instructions

product code:

US79620
US79625

DYEnamic Direct Cycle Sequencing Kit with -21 M13 Forward Primer

Warning
For research use only.
Not recommended or intended for the diagnosis of disease in humans or animals.
Do not use internally or externally in humans or animals.

US79620PL Rev A 2003

Amersham Biosciences

Handling

Storage
Store at -15 ˚C to -30 ˚C.

Page finder

Quality control

All batches of the DYEnamic Direct Cycle Sequencing Kit are assayed according to the recommended starting point protocol described in this booklet. Reactions are analyzed on an ABI™ 373A DNA Sequencer Stretch or ABI Prism™ 377 DNA Sequencer. Specifications for release are based on assessment of sequence by length of read (> 500 bases), accuracy and signal quality.

Materials not supplied

Reagents

- **Water**—Use only deionized, distilled water for the sequencing reactions.
- **Ethanol (100% and 70%)**—For sequencing reaction cleanup (optional).

Components of the kit

Solutions included in the DYEnamic™ Direct Cycle Sequencing Kit have been carefully formulated for optimal sequencing results. Each reagent has been tested extensively and its concentration adjusted to meet rigorous standards set by Amersham Biosciences. It is strongly recommended that all reagents supplied in the kit be used exactly as described in this protocol.

Note: Depending on the kit format, 7-deaza-dGTP**, a dGTP nucleotide analog, is incorporated into the reagent mixes for the purpose of resolving compressions.

The following components are included in this product:

The C, A, G, and T reagents listed below all contain 0.47 mM each dCTP, dATP, 7-deaza-dGTP or dGTP, and dTTP, 125 mM Tris-HCl, pH 9.5, 6.25 mM $MgCl_2$, Thermo Sequenase™ DNA polymerase with *Thermoplasma acidophilum* inorganic pyrophosphatase in addition to the specific dideoxynucleotide terminator and labelled primer.

C reagent (blue-capped tube): components as listed above plus 1.55 µM ddCTP and 0.2 µM -21 M13 forward primer-FAM™

A reagent (green-capped tube): components as listed above plus 1.55 µM ddATP and 0.2 µM -21 M13 forward primer-REG

G reagent (yellow-capped tube): components as listed above plus 1.55 µM ddGTP and 0.2 µM -21 M13 forward primer-TAMRA™

Materials not supplied *(continued)*

- **7.5 ammonium acetate—** For sequencing reaction cleanup (optional).

Equipment

- **Liquid-handling supplies**—Vials, pipettes, microcentrifuge, vortex mixer, and vacuum-dry centrifuge (optional). Perform all sequencing reactions in plastic microcentrifuge tubes (typically 0.5 ml), 96-well or 384-well plates suitable for thermal cycling.
- **Instrument**—This kit is designed for optimal performance with ABI 373A DNA Sequencer Stretch or ABI Prism 377 DNA Sequencer.
- **Thermal cycler**—For thermally cycled incubations between 45 °C and 95 °C (1–30 cycles).

Components of the kit *(continued)*

T reagent (red-capped tube): components as listed above plus 1.55 µM ddTTP and 0.2 µM -21 M13 forward primer-ROX™

Formamide loading dye: Deionized formamide containing a proprietary dye

Control DNA: double-stranded pUC19 DNA (0.05 µg/µl)

Mobility file disk

	Kit format	
	US79620	**US79625**
Nucleotide analog	dGTP	7-deaza-dGTP
Number of reactions	100	100
Reagent mix	200 µl	200 µl
Formamide loading dye	1 200 µl	1 200 µl
Control DNA	50 µl	50 µl

Store the kit reagents at -15 °C to -30 °C (not in a frost-free freezer). Keep all reagents on ice when removed from storage for use.

World Wide Web address

http://www.amersham.com

Visit the Amersham Biosciences home page for regularly updated product information.

Safety warnings and precautions

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle this product. Suitable protective clothing such as laboratory overalls, safety glasses and gloves should be worn. Care should be taken to avoid contact with the skin or eyes; if contact should occur, wash immediately with water (see Material Safety Data Sheet for specific recommendations).

Warning: This product contains formamide. Gel reagents may contain acrylamide, a neurotoxin and suspected carcinogen. An optional step in the protocol requires the use of ethanol, a flammable liquid. Please follow the manufacturer's Material Safety Data Sheet regarding safe handling and use of these materials.

Introduction

The DYEnamic Direct Cycle Sequencing Kit with -21 M13 Forward Primer is formulated for direct loading of the sequencing products onto the sequencing gel. Because the DYEnamic ET dye primers absorb and emit light with high efficiency, there is no need to concentrate the sequencing reaction products by ethanol precipitation. By removing the ethanol precipitation step, preparation time is reduced by at least one hour.

Energy transfer dye primers

Energy transfer (ET) dye primers are uniquely designed oligonucleotides with two fluorescent dyes—a donor and an acceptor—attached to each primer molecule for improved detection (1). The donor dye absorbs light of the wavelength of the argon ion laser in the sequencing instrument and transfers this absorbed light energy to the acceptor dye. The acceptor dye then emits the absorbed energy as fluorescence at its characteristic emission wavelength for detection by the sequencing instrument. Since ET primers absorb laser light efficiently, their effective fluorescence intensity is two to twelve times greater than primers with single-dye labels. Depending on the application, less template may be required, as well as decreased preparation and cycling times due to the higher signal strength that can be achieved.

DYEnamic ET primers use 5-carboxy-fluorescein (FAM) as the donor dye and FAM (C reagent), 6-carboxyrhodamine (R6G, also known as REG) (A reagent), N,N,N',N',-tetramethyl-5-carboxyrhodamine (TMR, also known as TAMRA) (G reagent) and 5-carboxy-X-rhodamine (ROX) (T reagent) as the acceptor dyes.

Thermo Sequenase DNA polymerase

Thermo Sequenase is an exonuclease-free, thermostable DNA polymerase (2, 3) engineered by Amersham Biosciences specifically for sequencing. The enzyme readily accepts dideoxynucleotide terminators (4) and generates bands of uniform intensity, much like T7 Sequenase™ DNA polymerase (5, 6). These characteristics, along with its thermal stability, make it ideal for cycle sequencing and greatly facilitate data interpretation and accurate base-calling using automated sequencing software.

The enzyme formulation includes thermostable inorganic pyrophosphatase (TAP) cloned from *Thermoplasma acidophilum*. TAP hydrolyzes the inorganic pyrophosphate product of nucleotide polymerization, thus preventing pyrophosphorolysis and subsequent loss of peaks from sequence data.

Cycle sequencing

In traditional dideoxynucleotide sequencing, synthesis of a new strand of DNA is initiated at a specific priming site by a DNA polymerase in the presence of deoxynucleotide triphosphates (dNTPs). Extension of the strand continues until a chain-terminating dideoxynucleoside triphosphate (ddNTP) is incorporated (7). By controlling the relative concentrations of dNTPs and ddNTPs, the majority of the DNA fragments generated will terminate at lengths within the resolving capacity of the gel matrix used for separation. When the fragments are labelled with dyes, fluorescent sequencing instruments can derive sequence information from the fluorescence emitted by the fragments as they move through a particular area of the gel.

When thermostable DNA polymerases are used, sequencing reactions can be cycled through alternating periods of thermal denaturation, primer annealing and extension/termination to increase the signal levels generated from template DNA (3, 8–14). For each cycle, the amount of sequencing product available for analysis will be equivalent to the amount of primed template, with the theoretical yield roughly equal to the number of cycles (in practice, each cycle is not 100% efficient and the amplification will be somewhat lower). By using a thermostable enzyme such as Thermo Sequenase DNA polymerase, programmed cycling can be performed with no need for additional enzyme. A cycling protocol is especially useful when the amount of template is limiting or the sensitivity of the detection system is low.

The use of DYEnamic ET primers and Thermo Sequenase DNA polymerase in conjunction with a cycle sequencing format yields the highest quality sequence data with minimal reagent, template and labor requirements.

Protocols

Preliminary preparation and general handling instructions

Primer-specific mobility files and instrument-specific color matrix files must be installed on the computer system in use. A mobility file for each individual DYEnamic ET primer is provided on the diskette supplied with the kit. Although the electrophoretic mobilities of the DYEnamic ET primers are much better matched than ordinary dye-labelled primers (15), a primer specific mobility file is still necessary for optimal base-calling accuracy. Please refer to Appendixes 1 and 2 on pages 14 and 15 for additional information.

Preprogram a thermal cycler as described in step 1.4 (below). For additional information concerning cycling parameters, see Appendix 3 on page 15.

Thaw the following reagents on ice prior to use:

- DYEnamic Direct Cycle Sequencing Kit reagents: C, A, G, and T reagents and formamide loading dye.
- DNA template: 200 ng (100 fmol) of single-stranded DNA or 500 ng (250 fmol) of double-stranded DNA or PCR product in water. Sample volume must be 12 µl or less.

Whenever possible, keep tubes capped and on ice to minimize evaporation of the small volumes of reagents in use. Dispense reagents using disposable-tip micropipettes, and exercise care to avoid contaminating stock solutions. Reaction mixtures must be thoroughly mixed after each addition, typically by "pumping" the solution two or three times with a micropipettor without creating air bubbles. Centrifuge tubes/plates briefly to collect the reaction mixtures at the bottoms of the tubes/wells.

Sequencing with DYEnamic Direct Cycle Sequencing Kit with -21 M13 Forward Primer

The protocol below is recommended as a starting point. Further experiments to optimize template DNA concentrations and conditions for thermal cycling may be required. Please refer to Appendix 3 on page 19 for additional information.

1 Preparation of sequencing reaction mixes

	Termination reaction			
	A	**C**	**G**	**T**
Reagent mix	2 µl	2 µl	2 µl	2 µl
Template DNA	3 µl	3 µl	3 µl	3 µl
Total volume	5 µl	5 µl	5 µl	5 µl

1.1 Label four microcentrifuge tubes "C", "A", "G", and "T". Pipette 2 µl of C, A, G, and T reagent mix into the appropriately labelled tube.

1.2 Add 3 µl of the DNA template to each tube/well for a total volume of 5 µl. When all the reagents have been dispensed, mix thoroughly by gentle pipetting.

1.3 When all reagents have been prepared, treat the sequencing reaction mixes appropriately for the thermal cycler in use. Cap the tubes or seal the plates and place the reactions into the preprogrammed thermal cycler.

1.4 Start the cycling program. The cycle parameters shown below are general guidelines. The optimal cycling parameters will depend on the amount and purity of the template DNA. Please refer to Appendix 3 for more information.

95 °C, 30 s
45 °C, 15 s
70 °C, 30–60 s
Repeat for 30 cycles.

1.5 After cycling is complete, centrifuge the tubes/plate briefly to collect the reaction mixtures at the bottoms of the tubes/wells.

1.6 Pool the contents of the "C", "A", "G", and "T" tubes/wells and place on ice.

❷ Preparation of sequening reaction mixes for direct loading onto the gel

Sequencing reaction mixes can be loaded directly onto the sequencing gel when sufficient template (~ 500 ng of double-stranded DNA or 200 ng of single-stranded DNA) is available or when 30 thermal cycles are applied.

2.1 Select the correct mobility file for the primer in use and the well-to-read (WTR)-length prior to running the gel.

	WTR length	Mobility file
ABI 373A Stretch	34 cm	standard
	48 cm	"longreader"
ABI 377 Prism	36 cm	standard
	48 cm	"longreader"

2.2 Add 6–8 µl of formamide loading dye to the sample (~ 20 µl) and mix well.

2.3 Heat the samples to 70 °C for 2–3 min to denature, then place on ice.

Note: DNA sequencing reactions that include 7-deaza-dGTP may produce inferior results when overheated, especially at temperatures > 80 °C. Therefore, these samples should be heated briefly prior to gel loading, as recommended above. Signal intensity and resolution will not be affected.

2.4 Load 1.5–6 µl of the sample directly onto the gel (volume loaded depends on whether an ABI Prism 377 or 373A Stretch instrument is in use).

2.5 Proceed with gel electrophoresis and data analysis as usual for the ABI 373A or 377 instruments.

Note: The formamide loading dye will migrate toward the cathode (up out of the well) during electrophoresis.

Note: It is critical that the mobility file and color matrix are appropriate for the DYEnamic ET primer and gel well-to-read (WTR)-length. Although signal strengths may be ~ 50% lower than for precipitated DYEnamic ET primer reactions, directly loaded samples show accurate sequence to a length comparable to precipitated reactions.

❸ Post-reaction cleanup (optional step)

In some cases where maximal detection sensitivity is required, it may be necessary to ethanol precipitate the sequencing reaction products. For example, include an ethanol precipitation step when template quantity is limiting or unknown, when only a few thermal cycles are performed, or if very long read-lengths are desired.

3.1 Add 1.5 µl of 7.5 M ammonium acetate to each pooled reaction mix.

3.2 Add 55 µl (~ 2.5 times the pooled reaction volume) of 100% ethanol to the pooled samples and mix well on a vortex mixer.

3.3 Centrifuge the tubes in a microcentrifuge (~ 12 000 rpm) for 15 min at either room temperature or 4 °C. Centrifuge 96-well or 384-well plates for at least 30 min at 2 500 ∞ *g* or greater.

Note: We routinely centrifuge for 30 min at 3 100 ∞ *g*.

3.4 Remove as much supernatant as possible by aspiration. For plates, a brief inverted spin (< 1 min at 300 ∞ *g*) is sufficient for supernatant removal. Wash the pellet with cold 70% ethanol. Vortex briefly.

Note: We routinely use 250–500 µl of 70% ethanol for 0.5 ml microcentrifuge tubes, 100 µl for 96-well plates, and 50 µl for 384-well plates.

3.5 Remove the supernatant by aspiration or by an inverted spin. Vacuum-dry the DNA pellets in a vacuum centrifuge for 2–3 min. Alternatively, air-dry the pellets for 10–15 min. Do not overdry the DNA pellets since this can make resuspension difficult.

3.6 Dissolve each pellet in 4 µl of formamide loading dye and vortex vigorously for 10–20 s to ensure complete resuspension. Centrifuge briefly to collect the samples at the bottoms of the tubes/wells.

Note: The DNA pellet must be completely dissolved at this step for optimal sequencing results. If a fixed-angle rotor was used for centrifugation, the DNA pellet will be on the side of the microcentrifuge tube. This material must be washed to the bottom of the tube to ensure that the entire reaction product is loaded onto the gel.

3.7 Heat the samples for 2–3 min at 70 ˚C to denature, then place on ice.

Note: DNA sequencing reactions that include 7-deaza-dGTP may produce inferior results when overheated, especially at temperatures > 80 ˚C. Therefore, briefly heat these samples as recommended above, prior to gel loading. Signal intensity and resolution will not be affected.

3.8 Immediately load the entire sample onto a lane of the sequencing gel.

Appendix 1:
Installation of mobility files

A mobility file for each individual DYEnamic ET primer is provided on a diskette supplied with the kit.

Note: Please refer to www.amershambiosciences.com for mobility file updates.

Although the electrophoretic mobilities of the DYEnamic ET primers are much better matched than ordinary dye-labelled primers (15), a primer-specific mobility file is still necessary for optimum base-calling accuracy. Attaching four different dyes to DNA fragments alters their mobility relative to each other. Altered mobility depends partly on the nucleotide sequence near the dye attachment site. Therefore, a different mobility file is required for each primer sequence.

To install the mobility file(s) onto the sequencing instrument:

1. Locate the ABI folder in the System folder of your computer.
2. Locate the file named **ET{-21M13}** in the DYEnamic ET primer start-up disk.
3. Select **ET{-21M13}** primer mobility file and drag it into the ABI folder on your computer. When you choose the settings on your sample sheet, select the appropriate DYEnamic ET primer mobility file appropriate for the dye primer set in use.

Appendix 2:
Creating a color matrix

DYEnamic ET primers have fluorescence spectra that differ from ordinary dye-labelled primers and from each other. For this reason, a specific color matrix file must be created for each DYEnamic ET primer to be analyzed by the base-calling software. For your convenience, sample color matrices have been provided for both the ABI 373A and 377 sequencing instruments. These can be installed the same way the mobility file is installed—by selecting the matrix file found in the DYEnamic ET primer start-up disk and dragging it into the ABI folder. However, because of instrument-to-instrument variation in the detection systems, it is strongly recommended that color matrix files be created for each primer on each sequencing instrument. It is essential also to create a new matrix whenever the filter wheel in the ABI 373A sequencing instrument is changed. Sequencing reactions can be electrophoresed on the same gel in parallel with the color matrix samples. Analysis is then performed after the new matrix is created.

To make a color matrix, dye primer cycle sequencing reactions are performed as usual. After cycling is complete, the four termination reactions are not combined. Instead, the individual reactions are ethanol precipitated and loaded into separate lanes of the sequencing gel (as in radioactive sequencing). Data from these four lanes are then evaluated by the ABI utility software to create the color matrix file. This file is specific for the particular instrument that will analyze the samples.

1. Color matrix preparation using the DYEnamic Direct Cycle Sequencing Kit reagents

   1.1 Assemble the following reagents from the DYEnamic Direct Cycle Sequencing Kit with -21 M13 Forward Primer or from laboratory stock. Place on ice.

   - C, A, G, and T reagents containing nucleotides, primer, buffer and enzymes
   - Formamide loading dye
   - DNA template: Use 0.6 µg of double-stranded plasmid DNA (pUC19 control template in the DYEnamic Direct Cycle Sequencing Kit) contained in a volume of 12 µl of 10 mM Tris, pH 8.0, 0.1 mM EDTA (TE).
   - 7.5 M ammonium acetate

2. Preparation of sequencing reaction mixtures

   2.1 Label four microcentrifuge tubes “C”, “A”, “G”, and “T”. Pipette 2 µl of C, A, G, and T reagent mix into the appropriately labelled tube.

   2.2 Add 3 µl of the DNA template to each tube/well for a total volume of 5 µl. After dispensing all the reagents, mix them thoroughly by gentle pipetting.

   2.3 Treat the sequencing reaction mixes appropriately for the thermal cycler in use. Cap the tubes or seal the plates and place the reactions into the preprogrammed thermal cycler.

2.4 Start the cycling program using the following parameters:

95 °C, 30 s
45 °C, 15 s
70 °C, 60 s
Repeat for 30 cycles.

2.5 After cycling is complete (typically 1.5–2 h), centrifuge the tubes briefly to collect the reaction mixtures at the bottoms of the tubes.

3. Preparation for loading reaction products onto the gel

3.1 Add 2 µl of 7.5 M ammonium acetate and 13 µl of sterile water to each reaction tube and place on ice. Do not combine the four reaction mixtures.

3.2 Add 50 µl of 100% ethanol to each reaction. Mix by vortexing.

3.3 Centrifuge the reactions in a microcentrifuge (~ 12 000 rpm) for 15 min at either room temperature or 4 °C.

3.4 Remove as much of the supernatant as possible by aspiration.

3.5 Add 250 µl of 70% ethanol to wash the pellet.

Note: Further centrifugation is not required at this point, but if used, will not be detrimental.

3.6 Remove the supernatant by aspiration and vacuum-dry the DNA pellets in a vacuum centrifuge for 2–3 min. Alternatively, air-dry the pellets for 10–15 min. Do not overdry the pellets because this can make resuspension difficult.

3.7 Dissolve each pellet in 8 µl of formamide loading dye and vortex vigorously for 10–20 s to ensure complete resuspension. Briefly centrifuge to collect the samples at the bottoms of the tubes.

Note: The DNA pellet must be dissolved completely at this step so that all of the DNA is used. If a fixed-angle rotor was used during centrifugation, the DNA pellet will be on the side of the microcentrifuge tube. This material must be washed to the bottom of the tube to ensure that the entire reaction product is loaded onto the gel.

3.8 Heat the samples to 70 °C for 2–3 min to denature; place them on ice.

Note: DNA sequencing reactions that include 7-deaza-dGTP may produce inferior results when overheated, especially at temperatures above 80 °C. Therefore, heat these samples briefly prior to gel loading, as recommended above. Signal intensity and resolution will not be affected.

3.9 Load eight wells with sample. With ABI 373A instruments, load 6 µl of the four reactions into four separate wells and load 1 µl of the four reactions into a second set of four wells. Be sure to skip lanes between each sample. When using an ABI 377 Prism instrument, use loading volumes of 1.5 µl and 0.5 µl.

3.10 Utilize the sequencing instrument as usual.

3.11 After completing electrophoresis, execute the **Make Matrix** utility program as specified in the ABI instrument manual. If the utility doesn't create a matrix with the data from the lanes loaded with 6 µl (or 1.5 µl for ABI 377 instruments), use the 1 µl or 0.5 µl lanes. Be sure to match the correct dye to the correct nucleotide.

The **Make Matrix** program will have difficulty locating good bands if the fluorescence signal is too strong or too weak. Two different volumes of the purified reaction products are loaded onto the gel for electrophoresis to ensure that at least one lane will have bands with appropriate intensities.

To run the **Make Matrix** utility program, you must quit the **Sequencing Analysis** program. Open the ABI 373 or 377 Software folder and then, the Utilities folder. Select **DataUtility** and choose **Make Matrix** under **Utilities** from the menu in the toolbar. The default values of **2 000** for the **Start at** parameter and **1 500** for the **Points** parameter are usually adequate. If creating a new matrix file, select **New File** in the **Make Matrix** dialog box. If changing an existing matrix, select **Update File** in the **Make Matrix** dialog box. When **Update File** is selected, the existing file will be overwritten. If you wish to save the existing matrix file, create a backup copy of this file using a different file name.

Appendix 3: Cycling parameters and quantity of template

The cycling temperatures of the sequencing reactions are based on characteristics of the sequencing primer; the number of required cycles depends on the quantity and quality of the template DNA. The following guidelines will help you select the most appropriate cycling parameters.

Cycling temperatures

For a typical sequencing primer of 15–25 bases and a G/C content of ~ 50%, the melting temperature will generally be 50–65 °C. We suggest

using a three-temperature cycling program, especially with PCR products—denaturation at 95 °C for no more than 30 s; annealing at 45–55 °C for 5–15 s; and extension at 70 °C for 30–120 s. If in doubt, choose a wide temperature range with brief pauses at the temperature extremes. It is best to determine the melting temperature of the primer using one of the many commercially available software packages. Annealing temperatures not more than 5 °C below the melting temperature usually produces specific priming.

Number of cycles and quantity of template

The optimal number of cycles depends on both the amount (fmol) and purity of template DNA and on the sensitivity of the fluorescent detection method. Using the protocol described in this booklet, the minimum quantities of highly purified DNA that have been successfully sequenced are ~ 5 fmol (10 ng) of M13mp18 DNA and ~ 25 fmol (50 ng) of pUC18 DNA.

For routine sequencing, at least 100 fmol (~ 200 ng) of M13 or 250 fmol (~ 500 ng) of plasmid DNA is sufficient. With minimum quantities of template DNA, or if the actual quantity of template is unknown, use 30 cycles. If read-lengths and signal strengths are consistently strong (e.g. all signal strengths are > 500), the number of cycles can be reduced by one-half to conserve use of the thermal cycler. We have obtained good sequence with as few as 5 cycles with 0.1 µg of CsCl-purified M13 or 1 µg of CsCl-purified double-stranded plasmid DNA. Results will vary with template purity. To avoid overloading the gel, use no more than 2.5 µg of plasmid.

Appendix 4:
Elimination of compressions

Some DNA sequences, especially those with dyad symmetries containing dG and dC residues, are not fully denatured during electrophoresis. When this occurs, the migration pattern of DNA fragments is interrupted. Peaks are spaced closer than normal (compressed) and just beyond the compression, they are unusually far apart. When this occurs, sequence information is lost.

Many of these gel artifacts can be eliminated by substituting 7-deaza-dGTP, a nucleotide analog which forms weaker secondary structure (17), in place of dGTP. DYEnamic Direct Cycle Sequencing Kits with -21 M13 Forward Primer are available with either 7-deaza-dGTP analog or dGTP.

Note: DNA sequencing reactions that include 7-deaza-dGTP may generate inferior results when overheated, especially at temperatures > 80 °C. Therefore, these samples should be heated briefly, as recommended in the "Protocols" section prior to gel loading. Signal intensity and resolution will not be affected.

For templates with strong compressions, gels containing up to 20% formamide may be effective for resolving these artifacts. However, some distortion of sequence, either early or late in the read depending on gel running conditions, may be encountered with formamide gels when electrophoresed on ABI sequencing instruments with standard or energy transfer mobility files.

Appendix 5:
Sequencing PCR products

We recommend the PCR Product Pre-sequencing Kit (product code US70995) to purify PCR products prior to sequencing. The kit includes exonuclease I and alkaline phosphatase for digestion of excess primer, single-stranded products, and excess nucleotides from the PCR.

Appendix 6:
Denaturing gel electrophoresis

Gels and buffers

Only use the highest quality sequencing grade acrylamide, urea and other gel components to prepare sequencing gels for fluorescent DNA sequencing analysis. Gels with 4–6% total acrylamide are typically used (19:1 acrylamide:bis ratio) with 6 M urea. Amersham Biosciences offers RapidGel™ and RapidGel-XL if premixed liquid acrylamide is preferred. Other commercially available gel mixes also work well with DYEnamic ET primer sequencing reactions. However, if the gel runs sufficiently faster or slower than typical acrylamide gels, a different mobility file may be required for sample analysis.

If the direct loading procedure is used, glycerol will be present in the material applied to the gel. With TBE-buffered gels, glycerol may interact with the boric acid in the buffer (16) to cause an artifact that appears as curvature of the lane, distortion, and loss of the signal as the glycerol complex passes through the detection window. This artifact is observed sufficiently far into the run so as not to interfere with most sequence analysis when running the acrylamide gel concentrations recommended in the "Gel composition and running conditions" section

below. The artifact will, however, interfere when higher concentration gels are used. In the typical 34 cm WTR 373A Stretch gel, the artifact is observed beyond 550 bases; in a 48 cm WTR 373A Stretch gel, it is observed beyond 800 bases. The artifact can be eliminated completely by using either a glycerol-tolerant Tris-taurine-EDTA (TTE) gel buffer or by ethanol precipitation of the samples.

To eliminate any glycerol gel artifact, substitute TTE (glycerol-tolerant gel buffer) for TBE in the gel and running buffer. This buffer can be used with samples containing glycerol at any concentration (16). If gels run too slowly with this buffer at 1∞ strength, reduce it to ~ 0.8∞ in the gel and running buffers. TTE is available in liquid and powder premix forms (product code US75827 and US71949, respectively). Always run glycerol-tolerant gels at the same power (wattage) as TBE-buffered gels to maintain normal temperature.

Gel composition and running conditions

The gel recipes below produce 100 ml of gel solution. Dissolve the ingredients in a minimal amount of water with gentle heating. Adjust the volume to 100 ml with distilled water and vacuum-filter the solution with a nitrocellulose filter unit.

ABI 373A Stretch Instrument

WTR	Gel %	Acrylamide	Bis	Urea	10∞ TBE
34 cm	4.75%	4.51 g	0.24 g	48 g (8 M)	10 ml
48 cm	4.00%	3.80 g	0.20 g	48 g (8 M)	10 ml

WTR	Formamide*	Power	Run time
34 cm	20 ml	30–32 W	14–16 h
48 cm	20 ml	40 W	18–24 h

ABI 377 Prism Instrument

WTR	Gel %	Acrylamide	Bis	Urea	10∞ TBE
36 cm	4.00%	3.80 g	0.20 g	48 g (8 M)	10 ml
48 cm	4.00%	3.80 g	0.20 g	48 g (8 M)	10 ml

WTR	Formamide*	Power	Run time
36 cm	20 ml	150 W	7 h
48 cm	20 ml	200 W	10 h

*Optional addition of formamide, see below.

Formamide gels

For any of the recipes listed above, substitute a portion of the water required for dissolution with 20 ml of deionized formamide to produce a 20% formamide gel. Heat formamide gel solutions very gently to approximately 50 °C. Once the mix is in solution, continue stirring until the temperature is ~ 30 °C. Adjust the volume to 100 ml with distilled water and vacuum-filter with a nitrocellulose filter unit. Pour the gel immediately. To prevent the urea from precipitating, do not allow the gel to cool below 25 °C. If the mixture precipitates, the process must be repeated from the beginning. An electrophoresis gel that has been redissolved will produce very poor results.

Add 1 ml of 10% ammonium persulfate and 25 µl of TEMED.

Note: Use this amount of TEMED when pouring the gel the night before it is needed. If the gel is to be used the same day, adding four to five times the amount of TEMED will polymerize the gel in one hour.

See Amersham Biosciences TECHtip #152 for more information.

Note: For fluorescent sequencing, use gels that contain no more than 20% formamide. Forty percent formamide gels will not produce useable data in an ABI 373 or 377 instrument.

Plate cleaning

Only use clean glass gel plates for fluorescent sequencing experiments. Dirty plates can decrease signal strength. If the direct loading procedure is used routinely, scrub the glass plates thoroughly with Alconox™ after each use and rinse thoroughly with deionized water. It may be necessary to occasionally soak the plates in 1 M NaOH for 30 min and then neutralize the solution with 1 M HCl for 30 min. Again, rinse the plates thoroughly before use.

Appendix 7: Control DNA sequence

pUC19 double-stranded DNA

```
   1  TCGCGCGTTT  CGGTGATGAC  GGTGAAAACC  TCTGACACAT  GCAGCTCCCG  GAGACGGTCA
  61  CAGCTTGTCT  GTAAGCGGAT  GCCGGGAGCA  GACAAGCCCG  TCAGGGCGCG  TCAGCGGGTG
 121  TTGGCGGGTG  TCGGGGCTGG  CTTAACTATG  CGGCATCAGA  GCAGATTGTA  CTGAGAGTGC
 181  ACCATATGCG  GTGTGAAATA  CCGCACAGAT  GCGTAAGGAG  AAAATACCGC  ATCAGGCGCC
 241  ATTCGCCATT  CAGGCTGCGC  AACTGTTGGG  AAGGGCGATC  GGTGCGGGCC  TCTTCGCTAT
 301  TACGCCAGCT  GGCGAAAGGG  GGATGTGCTG  CAAGGCGATT  AAGTTGGGTA

-40 M13 ForwardØ-21 M13 ForwardØ
      GT TTTCCCAGTC       ACGACG           TGT     AAAACGACGG         CCAGT
 351  ACGCCAGGGT  TTTCCCAGTC  ACGACGTTGT  AAAACGACGG  CCAGTGAATT  CGAGCTCGGT
 411  ACCCGGGGAT  CCTCTAGAGT  CGACCTGCAG  GCATGCAAGC  TTGGCGTAAT  CATGGTCATA
 471  GCTGTTTCCT  GTGTGAAATT  GTTATCCGCT  CACAATTCCA  CACAACATAC  GAGCCGGAAG
 531  CATAAAGTGT  AAAGCCTGGG  GTGCCTAATG  AGTGAGCTAA  CTCACATTAA  TTGCGTTGCG
 591  CTCACTGCCC  GCTTTCCAGT  CGGGAAACCT  GTCGTGCCAG  CTGCATTAAT  GAATCGGCCA
 651  ACGCGCGGGG  AGAGGCGGTT  TGCGTATTGG  GCGCTCTTCC  GCTTCCTCGC  TCACTGACTC
 711  GCTGCGCTCG  GTCGTTCGGC  TGCGGCGAGC  GGTATCAGCT  CACTCAAAGG  CGGTAATACG
 771  GTTATCCACA  GAATCAGGGG  ATAACGCAGG  AAAGAACATG  TGAGCAAAAG  GCCAGCAAAA
 831  GGCCAGGAAC  CGTAAAAAGG  CCGCGTTGCT  GGCGTTTTTC  CATAGGCTCC  GCCCCCCTGA
 891  CGAGCATCAC  AAAAATCGAC  GCTCAAGTCA  GAGGTGGCGA  AACCCGACAG  GACTATAAAG
 951  ATACCAGGCG  TTTCCCCCTG  GAAGCTCCCT  CGTGCGCTCT  CCTGTTCCGA
```

References

1. Ju, J., *et al.*, *Proc. Nat. Acad. Sci. USA* **92**, 4347–4351 (1995).
2. Reeve, M. A. and Fuller, C. W., *Nature* 376, 796–797 (1995).
3. Samols, S. B. and Fuller, C. W., *Comments* **21**, 57–78, United States Biochemical Corp., Cleveland, Ohio (1995).
4. Tabor, S. and Richardson, C. C., *Proc. Nat. Acad. Sci. USA* **92**, 6339–6343 (1995).
5. Tabor, S. and Richardson, C. C., *Proc. Nat. Acad. Sci. USA* **84**, 4767–4771 (1987).
6. Tabor, S. and Richardson, C. C., *J. Biol. Chem.* **264**, 6447–6458 (1989).
7. Sanger, F. *et al.*, *Proc. Nat. Acad. Sci. USA* **74**, 5463–5467 (1977).
8. Huibregtse, J. M. and Engelke, D. R., *DNA and Protein Engineering Techniques* **1**, 39–41 (1988).
9. McMahon, G. *et al.*, *Proc. Nat. Acad. Sci. USA* **84**, 4974–4978 (1987).
10. Carothers, A. M. *et al.*, *Biotechniques* **7**, 494–496, 498–499 (1989).
11. Murray, V., *Nucleic Acids Research* **17**, 8889 (1989).
12. Levedakou, E. N. *et al.*, *Biotechniques* **7**, 438–442 (1989).
13. Lee, J. S., *DNA Cell Biol.* **10**, 67–73 (1991).
14. Samols, S. B. and Fuller, C. W., *Comments* **18**, 23, United States Biochemical Corp., Cleveland, Ohio (1991).
15. Ju, J. *et al.*, *Anal. Biochem.* **231**, 131–140 (1995).
16. Pisa-Williamson, D. and Fuller, C. W., *Comments* **19**, 1–7, United States Biochemical Corp., Cleveland, Ohio (1992).
17. Mizusawa, S. *et al.*, *Nucleic Acids Research* **14**, 1319–1324 (1986).

All goods and services are sold subject to the terms and conditions of sale of the company within the Amersham Biosciences Group which supplies them. A copy of these terms and conditions is available on request.

NOTICE TO PURCHASER; LIMITED LICENSE

This kit is sold pursuant to Authorization from PE Applied Biosystems under one or more of the following US Patents: 4,849,513; 5,015,733; 5,118,800; 5,118,802; 5,151,507; 5,171,534; 5,332,666; 5,242,796; 5,306,618; 5,366,860; 4,855,225 and corresponding foreign patents and patent applications. The purchase of this kit includes limited non-transferrable rights (without the right to resell, repackage, or further sublicense) under such patent rights to use this kit for DNA sequencing or fragment analysis, solely when used in conjunction with an automated instrument for DNA sequencing or fragment analysis which has been authorized for such use by PE Applied Biosystems, or for manual sequencing. Purchase of this product does not itself convey to the purchaser a complete license or right to perform automated DNA sequence and fragment analysis under the subject patents. No other license is hereby granted for use of this kit in any other automated sequence analysis instrument. The rights granted hereunder are solely for research and other uses that are not unlawful. No other license is granted expressly, impliedly, or by estoppel.

Further information on purchasing licenses to perform DNA sequence and fragment analysis may be obtained by contacting the Director of Licensing at PE Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404.

AMERSHAM BIOSCIENCES IS LICENSED AS A VENDOR FOR AUTHORIZED SEQUENCING AND FRAGMENT ANALYSIS INSTRUMENTS.

NOTICE TO PURCHASER ABOUT LIMITED LICENSE

The purchase of this kit (reagent) includes a limited non-exclusive sublicense under certain patents* to use the kit (reagent) to perform one or more patented DNA sequencing methods in those patents solely for use with Thermo Sequenase DNA polymerase purchased from Amersham Biosciences for research activities. No other license is granted expressly, impliedly or by estoppel. For information concerning availability of additional licenses to practice the patented methodologies, contact Amersham Biosciences Corp, Director, Business Development, 800 Centennial Avenue, PO Box 1327, Piscataway, NJ 08855 USA..

* US Patent numbers 4,962,020; 5,173,411; 5,409,811 and 5,498,523. Patents pending.

Thermo Sequenase DNA polymerase – this product and/or its method of use is covered by one or more of the following patents : 4,962,020; 5,173,411: 5,409,811: 5,498,523 and foreign equivalents.

Glycerol-tolerant gel buffer—Purchase includes a non-exclusive sublicense for use under US Patent number 5,314,595. No other license is granted.

The Polymerase Chain Reaction (PCR) is covered by patents owned by Roche Molecular Systems and F. Hoffmann-La Roche Ltd. A license to use the PCR process for certain research and development activities accompanies the purchase of certain reagents from licensed suppliers such as Amersham Biosciences and Affiliates when used in conjunction with an authorized thermal cycler.

DYEnamic ET primers—Patent pending.

** 7-deaza-dGTP is sold pursuant to a license and is covered by patent number 4804748 issued by Boehringer Mannheim GmbH.

Trademarks

http://www.amersham.com

Amersham Biosciences UK Limited

Amersham Place Little Chalfont Buckinghamshire HP7 9NA UK

Amersham Biosciences AB

SE-751 84 Uppsala Sweden

Amersham Biosciences Corp

800 Centennial Avenue PO Box 1327 Piscataway NJ 08855 USA

Amersham Biosciences Europe GmbH

Munzinger Strasse 9 D-79111 Freiburg Germany

Amersham Biosciences