instructions

product code:

US79720
US79730
US79725
US79735

Thermo Sequenase DYEnamic Direct Cycle Sequencing Kit

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.

74004073 Ed. AA

Amersham Biosciences

Handling

Storage
Store at -15 ˚C to -30 ˚C.

Page finder

Quality control

All batches of DYEnamic Direct Cycle Sequencing Kits are assayed according to the recommended starting point protocol described below. Gels are run on an ABI® 373A Stretch or ABI Prism™ 377 fluorescent sequencing instrument. Release specifications are as follows: Sequence is assessed by length of read, accuracy and quality of the signal. Readable sequence beyond 500 bases is achieved.

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

The solutions included in the DYEnamic™ Direct Cycle Sequencing Kit have been carefully prepared to yield the best possible sequencing results. Each reagent has been tested extensively and its concentration determined to meet rigorous standards set at Amersham Biosciences. It is strongly recommended that the reagents supplied in the kit be used. The dGTP nucleotide or 7-deaza-dGTP* analog is incorporated in the appropriate kit.

The following solutions are included in the kit:

C reagent (blue-capped tube): 0.47 mM each dCTP, dATP, 7-deaza-dGTP or dGTP, dTTP, 1.55 µM ddCTP, 125 mM Tris-HCl, pH 9.5, 6.25 mM $MgCl_2$, thermostable pyrophosphatase and Thermo Sequenase™ DNA polymerase, 0.2 µM -28 M13 reverse primer-FAM™.

A reagent (green-capped tube): 0.47 mM each dCTP, dATP, 7-deaza-dGTP or dGTP, dTTP, 1.55 µM ddATP, 125 mM Tris-HCl, pH 9.5, 6.25 mM $MgCl_2$, thermostable pyrophosphatase and Thermo Sequenase DNA polymerase, 0.2 µM -28 M13 reverse primer-REG.

*See license information on page 35.

Materials not supplied *(continued)*

- **7.5 M ammonium acetate**—For sequencing reaction cleanup (optional).
- **DYEnamic ET Primers**
  - — -40 M13 forward 5'-GTT TTC CCA GTC ACG ACG-3'
  - — -21 M13 forward 5'-TGT AAA ACG ACG GCC AGT-3'
  - — -28 M13 reverse 5'-AGG AAA CAG CTA TGA CCA T-3'
  - — -28 M13 rev 2 5'-AGG AAA CAG CTA TGA CAT G-3'
  - — SP6 5'-ATT TAG GTG ACA CTA TAG-3'
  - — T7 5'-TAA TAC GAC TCA CTA TAG GG-3'
  - — T3 5'-ATT AAC CCT CAC TAA AGG GA-3'

Components of the kit *(continued)*

G reagent (yellow-capped tube): 0.47 mM each dCTP, dATP, 7-deaza-dGTP or dGTP, dTTP, 1.55 µM ddGTP, 125 mM Tris-HCl, pH 9.5, 6.25 mM $MgCl_2$, thermostable pyrophosphatase and Thermo Sequenase DNA polymerase, 0.2 µM -28 M13 reverse primer-TAMRA™.

T reagent (red-capped tube): 0.47 mM each dCTP, dATP, 7-deaza-dGTP or dGTP, dTTP, 1.55 µM ddTTP, 125 mM Tris-HCl, pH 9.5, 6.25 mM $MgCl_2$, thermostable pyrophosphatase and Thermo Sequenase DNA polymerase, 0.2 µM -28 M13 reverse primer-ROX™.

Formamide loading dye

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

Mobility file disk

Kit configuration	US79520	US79530
Product number	**Nucleotide analog**	**Number of reactions**
US 79720	dG	100
US 79730	dG	500
US 79725	7-deaza-dGTP	100
US 79735	7-deaza-dGTP	500
Reagent mix	**Loading dye**	**Control DNA**
200 µl	1200 µl	50 µl
5 × 200 µl	5 × 1 200 µl	50 µl
200 µl	1 200 µl	50 µl
5 × 200 µl	5 × 1 200 µl	50 µl

Materials not supplied *(continued)*

Equipment

- **Liquid-handling supplies**—Vials, pipettes, microcentrifuge, vortex mixer, and vacuum centrifuge (optional). Perform all sequencing reactions in either plastic microcentrifuge tubes (typically 0.5 ml), or in 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).

Primer/vector compatability

It is critical to confirm that the vector to be used with this kit is compatible with a standard -28 M13 reverse primer (see details below). If it is not, the kit will not work. Instead, select a DYEnamic Direct Kit without primer, and utilize the -28 M13 rev 2 primer.

Choosing a reverse primer

When using a reverse primer, care should be taken to determine whether -28 M13 reverse or -28 M13 rev 2 primer is appropriate for your vector. Many, if not most, vectors contain the "correct" sequence appropriate for use with the -28 M13 reverse primer, but some pUC-derived vectors contain a "C" deletion in the priming region (AGGAAACAGCTATGACC*AT). For these vectors, use the -28 M13 rev 2 primer. The Amersham Biosciences pUC18 vector contains a -28 M13 rev 2 priming site while pUC19 contains the standard -28 M13 reverse priming site. In our experience, Stratagene's pBluescript® and derivative vectors contain the standard site while certain Amersham Biosciences vectors contain the -28 M13 rev 2 site.

These are generally noted in their catalog information as having a non-standard sequence in the priming region. When using other vectors, the priming region should be examined closely to determine which of the two reverse primers is appropriate.

-28 M13 reverse primer sequence: 5'-AGGAAACAGCTATGACCAT-3'
-28 M13 rev 2 primer sequence: 5'-AGGAAACAGCTATGACAT-3'

World Wide Web address

http://www.amershambiosciences.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 skin or eyes; if contact should occur, wash immediately with water (see Material Safety Data Sheet for specific recommendations).

Warning: This kit 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

Energy transfer dye primers

Energy transfer dye primers are oligonucleotide DNA sequencing primers that have two fluorescent dyes attached for improved detection properties (1). One dye, called the donor dye, efficiently absorbs light of the wavelength of the argon ion laser. This dye transfers absorbed light energy to the second dye attached to the same primer molecule. This acceptor dye emits the absorbed energy as fluorescence at its normal emission wavelength for efficient detection by fluorescent sequencing instruments. Since all the primers absorb the laser light efficiently, the effective fluorescence intensity of the energy transfer primers is 2–12 times greater than primers having single-dye labels. The greater signal strength saves labor, time or template, depending on the application.

The DYEnamic ET primers use 5-carboxyfluorescein (FAM) as the donor dye. The acceptor dyes are FAM (C), 6-carboxyrhodamine (REG) (A), N,N,N',N'-tetramethyl-5-carboxyrhodamine (TAMRA) (G) and 5-carboxy-X-rhodamine (ROX) (T).

Thermo Sequenase DNA polymerase

Thermo Sequenase DNA polymerase is a thermostable enzyme engineered specifically for DNA sequencing. Amersham has applied the work of Tabor and Richardson (2) to construct this exonuclease-free thermostable DNA polymerase (3,4). Like T7 Sequenase™ DNA polymerase (5,6), Thermo Sequenase polymerase generates bands of uniform intensity, improving the data for interpretation by automated sequencing software. Thermo Sequenase polymerase combines thermal stability suitable for cycle sequencing with accuracy comparable to T7 Sequenase DNA polymerase.

The Thermo Sequenase DNA polymerase formulation contains thermostable inorganic pyrophosphatase (TAP) cloned from the thermophile *Thermoplasma acidophilum*. TAP hydrolyzes the inorganic pyrophosphate product of nucleotide polymerization. This prevents pyrophosphorolysis (the reversal of polymerization), which can result in sequence data with missing peaks.

Cycle sequencing

DNA sequencing relies on the synthesis of a new strand of DNA starting at a specific priming site and ending with the incorporation of a chain terminating nucleotide such as a dideoxynucleoside triphosphate (7). The relative concentrations of dNTPs and ddNTPs are such that the majority of chains will terminate at lengths within the resolving capacity of the gel. When these fragments are separated on a suitable gel matrix in a fluorescent sequencing instrument, sequence information can be extracted from the fluorescent emissions detected moving through a particular area of the gel.

Cycle sequencing (4, 8–14) uses repeated cycles of thermal denaturation, annealing and extension/termination to increase signal levels and therefore decrease the amount of template required. For each cycle, the amount of product DNA will be equivalent to the amount of primed template. Thus, after ten cycles there could be up to ten times as much product as template (in practice each cycle is not 100% efficient and the amplification is somewhat lower than this). If a thermostable enzyme such as Thermo Sequenase DNA polymerase is used, many cycles can be performed without the need for additional enzyme.

Cycle sequencing is therefore ideally suited for applications where the amount of template may be limiting or where the sensitivity of the detection system is not high. The combination of DYEnamic ET primers and Thermo Sequenase cycle sequencing produces the highest quality sequence data with minimal reagent, template and labor requirements.

DYEnamic Direct Cycle Sequencing Kit

This protocol provides the methods for sequencing with the DYEnamic Direct Cycle Sequencing Kit with -28 M13 reverse DYEnamic ET primer. This kit allows direct loading of the sequencing products onto the sequencing gel. The high efficiency light absorption and emission of the DYEnamic ET dye primers obviates the requirement to concentrate the sequencing reaction products by ethanol precipitation. Removing the ethanol precipitation step decreases sequencing reaction preparation time by at least one hour.

Initial setup

Prior to sequencing with DYEnamic ET primers it is necessary to perform a few preliminary tasks. Primer-specific mobility files and instrument-specific color matrix files must be placed on your computer system. A mobility file for each individual DYEnamic ET primer is provided on the diskette supplied. Although the electrophoretic mobilities of the DYEnamic ET primers are much better matched than the ordinary dye labeled primers (15), a primer-specific mobility file is still necessary for optimal base-calling accuracy. See Appendix 1 for details.

Protocols

Sequencing with the DYEnamic Direct Cycle Sequencing Kit with -28 M13 reverse primer.

Sequencing reaction products are analyzed on Applied Biosystems® models 373 or 377 instruments. All sequencing reactions can be performed in small plastic centrifuge tubes (typically 0.5 ml) or 96-well plates suitable for thermal cyclers.

This protocol is a recommended starting point. Further experiments to optimize template concentrations and conditions for thermal cycling may be required. Please refer to the "Supplementary information" section for more information.

Thaw the following on ice:

Nucleotide reagents (from the sequencing kit)—containing nucleotides, reaction buffer, enzymes, and primer; C reagent, A reagent, G reagent, T reagent.

Formamide loading dye (from the sequencing kit).

DNA template: 200 ng of single-stranded DNA or 500 ng of double-stranded DNA or PCR product. This quantity of DNA must be present in a volume of 12 µl or less (in water).

1 Preparation of sequencing reaction mixes

1.1 In four labeled microcentrifuge tubes, combine each reagent mix with the template DNA as follows.

	C	A	G	T
C reagent premix	2 µl			
A reagent premix		2 µl		
G reagent premix			2 µl	
T reagent premix				2 µl
DNA template	3 µl	3 µl	3 µl	3 µl
Total volume	5 µl	5 µl	5 µl	5 µl

2 Termination reactions

2.1 When all reagents have been prepared, treat the samples appropriately for your thermal cycler. If necessary, overlay each sample with light mineral oil. If using microcentrifuge tubes, cap the tubes and place them in the thermal cycler.

2.2 Start the cycling program.

Note: The specific cycling parameters will depend on the amount and purity of the template DNA. See the "Supplementary information" section for further information. The following parameters are general guidelines:

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

2.3 After cycling is complete, centrifuge briefly to collect any condensation.

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

❸ Preparation for loading reaction products on the gel

Mobility file selection

The correct mobility file for the primer used and the well-to-read (WTR) length must be selected 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”

4 Direct loading protocol

Load the samples directly when sufficient template (~ 500 ng ds DNA or 200 ng ss DNA) is available or when 30 thermal cycles are used.

4.1 If an oil overlay was used, remove ~ 15 µl of aqueous layer from beneath the oil. If no oil was used ~ 20 µl will be available for use. In either case, add 6–8 µl of formamide loading dye and mix well.

4.2 Heat 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 above 80 °C. Therefore, heat these samples briefly as recommended above, prior to gel loading. Signal intensity and resolution will not be affected.

4.3 Load 1.5–6 µl (depending on whether you are using an ABI Prism 377 or 373A Stretch instrument) directly onto the gel.

4.4 The gel electrophoresis and data analysis should proceed as usual for the ABI 373 or 377 instruments. Be certain to use the mobility file and color matrix appropriate for the DYEnamic ET primer and gel well-to-read length used. Signal strengths may be ~ 50% lower than for precipitated DYEnamic ET primer reactions, but produce results with accurate sequence to a read-length comparable to precipitated reactions.

5 Precipitation of sequencing reaction products

In cases where maximal detection sensitivity is required—for instance when template quantity is limiting or unknown, when only a few thermal cycles were performed, or if very long read-lengths are desired—it may be necessary to ethanol precipitate the sequencing reaction products.

Proceed through step 2.4 in the "Termination reaction" section of the sequencing protocol. After pooling the reaction:

5.1 Add 1.5 µl of 7.5M ammonium acetate to each pooled reaction tube.

5.2 Add 55 µl (approximately 2.5× the pooled reaction volume) of 100% ethanol to each reaction pool. Vortex and place on ice for 15–20 min to precipitate the DNA.

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

5.4 Draw off supernatant (aspiration is recommended if using microcentrifuge tubes), removing as much as possible.

5.5 Add 200 µl of 70% ethanol to wash the pellet. Further centrifugation is not required but will not be detrimental.

5.6 Draw off the supernatant and vacuum-dry the pellets (in a vacuum centrifuge) for 2–3 min. Air-drying for 10–15 min is also sufficient.

Note: Overdrying the DNA pellets can make resuspension difficult.

5.7 Resuspend each pellet in 4 µl of formamide loading dye. Be extremely careful to redissolve the DNA completely at this step so that the longest possible sequences can be determined. If the ethanol precipitation and wash steps were centrifuged in a fixed angle rotor, the DNA pellet will be on the side of the microcentrifuge tube. This material must be washed to the bottom of the tube to assure that the entire reaction product is loaded on the gel. Vortex vigorously (10–20 s) to ensure complete resuspension. Centrifuge briefly to collect the sample at the bottom of the tube.

5.8 Heat 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 above 80 ˚C. Therefore, heat these samples briefly as recommended above, prior to gel loading. Signal intensity and resolution will not be affected.

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

Supplementary information

The formamide loading dye migrates toward the cathode (up out of the well) upon electrophoresis.

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

Cycling temperatures

The melting temperature of a "typical" sequencing primer of 15–25 bases and approximately 50% G/C content will generally be 50–65 °C. We suggest a three temperature cycling routine (especially when the sample is a PCR product) with a denaturation temperature of 95 °C for no more than 30 s, an annealing temperature of 45–55 °C for 5–15 s, and extension at 70–75 °C for 30–120 s. If in doubt, choose a wide temperature range with brief pauses at the extremes of temperature. It is best to determine the melting temperature of your primer using commercially available software packages. Annealing at temperatures not greater than 5 °C below the melting temperature should produce specific priming.

Number of cycles and quantity of template

Cycle number depends primarily on the amount of template DNA (fmoles) used for sequencing. It also depends on the purity of the DNA and the sensitivity of the detection method. The minimum quantities of highly-purified DNA which we can sequence using these methods are about 5 fmoles (10 ng) of M13mp18 DNA and about 25 fmoles (50 ng) of pUC18 DNA. For routine sequencing, we recommend at least 100 fmoles (200 ng) of M13 and at least 250 fmoles (500 ng) of plasmid DNA. With minimal quantities of template DNA, or if the quantity of template is unknown, use 30 cycles. If read lengths and signal strengths are consistently strong (e.g. all signal strengths > 500) try reducing the number of cycles by half to save time in the thermal cycler.

We have obtained good sequence with as few as 5 cycles when using 0.1 µg CsCl purified M13 or 1 µg CsCl purified double-stranded plasmid DNA. Results vary with template purity. Use no more than 2.5 µg of plasmid or the gel may be overloaded.

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 regular pattern of migration of DNA fragments is interrupted; peaks are spaced closer than normal (compressed) and just beyond the compression, farther apart. When this occurs, sequence information is lost. The substitution of a nucleotide analog for dGTP (7-deaza-dGTP) that forms weaker secondary structure has successfully eliminated many of these gel artifacts (17). DYEnamic Direct Cycle Sequencing Kits with -28 M13 reverse primer containing either 7-deaza-dGTP analog or dGTP are available to eliminate band compression.

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

For templates with strong compressions, we observe that gels with up to 20% formamide are effective. When run on Applied Biosystems sequencing instruments with standard or energy transfer mobility files, some distortion of sequence, either early or late in the read depending on gel running conditions, may be encountered when using formamide.

Sequencing PCR products

For cleanup of PCR* products prior to sequencing we recommend the use of the PCR Product Pre-Sequencing Kit (US70995). Supplied in this pre-sequencing kit are exonuclease I and alkaline phosphatase for digestion of excess primer and single-stranded products and excess nucleotides from the PCR. Please also refer to TECHtip #160.

Denaturing gel electrophoresis

Use only the highest quality sequencing grade acrylamide, urea and other gel components to prepare the sequencing gel for fluorescent DNA sequencing analysis. Gels with 4–6% total acrylamide are typically used (19:1 acrylamide:bis ratio) with 6M 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, different mobility file might be required for sample analysis.

*See license information on page 35.

When using the direct loading procedure, glycerol will be present in the material applied to the gel. With TBE-buffered gels, an artifact may be observed that is caused by the interaction of glycerol with the boric acid in the buffer (16). This artifact is observed sufficiently far into the run so as not to interfere with most sequence analysis using the acrylamide gel concentrations recommended in the "Gel composition and running conditions" section (below). The artifact (curvature of the lane, distortion and loss of the signal as the glycerol complex passes through the detection window) will 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 may be eliminated completely by the use of a glycerol tolerant Tris-taurine-EDTA (TTE) gel buffer or by ethanol precipitating 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. This buffer is available in liquid and powder premix forms, product numbers US75827 and US71949, respectively. Be certain to run glycerol tolerant gels at the same power (wattage) as TBE-buffered gels to maintain normal gel temperature.

Gel composition and running conditions

The gel recipes below will 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 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 above recipes, substitute a portion of the water required for dissolution with 20 ml deionized formamide to produce a 20% formamide gel. Heat formamide gel solutions very gently to about 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 mix precipitates, the process must be repeated from the beginning. Electrophoresis on a gel that has been redissolved will generate 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 you plan to use it. If the gel is to be used the same day, adding four to five times the amount of TEMED will polymerize the gel in 1 hour.

See Amersham TECHtip #152 for more information.

Note: For use in fluorescent sequencing, use no more than a 20% formamide gel. The 40% formamide gel described will not produce useable data in an ABI 373 or 377 instrument.

Plate cleaning

It is extremely important to use clean glass gel plates during fluorescent sequencing. Dirty plates may lead to decreased signal strength. If you routinely use the direct loading procedure, 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 1M NaOH for 30 min and to then neutralize the solution with 1M HCl for 30 min. Again, rinse plates thoroughly before use.

Appendix 1:
Installation of mobility files

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

Note: Check the Amersham Biosciences homepage on the worldwide web 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 is partly dependent 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 files named **ET{-28M13 Reverse}** in the DYEnamic ET primer start-up disk.
3. Select **ET{-28M13 Reverse}** primer mobility files 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 for the dye primer set used.

Appendix 2: Making 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 used by the base-calling software. For your convenience, sample color matrices have been provided for both the ABI 373 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 there is instrument-to-instrument variation in the detection systems of these machines, it is strongly recommended that color matrix files be created for each primer on each instrument. A new matrix must also be made whenever the filter wheel in the ABI 373A sequencing instrument is changed. Sequencing reactions may be placed on the same gel as the color matrix samples, with analysis 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 subjected to gel electrophoresis in separate lanes (as in radioactive sequencing). Data from these four lanes are then analyzed by the ABI utility software to create the color matrix file. This file is specific for the particular instrument that ran the samples.

Color matrix preparation using the DYEnamic Direct Cycle Sequencing Kit reagents

1. Assemble the following reagents from the DYEnamic Direct Cycle Sequencing Kit with -28 M13 reverse primer or laboratory stock.
   - Nucleotide reagents with -28 M13 reverse primer C reagent, A reagent, G reagent, and T reagents.
   - 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).
   - Ammonium acetate, 7.5 M.

Reaction mixtures

1. In four labelled microcentrifuge tubes, mix the following reagents listed below:

	C	A	G	T
C reagent premix	2 µl			
A reagent premix		2 µl		
G reagent premix			2 µl	
T reagent premix				2 µl
DNA template	3 µl	3 µl	3 µl	3 µl
Total volume	5 µl	5 µl	5 µl	5 µl

Termination reactions

1. When all reagents have been prepared, treat the samples appropriately for your thermal cycler. If necessary, overlay each sample with light mineral oil. If using microcentrifuge tubes, cap the tubes and place them in the thermal cycler.
2. Start the cycling program using the following parameters:

   95 °C, 30 s
   45 °C, 15 s
   70 °C, 60 s
   30 cycles
3. After cycling is complete (typically 1.5–2 h), centrifuge briefly to collect any condensation.

Preparation for loading reaction products onto the gel

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.
2. Add 50 µl of 100% ethanol to each reaction. Vortex and place on ice for 15–20 min to precipitate the DNA.
3. Centrifuge in a microcentrifuge (~ 12 000 rpm) for 15 min at either room temperature or 4 °C.
4. Draw off the supernatant (aspiration is recommended if using microcentrifuge tubes), removing as much as possible.
5. Add 250 µl of 70% ethanol to wash the pellet. Further centrifugation is not required but will not be detrimental.

6. Draw off the supernatant and vacuum-dry the DNA pellets (in a vacuum centrifuge) for 2–3 min. Air-drying for 10–15 min is also sufficient.

   Note: Overdrying the DNA pellets can make resuspension difficult.

7. Resuspend each pellet in 8 µl of formamide loading dye. It is crucial to redissolve the DNA completely at this step so that all of the DNA is present. If the ethanol precipitation and wash steps were centrifuged in a fixed angle rotor, the DNA pellet will be on the side of the microcentrifuge tube. This material must be washed to the bottom of the tube to assure that the entire reaction product is loaded on the gel. Vortex vigorously (10–20 s) to ensure complete resuspension. Briefly centrifuge to collect the sample at the bottom of the tube.

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

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

9. A total of eight lanes will be loaded—when using a 373A instrument load, four lanes with 6 µl and four with 1 µl. Load 6 µl of each sample onto the gel, skipping lanes in between samples. Next, load 1 µl of each sample onto the gel, again skipping lanes between samples. When using an ABI 377 Prism instrument, load four lanes with 1.5 µl and four lanes with 0.5 µl.

10. Run the sequencing instrument as usual.

11. After completing electrophoresis, run the Make Matrix utility program as specified in the ABI instrument manual. If the utility fails to create a matrix with the data from the lanes loaded with 6 µl (or 1.5 µl for 377 instruments), try 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 finding 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 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. When 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, make a backup copy of this file with a different name.

Appendix 3:
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  ACCCGGGGAT
 361 TTTCCCAGTC  ACGACGTTGT  AAAACGACGG  CCAGTGAATT  CGAGCTCGGT  ACCCGGGGAT
                                                    <--- -28 M13 Reverse
                                                    TACCAGTAT CGACAAAGGA
 421 CCTCTAGAGT  CGACCTGCAG  GCATGCAAGC  TTGGCGTAAT  CATGGTCATA  GCTGTTTCCT
 481 GTGTGAAATT  GTTATCCGCT  CACAATTCCA  CACAACATAC  GAGCCGGAAG  CATAAAGTGT
 541 AAAGCCTGGG  GTGCCTAATG  AGTGAGCTAA  CTCACATTAA  TTGCGTTGCG  CTCACTGCCC
 601 GCTTTCCAGT  CGGGAAACCT  GTCGTGCCAG  CTGCATTAAT  GAATCGGCCA  ACGCGCGGGG
 661 AGAGGCGGTT  TGCGTATTGG  GCGCTCTTCC  GCTTCCTCGC  TCACTGACTC  GCTGCGCTCG
 721 GTCGTTCGGC  TGCGGCGAGC  GGTATCAGCT  CACTCAAAGG  CGGTAATACG  GTTATCCACA
 781 GAATCAGGGG  ATAACGCAGG  AAAGAACATG  TGAGCAAAAG  GCCAGCAAAA  GGCCAGGAAC
 841 CGTAAAAAGG  CCGCGTTGCT  GGCGTTTTTC  CATAGGCTCC  GCCCCCCTGA  CGAGCATCAC
 901 AAAAATCGAC  GCTCAAGTCA  GAGGTGGCGA  AACCCGACAG  GACTATAAAG  ATACCAGGCG
 961 TTTCCCCCTG  GAAGCTCCCT  CGTGCGCTCT  CCTGTTCCGA
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With 7-deaza	100 templates 500 templates	US79525 US79535
With 7-deaza-dGTP and -21 M13 forward primer	100 templates 500 templates	US79625 US79635

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-28 M13 rev 2 primer set	US79324	US79325	US79330
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RapidGel-4.75%	100 ml 500 ml	US75859-100 ml US75859-500 ml
RapidGel-5%	100 ml 500 ml	US75842-100 ml US75842-500 ml
Ammonium persulfate	100 g 1 kg	US76322-100 g US76322-1 kg
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