ABI PRISM® dRhodamine Terminator 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

New Dye Terminators

Applied Biosystems has designed new dichlororhodamine (dRhodamine) dye terminators to give more even peak heights than the original rhodamine dye terminators. The new dyes have narrower emission spectra, giving less spectral overlap and therefore less noise.

The new dRhodamine dye terminators have the following dye labels:

Terminator	Dye Label	Color of Raw Data on ABI PRISM 310 Electropherogram	Color of Raw Data on ABI PRISM 377 Gel Image
A	dichloro[R6G]	green	green
C	dichloro[TAMRA]	black	yellow
G	dichloro[R110]	blue	blue
T	dichloro[ROX]	red	red

Data collected in Applied Biosystems laboratories shows more uniform signal intensities with the new dyes and a reduction of the weak G after

A pattern that is characteristic of the original rhodamine dye terminators.

With less noise, better signal uniformity, and a reduced weak G after A pattern, the new dRhodamine dye terminators can give better sequencing results than the rhodamine dye terminators (Figure 1 and Figure 2).

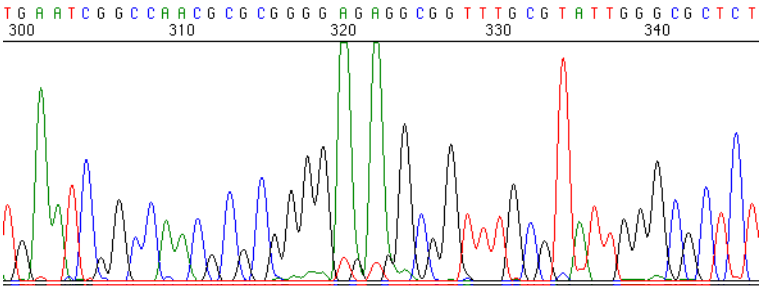

Figure 1 Region of pGEM-3Zf(+) sequenced with rhodamine dye terminators

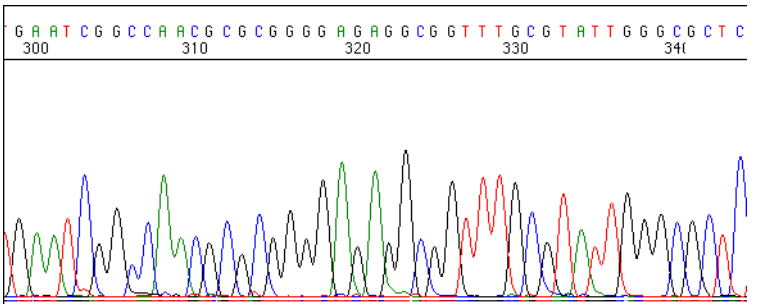

Figure 2 Region of pGEM-3Zf(+) sequenced with dRhodamine dye terminators

Note in particular that the G nucleotides after A nucleotides near base 320 are much stronger with the dRhodamine dye terminators than with the rhodamine dye terminators.

dRhodamine Dye Terminator Structures

The structures of the dRhodamine dye terminators are shown in Figure 3.

Note Uracil is used in place of thymine in the T-Dye Terminator.

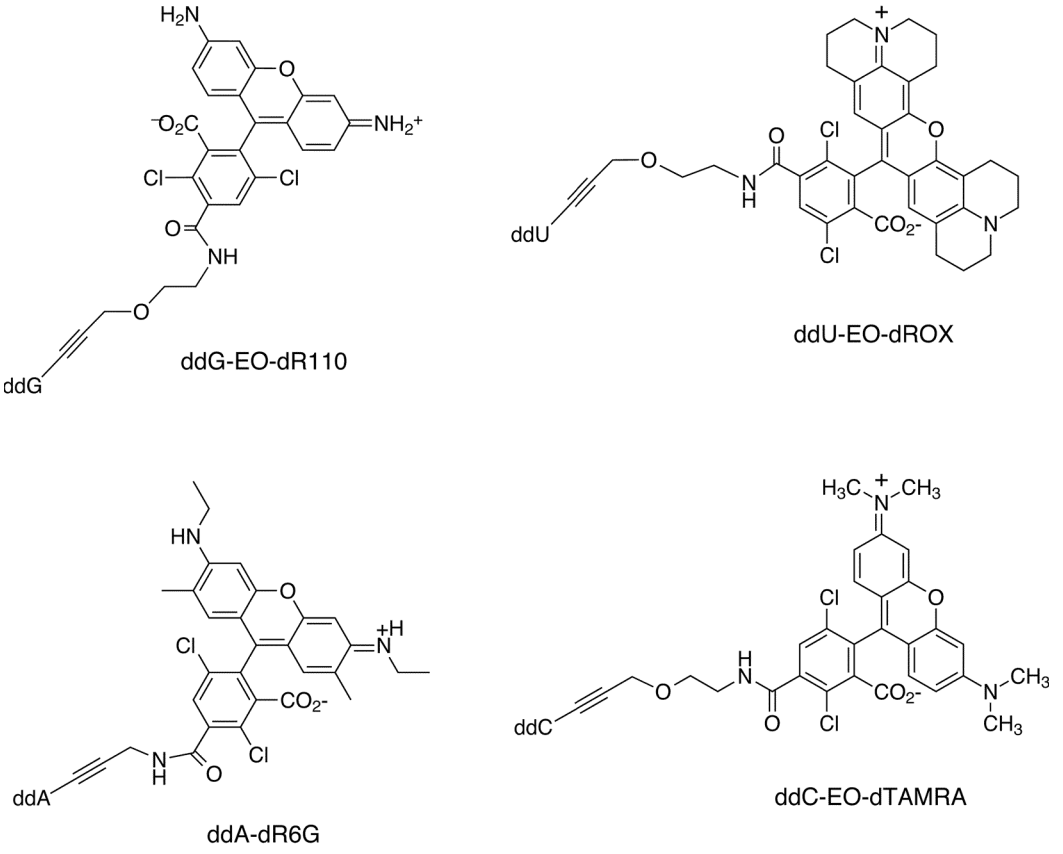

Figure 3. dRhodamine dye terminators

Dye Spectra

The normalized absorption and emission spectra of the dRhodamine dyes are shown in Figure 4 and Figure 5, respectively.

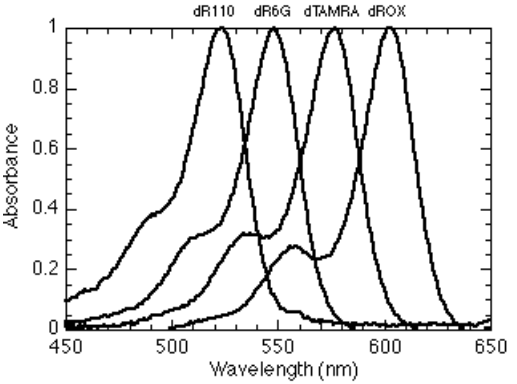

Figure 4. Absorption spectra of dRhodamine dyes

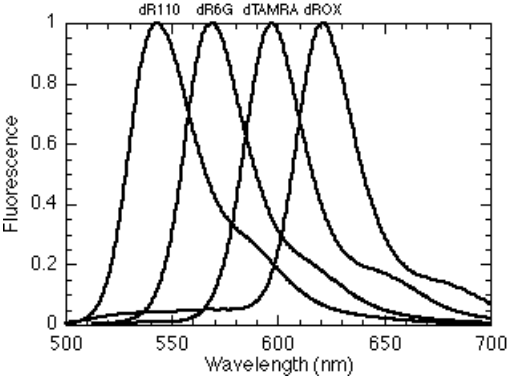

Figure 5. Emission spectra of dRhodamine dyes

dRhodamine Terminator Ready Reaction Kit

The ABI PRISM® dRhodamine Terminator Cycle Sequencing Ready Reaction Kit combines the unique properties of AmpliTaq DNA Polymerase, FS and the new dRhodamine dye terminators with the convenience of the Ready Reaction format.

In this format, the dye terminators, deoxynucleoside triphosphates, enzymes, magnesium chloride, and buffer are premixed into a single tube of Ready Reaction Mix and are ready to use. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates or on polymerase chain reaction (PCR) fragments.

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.

General instructions are given for using the kit reagents to generate samples for the ABI PRISM® 310 Genetic Analyzer, the ABI PRISM® 377 DNA Sequencer, and the ABI PRISM 377 DNA Sequencer with XL Upgrade ("ABI PRISM 377XL"). For more detailed instructions, refer to the appropriate instrument user's manual.

IMPORTANT You must install new dye set/primer (mobility) files and run modules (see "Sample Electrophoresis" on page 25) and make new instrument (matrix) files to use the dRhodamine dye terminators (see Appendix A on page 29).

Instrument Platforms

The ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit is for use with the ABI PRISM 310 Genetic Analyzer, the ABI PRISM 377 DNA Sequencer, and the ABI PRISM 377 DNA Sequencer with XL Upgrade.

IMPORTANT This kit is not designed for use with the ABI™ 373 DNA Sequencer or the ABI 373 DNA Sequencer with XL Upgrade.

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 dRhodamine Terminator Cycle Sequencing Ready Reaction Kit.

Materials

Ready Reaction Kit Reagents

The ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kits contain sufficient reagents to sequence 100 or 1000 templates (P/N 403044 and 403045, respectively). Enough standard primer and double-stranded template for 12 control reactions are included in the 100-reaction kits. The 1000-reaction kits contain enough standard primer and template for at least 50 reactions. The kit reagents are listed below.

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

Storage and Use of the Kit

The ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit should be stored at –15 to –25 °C. Prior to 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.

Reagents and Equipment Not Included

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 dRhodamine terminator 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.

Reagents and Equipment

Table 1. Reagents Supplied by the User

Reagent	Source
ABI PRISM dRhodamine Matrix Standards Kit	Applied Biosystems (P/N 403047)
dRhodamine Terminator Cycle Sequencing Standard with AmpliTaq DNA Polymerase, FS	Applied Biosystems (P/N 4303120, available September 1997)
DNA Sequencing Polymer with Template Suppression Reagent (TSR)	Applied Biosystems (P/N 402091)
ABI PRISM 310 Genetic Analyzer Buffer (for use with DNA Sequencing Polymer)	Applied Biosystems (P/N 401884)
Performance Optimized Polymer 6 (POP-6™) with TSR	Applied Biosystems (P/N 402844)
ABI PRISM 310 Genetic Analyzer Buffer with EDTA (for use with POP-6)	Applied Biosystems (P/N 402824)
Deionized formamide	Major laboratory suppliers (MLS)
Deionized water	MLS
25 mM EDTA (ethylenediaminetetraacetate) with 50 mg/mL blue dextran, pH 8.0	Applied Biosystems (P/N 402055)
Ethanol (EtOH), non-denatured, 95% or 70%	MLS
Magnesium chloride ($MgCl_2$), 0.5 M or 2 mM	MLS
Mineral oil, for the DNA Thermal Cycler (TC1) and the DNA Thermal Cycler 480	Applied Biosystems (P/N 0186-2302)

Table 1. Reagents Supplied by the User *(continued)*

Reagent	Source
Shrimp alkaline phosphatase (SAP), 1 U/µL, and 10X SAP buffer (200 mM Tris-HCl, 100 mM $MgCl_2$, pH 8.0)	Amersham Life Science (P/N E 70092 Z)
Sodium acetate (NaOAc), 3 M, pH 4.6	Applied Biosystems (P/N 400320)

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)
DNA Sequencing Capillaries, internally coated (for use with the DNA Sequencing Polymer)	Applied Biosystems (P/N 401821)
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
Spin column, Centri-Sep, 1-mL	
♦ in North America:	Princeton Separations (P/N CS-901)
♦ outside North America:	Applied Biosystems:
32 columns	P/N 401763
100 columns	P/N 401762
Table top centrifuge, with 96-tube tray adaptor	MLS
Thermal cycler	Applied Biosystems

Table 2. Equipment Supplied by the User *(continued)*

Item	Source
Vacuum centrifuge	Savant Speedvac (P/N DNA100) or equivalent
Vortexer	MLS

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Preparation of Templates

Control DNA Templates

Always use a control template. We recommend M13mp18 as a single-stranded control, pGEM-3Zf(+) as a double-stranded control.

Single-stranded DNA Templates

A protocol for preparing M13 templates is provided in the *ABI PRISM DNA Sequencing Guide* (P/N 903563). Prepare adequate template to check purity and to quantitate the DNA accurately. The recommended concentrations and quantities are shown in Table 3 on page 13.

Double-stranded DNA Templates

The quality of your sequencing results will be directly proportional to the quality of your starting DNA template. The optimal procedure for preparing a particular plasmid depends on the particular bacterial strain and the yield of each construct. Good sequencing data has been obtained from plasmids isolated by cesium-banding methods and alkaline lysis “miniprep” methods, such as the ABI PRISM Plasmid Miniprep Kit (P/N 402790 or 402791). The recommended concentrations and quantities are shown in Table 3 on page 13.

Transposons

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

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

The Primer Island Transposition Kit (P/N 402984) provides reagents for generating artificial transposon insertions into target DNA *in vitro*. The artificial transposon contains the PI(+) and PI(–) priming sites. The Primer Island reagents are combined with a target DNA of choice and used to transform *Escherichia coli*.

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

The target DNA can be isolated for sequencing or PCR analysis. Refer to the *Primer Island Transposition Kit Protocol* (P/N 402920) for more information.

Symmetric PCR Templates

Cycle sequencing has been found to provide the most reproducible results when sequencing symmetric templates. Although symmetric 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” below.

Purifying PCR Fragments

To purify PCR fragments:

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.

DNA Quantity

If possible, quantitate the amount of purified DNA by measuring the absorbance at 260 nm or by some other method. The recommended concentrations and amounts are shown in Table 3.

Table 3. Recommended DNA Concentrations and Quantities

DNA	Concentration	Quantity
single-stranded DNA	50–100 ng/µL	50–100 ng
double-stranded DNA	100–200 ng/µL	200–500 ng
PCR product DNA	5–15 ng/µL	30–90 ng

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.

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

Cycle Sequencing

Overview

This protocol has 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 and 2400. The protocols contained in this document should work for all six 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 °/sec), poor (noisy) data may result.

Mixing the Reagents

Note The type of tube required depends on the type of thermal cycler used. For the DNA Thermal Cycler (TC1) and DNA Thermal Cycler 480, use 0.5-mL GeneAmp Thin-Walled PCR tubes. For the GeneAmp PCR Systems 9600 and 2400, use 0.2-mL MicroAmp® PCR tubes. If using the CATALYST 800 or ABI PRISM 877, refer to your instrument user's manual for reaction setup.

To prepare the reaction mixtures:

Step	Action	
1	For each reaction, add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix	8.0 µL
	Template	–
	single-stranded DNA	50–100 ng
	double-stranded DNA	200–500 ng
	PCR product	30–90 ng
	Primer	3.2 pmol
	Deionized water	q.s.
	Total Volume	20 µL
2	Mix well and spin briefly.	
3	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**	
	Overlay the reaction mixture with 40 µL of light mineral oil.	

Cycle Sequencing on the GeneAmp 9600 or 2400

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

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 20 µL.
2	Repeat the following for 25 cycles: ♦ Rapid thermal ramp 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.
3	Rapid thermal ramp to 4 °C and hold until ready to purify.
4	Spin down the contents of the tubes in a microcentrifuge.
5	Proceed to “Purifying Extension Products” on page 18.

Cycle Sequencing on the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480

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

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 20 µL.
2	Repeat the following for 25 cycles: ♦ Rapid thermal ramp to 96 °C ♦ 96 °C for 30 sec. ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 15 sec. ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 4 min.
3	Rapid thermal ramp to 4 °C and hold until ready to purify.
4	Spin down the contents of the tubes in a microcentrifuge.
5	Proceed to “Purifying Extension Products” on page 18.

Cycle Sequencing on the CATALYST 800

Templates that have been prepared as described on page 11 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* to set up your reactions.

CATALYST Turbo offers Primer and Terminator Sequencing options. Predefined temperature profiles are provided for Double-Stranded Forward (Universal) Primer, Double-Stranded Reverse (Universal) Primer, Single-Stranded Forward Primer, Quick Cycle (for primer sequencing), and Terminator. These are chosen during the pre-run dialogue, and can be edited to make custom profiles.

Terminator Sequencing has two options:

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

  This eliminates the requirement for premixing samples and primers.

Ethanol precipitation is not available for Terminator Sequencing protocols on the CATALYST 800 Molecular Biology LabStation. Ethanol precipitation or spin-column purification must be performed manually. See "Purifying Extension Products" on page 18.

Note Ethanol precipitation is available for Terminator Sequencing protocols on the ABI PRISM 877 Integrated Thermal Cycler (see page 17).

Cycle Sequencing on the ABI PRISM 877 ITC

The ABI PRISM 877 Integrated Thermal Cycler offers Primer, Terminator, and Terminator Automix Sequencing options.

Predefined temperature profiles are provided for Terminator Sequencing. The profile is chosen on the Chemistry page of the Sequencing Notebook and can be edited to make custom profiles. Refer to Chapter 4, "Using the ABI PRISM 877 Software," in the ABI PRISM 877 *Integrated Thermal Cycler User's Manual* for instructions on editing temperature profiles.

Terminator Sequencing uses a reaction premix containing the sequencing primer, or else requires premixing template with primer in the sample tube. Terminator Automix Sequencing combines reaction cocktail (lacking primers), water, primer from one tube, and template from another tube. This eliminates the requirement for premixing of samples and primers. Refer to Chapters 2 and 4, "Getting Started" and "Using the ABI PRISM 877 Software," respectively, in the ABI PRISM 877 *Integrated Thermal Cycler User's Manual*.

Ethanol precipitation can be chosen for dye terminator sequencing. The proportions of ethanol and precipitation additive are set for default reaction volumes. These volumes can be changed, especially if the reaction volume is modified. After the program is completed, proceed to "Purifying Extension Products" on page 18.

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

Various options are available for purge of the thermal cycler plate:

- "Quick purge" rinses the plate twice with water.
- "Standard purge" and "Terminator purge" rinse the plate with water, Tris/Tween-20 solution, then water again, and heat the plate during the first two rinses.
- "Bleach purge" rinses the plate with sodium hypochlorite solution, water, Tris/Tween-20 solution, and water again.

  "Bleach purge" is the most extensive and most time-consuming purge, but is recommended for regular (*e.g.*, weekly) complete cleaning of plates, especially when PCR products are used as templates.

Purifying Extension Products

Overview

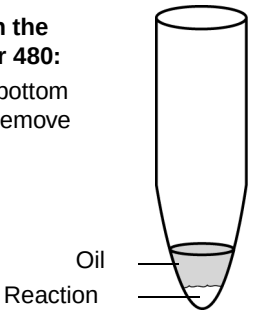
The ABI PRISM Dye Terminator Cycle Sequencing Kits with AmpliTaq DNA Polymerase, FS require much lower amounts of dye terminators than earlier kits with AmpliTaq DNA Polymerase. As a result, excess dye terminators can be removed either by ethanol precipitation or by using a spin column.

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

Note These protocols are for use only with AmpliTaq DNA Polymerase, FS, dRhodamine dye terminator chemistry. For purifying extension products of other chemistries, refer to the appropriate protocol or chemistry guide.

Ethanol/Sodium Acetate Precipitation Procedure

To remove excess dye terminators:

Step	Action
1	For each sequencing reaction, prepare a 1.5-mL microcentrifuge tube containing the following: ♦ 2.0 µL of 3 M sodium acetate (NaOAc), pH 4.6 ♦ 50 µL of 95% ethanol (EtOH)
2	Pipet the entire contents of each extension reaction into a tube of sodium acetate/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.  **IMPORTANT** Transfer as little oil as possible.
3	Vortex the tubes and place on ice for 10 minutes to precipitate the extension products.

To remove excess dye terminators: *(continued)*

Step	Action
4	Spin the tubes in a microcentrifuge for 15–30 minutes at maximum speed.
5	Carefully aspirate the supernatant with a pipette and discard.
6	Rinse the pellet with 250 µL of 70% ethanol.
7	Spin for five minutes in a microcentrifuge at maximum speed. Again, carefully aspirate or decant the supernatant and discard.
8	Dry the pellet in a vacuum centrifuge for 10–15 minutes, or until dry. Do not over-dry.

Simplified Ethanol Precipitation Procedures

These procedures require 70% ethanol (EtOH) containing 0.5 mM $MgCl_2$. This reagent can be prepared *in situ* or as a stock solution.

To prepare the 70% EtOH/0.5 mM $MgCl_2$ stock solution:

Step	Action
1	Combine the following in a 1.5-mL microcentrifuge tube: ♦ 1 mL 70% EtOH ♦ 1 µL 0.5 M $MgCl_2$
2	Vortex briefly to mix.

Precipitation in microcentrifuge tubes

Step	Action
1	Pipet the entire contents of each extension reaction into a 1.5-mL microcentrifuge tube. **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 2 of the EtOH/NaOAc procedure on page 18.
2	Add 74 µL of 70% EtOH/0.5 mM $MgCl_2$ to each tube. (Alternatively, add 20 µL of 2 mM $MgCl_2$ and then 55 µL of 95% ethanol.)
3	Close the tubes and vortex briefly.

Precipitation in microcentrifuge tubes *(continued)*

Step	Action
4	Leave the tubes at room temperature for 10–15 minutes to precipitate the extension products. **Note** Precipitation times less than 5 minutes will result in the loss of very short extension products. Precipitation times greater than 24 hours will increase the precipitation of unincorporated dye terminators.
5	Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 10–20 minutes at maximum speed. **IMPORTANT** Proceed to the next step immediately.
6	Carefully aspirate the supernatants with a separate pipette for each sample and discard. Pellets may or may not be visible. **IMPORTANT** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.
7	Visually inspect the sample tubes for residual supernatants. If there are any residual supernatants: a. Place the tubes in the microcentrifuge in the same orientation as in step 5 and spin for 5–10 seconds. b. Aspirate the supernatants carefully as in step 6.
8	Dry the samples in a vacuum centrifuge for 10–15 minutes or to dryness. (Alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90 °C for 1 minute.)

Precipitation in MicroAmp Trays

Step	Action
1	Remove the MicroAmp Tray from the thermal cycler. Remove the caps from each tube.
2	Add 74 µL of 70% EtOH/0.5 mM $MgCl_2$ to each tube. (Alternatively, add 20 µL of 2 mM $MgCl_2$ and then 55 µL of 95% ethanol.)
3	Seal the tubes by applying a piece of 3M Scotch Tape 425-3 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
4	Invert the tray a few times to mix.

Precipitation in MicroAmp Trays *(continued)*

Step	Action
5	Leave the tray at room temperature for 10–15 minutes to precipitate the extension products. **Note** Precipitation times less than 5 minutes will result in the loss of very short extension products. Precipitation times greater than 24 hours will increase the precipitation of unincorporated dye terminators.
6	Place the tray in a table-top centrifuge with tube-tray adaptor for 20–30 minutes at the maximum speed. This should be at least 1400 × *g* but less than 3000 × *g*. **Note** A MicroAmp tube in a MicroAmp Tray can withstand 3000 × *g* for 30 minutes. **IMPORTANT** Proceed to the next step immediately.
7	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the tray onto a paper towel folded to the size of the tray.
8	Place the inverted tray with the towel into the table-top centrifuge and spin at 500–1000 × *g* for 1 minute.
9	Remove the tray and discard the paper towel. **Note** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.

Shrimp Alkaline Phosphatase Digestion (Optional)

The following shrimp alkaline phosphatase (SAP) digestion procedure is optional. It can be used for more efficient removal of unincorporated dye terminators.

To perform shrimp alkaline phosphatase (SAP) digestion:

Step	Action
1	At the end of thermal cycling, add 2 µL of SAP (1 U/µL) and 18 µL of 1X SAP buffer to each tube. Seal each tube and incubate at 37 °C for 30 minutes.
2	**For precipitation in microcentrifuge tubes:**
	a. Transfer the contents of each tube to a 1.5-mL microcentrifuge tube. b. Add 150 µL of 70% EtOH/0.5 mM $MgCl_2$ to each tube. (Alternatively, add 40 µL of 2 mM $MgCl_2$ and then 110 µL of 95% ethanol.) c. Proceed to step 3 of “Precipitation in microcentrifuge tubes” on page 19.
	For precipitation in MicroAmp Trays:
	a. Add 150 µL of 70% EtOH/0.5 mM $MgCl_2$ to each tube. (Alternatively, add 40 µL of 2 mM $MgCl_2$ and then 110 µL of 95% ethanol.) b. Proceed to step 3 of “Precipitation in MicroAmp Trays” on page 20.

Spin Column Purification

We recommend Centri-Sep spin columns from Princeton Separations (P/N CS-901). Refer to the manufacturer's instructions and *User Bulletin Number 20* for more details.

Tips for optimizing spin column purification:

- Use one column for each sample. Do not process more columns than you can handle conveniently at one time.
- Load the sample in the center of the column bed. Make sure that the sample does not touch the sides of the column.
- Spin the column at 325–1300 × *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

  rpm = revolutions per minute

  r = radius of the rotor in cm
- The entire spin column procedure should be performed without interruption to ensure optimal results. Do not allow the column to dry out.

To perform spin column purification:

Step	Action
1	Gently tap the column to cause the gel material to settle to the bottom of the column.
2	Remove the upper end cap and add 0.8 mL of deionized water.
3	Replace the upper end cap and invert the column a few times to mix the water and gel material.
4	Allow the gel to hydrate at room temperature for at least 30 minutes. **Note** Rehydrated columns can be stored for a few days at 2–6 °C. Longer storage in water is not recommended. Allow columns that have been stored at 2–6 °C to warm to room temperature before use.
5	Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.

To perform spin column purification: *(continued)*

Step	Action
6	Remove the upper end cap first, then remove the bottom cap. Allow the column to drain completely by gravity. **Note** If flow does not begin immediately, apply gentle pressure to the column with a pipette bulb.
7	Insert the column into the wash tube provided.
8	Spin the column in a microcentrifuge at 1300 × *g* for 2–4 minutes to remove the interstitial fluid.
9	Remove the column from the wash tube and insert it into a sample collection tube (*e.g.*, a 1.5-mL microcentrifuge tube).
10	Remove the extension reaction mixture from its tube and load it carefully on top of the gel material. **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 2 of the EtOH/NaOAc procedure on page 18.
11	Spin the column in a microcentrifuge at 1300 × *g* for 2–4 minutes. **Note** If using a centrifuge with a fixed-angle rotor, place the column in the same orientation as it was in for the first spin. This is important because the surface of the gel will be at an angle in the column after the first spin.
12	Discard the column. The sample is in the sample collection tube.
13	Dry the sample in a vacuum centrifuge for 10–15 minutes, or until dry. Do not over-dry.

Sample Electrophoresis

Overview

Follow the procedure below for the sequencer you use. For more information about using a specific instrument, refer to the user's manual for that instrument.

Cycle sequencing with the dRhodamine dye terminators requires new run modules and dye set/primer (mobility) files that are found on the diskette supplied with the dRhodamine Matrix Standards Kit (P/N 403047). They can also be obtained from the Applied Biosystems site on the World Wide Web (www.appliedbiosystems.com/techsupport), from Applied Biosystems Technical Support, or from your local field applications specialist (call your local sales office for more information).

IMPORTANT You cannot use dRhodamine dye terminators and rhodamine dye terminators on the same gel. The two types of terminators use different run modules, dye set/primer (mobility) files, and instrument (matrix) files.

Run Modules

Use the appropriate run module as shown in Table 4.

Table 4. Run Modules

Instrument	Configuration	Run Module
ABI PRISM 310	DNA Sequencing Polymer, 250-µL syringe, DNA Sequencing Capillary	Seq Run (250 uL) 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 (1 mL) Rapid E
ABI PRISM 377	36-cm wtr, 2X (1200 scans/hr), any comb	Seq Run 36E-1200
	36-cm wtr, 4X (2400 scans/hr), any comb	Seq Run 36E-2400
	48-cm wtr, 2X (1200 scans/hr), any comb	Seq Run 48E-1200
ABI PRISM 377 with XL Upgrade	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

Dye Set/Primer Files

Use the correct dye set/primer (mobility) file for your instrument as shown in Table 5.

Table 5. Dye Set/Primer (Mobility) Files

Instrument	Dye Set/Primer File
ABI PRISM 310	DT DSP{dR Set-AnyPrimer}
ABI PRISM 310, POP-6 polymer	DT POP6{dR Set-Any Primer}
ABI PRISM 310, POP-6 polymer, Rapid Sequencing	DT POP6{dR Set-Any Primer}
ABI PRISM 377[a]	DT {dR Set Any-Primer}
ABI PRISM 377 with XL Upgrade[b]	DT {dR Set Any-Primer}

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 Table 4 on page 25), dye set/primer (mobility) file (see Table 5), and a matrix file prepared as in Appendix A on page 29.

Note The first time you run sequencing reactions with the new dRhodamine dye terminators, any matrix file (or none) can be used for the run. When the run is completed, you must make a matrix file with the new matrix standards as detailed in Appendix A on page 29.

To run the samples on the ABI PRISM 310:

Step	Action
1	Resuspend each sample pellet in 12 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 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.

Electrophoresis on the ABI PRISM 377

Electrophoresis on the ABI PRISM 377 DNA Sequencer requires the appropriate run module (see Table 4 on page 25), dye set/primer (mobility) file (see Table 5 on page 26), and an instrument (matrix) file prepared as in Appendix A on page 29. You can use any plate check and prerun modules.

Note The first time you run sequencing reactions with the new dRhodamine dye terminators, any matrix file (or none) can be used for the run. When the run is completed, you must make an instrument (matrix) file with the new matrix standards as detailed in Appendix A on page 29.

To run samples on the ABI PRISM 377:

<table>
<tr><th>Step</th><th colspan="2">Action</th></tr>
<tr><td>1</td><td colspan="2">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.</td></tr>
<tr><td>2</td><td colspan="2">Resuspend each sample pellet in 4 µL of loading buffer.</td></tr>
<tr><td>3</td><td colspan="2">Vortex and spin the samples.</td></tr>
<tr><td>4</td><td colspan="2">Heat the samples at 95 °C for two minutes to denature. Place on ice until ready to load.</td></tr>
<tr><td>5</td><td colspan="2">Load each sample into a separate lane of the gel as shown below.</td></tr>
<tr><td></td><td>Purification Method</td><td>Loading Volume (µL)</td></tr>
<tr><td></td><td>ethanol precipitation</td><td>2</td></tr>
<tr><td></td><td>spin column</td><td>1</td></tr>
</table>

Electrophoresis on the ABI PRISM 377 with XL Upgrade

Electrophoresis on the ABI PRISM 377 DNA Sequencer with XL Upgrade requires the appropriate run module (see Table 4 on page 25), dye set/primer (mobility) file (see Table 5 on page 26), and an instrument (matrix) file prepared as in Appendix A on page 29. You can use any plate check and prerun modules.

Note The first time you run sequencing reactions with the new dRhodamine dye terminators, any matrix file (or none) can be used for the run. When the run is completed, you must make an instrument (matrix) file with the new matrix standards as detailed in Appendix A on page 29.

To run samples on the ABI PRISM 377 with XL Upgrade:

<table>
<tr><th>Step</th><th colspan="3">Action</th></tr>
<tr><td>1</td><td colspan="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.</td></tr>
<tr><td rowspan="5">2</td><td colspan="3">If ethanol precipitation was used for purification:</td></tr>
<tr><td colspan="3">Resuspend each sample pellet in loading buffer as follows:
♦ 4 µL for a 36-well gel
♦ 2 µL for a 48-well or 64-well gel</td></tr>
<tr><td colspan="3">If spin columns were used for purification:</td></tr>
<tr><td colspan="3">Resuspend each sample pellet in 4 µL of loading buffer.</td></tr>
<tr><td rowspan="6"></td></tr>
<tr><td>3</td><td colspan="3">Vortex and spin the samples.</td></tr>
<tr><td>4</td><td colspan="3">Heat the samples at 95 °C for 2 minutes to denature. Place on ice until ready to load.</td></tr>
<tr><td>5</td><td colspan="3">Load each sample into a separate lane of the gel as shown below.</td></tr>
<tr><td></td><td>Comb Size</td><td>Purification Method</td><td>Loading Volume (µL)</td></tr>
<tr><td></td><td rowspan="2">36-well</td><td>ethanol precipitation</td><td>2</td></tr>
<tr><td></td><td>spin column</td><td>1</td></tr>
<tr><td></td><td rowspan="2">48-well
64-well</td><td>ethanol precipitation</td><td>1</td></tr>
<tr><td></td><td>spin column</td><td>1</td></tr>
</table>

Appendix A. Making Instrument (Matrix) Files

Multicomponent Analysis

Multicomponent analysis is the process that separates the four different fluorescent dye colors into distinct spectral components. The four dyes used in the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit are dR110, dR6G, dTAMRA, and dROX.

Each of these fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection on the ABI PRISM 310 and ABI PRISM 377 instruments, the ABI PRISM Collection software collects light intensities from four specific areas on the CCD camera, each area corresponding to the emission wavelength of a particular fluorescent dye. Each of these areas on the CCD camera is referred to as a “virtual” filter, since no physical filtering hardware (like band-pass glass filters) is used. The information that specifies the appropriate virtual filter settings for a particular set of fluorescent dyes is contained in each appropriate ABI PRISM Collection module file.

In the dRhodamine dyes, dR110 emits at the shortest wavelength and is detected as blue, followed by dR6G (green), dTAMRA (yellow) and ROX (red). Although each of these dyes emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra between the four dyes (Figure 5 on page 4). The goal of multicomponent analysis is to isolate the signal from each dye so that there is as little noise in the data as possible.

The precise spectral overlap between the four dyes is measured by running DNA fragments labeled with each of the dyes in separate lanes of a gel or in separate injections on a capillary. These dye-labeled DNA fragments are called matrix standard samples.

The Data Utility software (see page 30) then analyzes the data from each of these four samples and creates an instrument (matrix) file. The instrument file contains tables of numbers with four columns and four rows. These numbers are normalized fluorescence intensities and represent a mathematical description of the spectral overlap that is observed between the four dyes.

Multicomponent analysis of sequencing data is performed automatically by the Sequencing Analysis software, which applies a mathematical matrix calculation (using the values in the instrument file) to all sample data.

Using the new dRhodamine-based sequencing chemistries requires making instrument files from the new matrix standards found in the dRhodamine Matrix Standards Kit (P/N 403047). The instrument file is prepared to be consistent with current base-calling conventions, *i.e.*, C is blue, A is green, G is black, and T is red in the output files.

Data Utility Software

The Data Utility software is used to make instrument files. You must put the correct data file for each matrix standard into the correct "box" in the Data Utility application (Table 6).

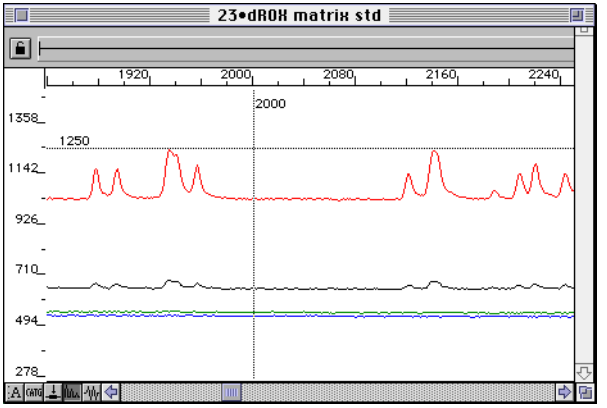
Table 6. Placement of Standards in the Data Utility Application

Box	Dye Primer Matrix	Taq Terminator Matrix	T7 Terminator Matrix
C…	dR110	dROX	dR6G
A…	dR6G	dR6G	dTAMRA
G…	dTAMRA	dR110	dROX
T…	dROX	dTAMRA	dR110

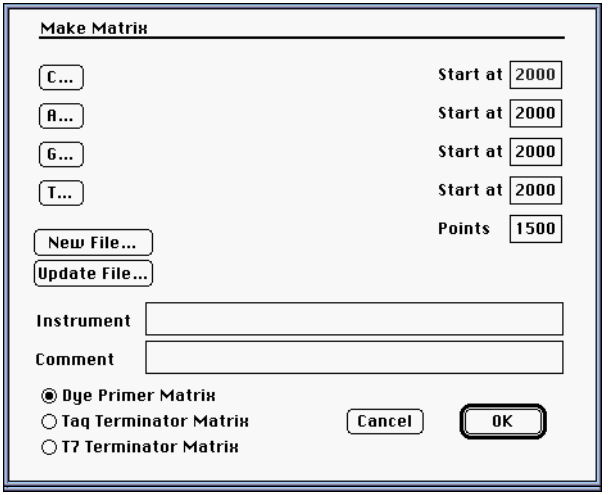
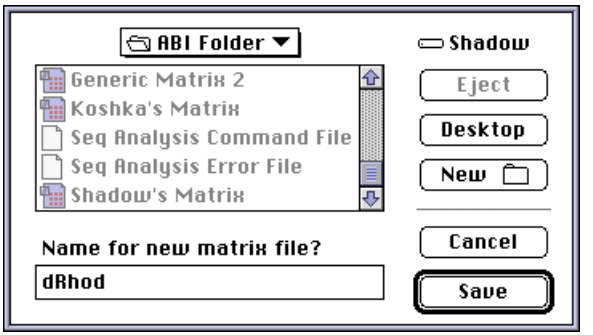
You need to make all three matrix files if you wish to use the other dRhodamine sequencing chemistries. For example, the Collection software will not run with only a terminator matrix in the file. An error message will appear saying, "Tag not found. Cannot start the run."

Make the Instrument File

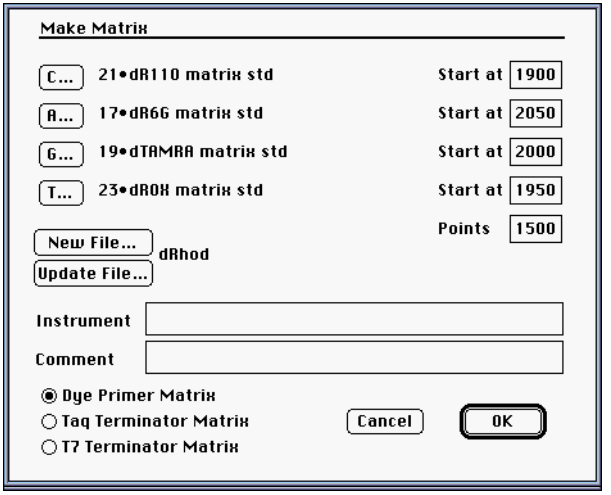
To make the Dye Primer Matrix:

Step	Action
1	Set the analysis start point and the number of data points to analyze. a. In the Sequencing Analysis software, examine the raw data for one of the matrix standard samples as shown below. b. Select a starting point where there are no peaks and the baseline is flat. c. Select a number of data points to analyze such that no peaks in the range are off-scale, *i.e.*, above 4000 relative fluorescence units (RFU) and where the baseline at the end of the range is flat. A typical number of data points is 1500. 
2	Repeat step 1 for each matrix standard sample. Record the results for later use. **IMPORTANT** The number of data points analyzed is the same for each matrix standard. Choose starting points for each sample such that all peaks are less than 4000 RFU and where both the starting and ending points have flat baselines and no peaks.
3	Launch the Data Utility software.

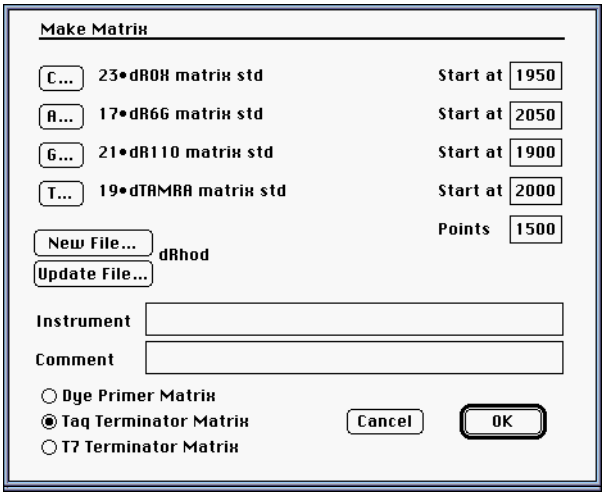
To make the Dye Primer Matrix: *(continued)*

Step	Action
4	From the Utilities menu, choose Make Matrix… The Make Matrix dialog box appears as shown below. Verify that the Dye Primer Matrix button at the lower left is selected. 
5	Click on the box for each nucleotide base and enter the data file that corresponds to the correct matrix standard as shown in Table 6 on page 30.
6	Enter the analysis start point for each matrix standard sample as determined in step 1 on page 31.
7	Click New File… A dialog window appears as shown below. Name the file dRhod and save it in the ABI folder within the System folder. 

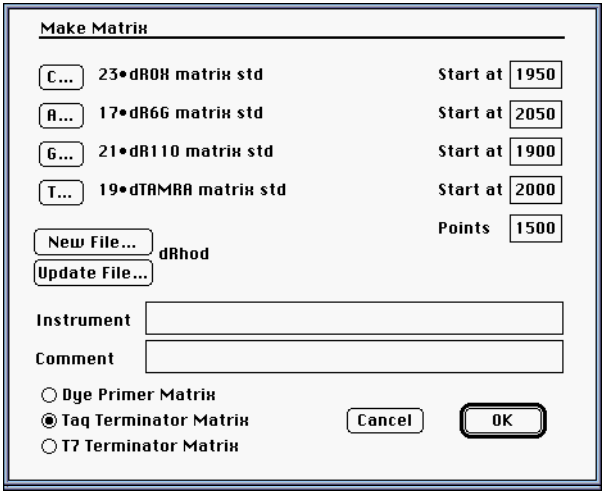
To make the Dye Primer Matrix: *(continued)*

Step	Action
8	The Make Matrix dialog box should look like that shown below. **Note** The numbers in the Start at and Points boxes below are typical values. Your numbers may vary. 
9	a. Click OK. The computer makes the matrix. When finished, a dialog window appears with the message “Make matrix successfully completed.” b. Click OK.
10	If the computer is unable to make a matrix, examine the raw data again in the Sequencing Analysis software. If many peaks are off-scale, dilute the matrix standards and rerun them.

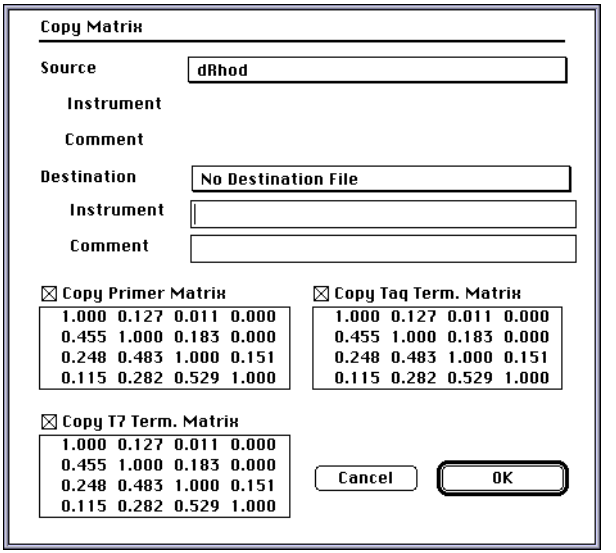
To make the Taq Terminator Matrix:

Step	Action
1	In the Data Utility application, choose Make Matrix… from the Utilities menu. The Make Matrix dialog box appears.
2	In the Make Matrix dialog box, click the Taq Terminator Matrix button at the lower left.
3	Click on the box for each nucleotide base and enter the data file that corresponds to the correct matrix standard as shown in Table 6 on page 30. **IMPORTANT** The order of matrix standard data files is different from that in the Dye Primer Matrix (see Table 6 on page 30).
4	Enter the same numbers for each matrix standard sample in the Start at and Points boxes as for the Dye Primer Matrix.
5	Click Update File… A dialog window appears.
6	Choose dRhod from the ABI folder within the System folder and click Save. The Make Matrix dialog box should look like that shown below. 
7	a. Click OK. The computer makes the matrix. When finished, a dialog window appears with the message “Make matrix successfully completed.” b. Click OK.

To make the T7 Terminator Matrix:

Step	Action
1	In the Data Utility application, choose Make Matrix… from the Utilities menu. The Make Matrix dialog box appears.
2	In the Make Matrix dialog box, click the T7 Terminator Matrix button at the lower left.
3	Click on the box for each nucleotide base and enter the data file that corresponds to the correct matrix standard as shown in Table 6 on page 30 (note the order of the matrix standard files).
4	Enter the same numbers for each matrix standard sample in the Start at and Points boxes as were used for the Dye Primer Matrix and Taq Terminator Matrix.
5	Click Update File… A dialog window appears.
6	Choose dRhod from the ABI folder within the System folder and click Save. The Make Matrix dialog box should look like that shown below. 
7	a. Click OK. The computer makes the matrix. When finished, a dialog window appears with the message “Make matrix successfully completed.” b. Click OK.

To check the instrument file:

Step	Action
1	From the Utilities menu, choose Copy Matrix…
2	Under Source, select Instrument file and choose dRhod from the ABI folder within the System folder. The three matrix files within the dRhod instrument file appear as shown below. 
3	Make sure that all three matrix files have numbers that range from 0–1. The numbers on the diagonals from top left to bottom right should be 1. If not, then repeat the matrix-making procedure starting with “To make the Dye Primer Matrix:” on page 31. **Note** The corresponding numbers for all three matrix files will be the same.
4	Click Cancel.
5	Restart the Sequencing Analysis software and use dRhod as the instrument file to analyze your sequencing data.

Appendix B. 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.

Recommendations

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. This is especially true for 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.

Appendix C. Control Sequence

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

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