CAACCCTATC	TCGGTCTATT	CTTTTGATTT	640
ATAAGGGATT	TTGCCGATTT	CGGCCTATTG	GTTAAAAAAT	680
GAGCTGATTT	AACAAAAATT	TAACGCGAAT	TTTAACAAAA	720
TATTAACGCT	TACAATTTCC	TGATGCGGTA	TTTTCTCCTT	760
ACGCATCTGT	GCGGTATTTC	ACACCGCATA	TGGTGCACTC	800
TCAGTACAAT	CTGCTCTGAT	GCCGCATAGT	TAAGCCAGCC	840
CCGACACCCG	CCAACACCCG	CTGACGCGCC	CTGACGGGCT	880
TGTCTGCTCC	CGGCATCCGC	TTACAGACAA	GCTGTGACCG	920
TCTCCGGGAG	CTGCATGTGT	CAGAGGTTTT	CACCGTCATC	960
ACCGAAACGC	GCGAGACGAA	AGGGCCTCGT	GATACGCCTA	1000

Appendix B. Technical Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then 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 access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

ABI PRISM® DNA Sequencing Kits

To order ABI PRISM DNA Sequencing Kits, please contact Applied Biosystems at one of the regional sales offices listed in Appendix B, Technical Support. 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	–
403047	dRhodamine Matrix Standards Kit	–

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	–
403047	dRhodamine Matrix Standards Kit	–

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

P/N	Kit	Reactions
4303573	Ready Reaction	24
4303149	Ready Reaction	100
4303150	Ready Reaction	1000
4303151	Ready Reaction	5000
4303237	Protocol	–
403047	dRhodamine Matrix Standards Kit	–

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