GE Healthcare

DYEnamic ET Terminator Cycle Sequencing Kit

Product Booklet

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US81070

Page finder

1. Legal 3
2. Handling 6
   - 2.1. Safety warnings and precautions 6
   - 2.2. Storage 6
   - 2.3. Quality control 6
3. Components of the kit 7
4. Materials not supplied 8
5. Introduction 9
6. Protocols 11
   - 6.1. Preliminary preperation and general handling instructions 11
   - 6.2. Preparation of sequencing reactions 12
   - 6.3. Resuspension of samples and electrophoresis 15
7. Appendixes 19
   - 7.1. Setting up the instrument to run and analyze DYEnamic ET Terminators 19
   - 7.2. Template DNA–general considerations 46
   - 7.3. Primers–general considerations 49
   - 7.4. Dilution and reduced reaction volumes 50
   - 7.5. Cycling conditions 52
   - 7.6. Optimal solutions for sample resuspension and injection for the ABI 3730, ABI 3730XL, ABI 3700 and ABI 3100 54
   - 7.7. Sequencing BACs and other large templates 54
   - 7.8. Considerations for post-reaction cleanup 56
8. Troubleshooting (all instruments) 59
9. References 64

1. Legal

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This kit is sold pursuant to Authorization from PE Applied Biosystems under one or more of the following US Patent numbers: 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-transferable 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.

GE HEALTHCARE 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 II DNA polymerase purchased from GE Healthcare 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 GE Healthcare Bio-Sciences 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, 5,498,523, 5,614,365 and 5,674,716. Patents pending

†This product is sold under licensing arrangements with Roche Molecular Systems, F Hoffmann-La Roche Ltd and the Perkin-Elmer Corporation. Purchase of this product is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) process for research in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Perkin-Elmer or as purchased, i.e. an authorized thermal cycler.

Energy Transfer dyes and primers—US Patent numbers: 5,654,419, 5,688,648, and 5,707,804.

Thermo Sequenase is a heat-stable DNA polymerase producing even band intensities in DNA sequencing.

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GE Healthcare reserves the right, subject to any regulatory and contractual approval, if required, to make changes in specification and features shown herein, or discontinue the product described at any time without notice or obligation.

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2. Handling

2.1. 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. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

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

2.2. Storage

Store at -15°C to -30°C.

2.3. Quality control

All batches of DYEnamic ET Terminator Cycle Sequencing Kit are assayed according to the recommended starting point protocol described in this booklet. Reactions are analyzed on ABI™ Prism™ 377 fluorescent sequencing instrument. Specifications for release are based on assessment of sequence by length of read (> 500 bases), accuracy, and signal quality.

3. Components of the kit

Solutions included in DYEnamic™ ET Terminator Cycle Sequencing Kit have been carefully formulated for optimal sequencing results. Each reagent has been tested extensively and its concentration adjusted to meet high standards set by GE Healthcare. It is strongly recommended to use all reagents supplied in the kit exactly as described in this protocol.

The following components are included in the kits:

Kit components	**US81050 (100 rxns)**	**US81060 (1000 rxns)**	**US81070 (5000 rxns)**
Sequencing reagent premix	1 x 800 µl	1 x 8 ml	1 x 40 ml
Control M13mp18 DNA (0.2 µg/µl)	1 x 50 µl	1 x 50 µl	Not included
Control primer (Universal cycle primer) (0.5 pmol/µl)	1 x 100 µl	1 x 100 µl	Not included
Sodium acetate/EDTA buffer	1 x 400 µl	1 x 4 ml	1 x 20 ml
Formamide loading dye	1 x 1200 µl	5 x 1200 µl	Not included

Store these kits and their components at -15°C to -30°C (**not** in a frost-free freezer). When the reagent is not in a freezer, keep it on ice prior to use. For convenience, the kit can be stored at 2–4°C for up to three months with no loss of performance; however, avoid this if the reagents will not be consumed completely within three months.

4. Materials not supplied

Reagents

- **Water**—Use only deionized, distilled water for the sequencing reactions.
- **Sequencing primers**—Use primers appropriate for the template being sequenced. For most applications, 5 pmol of primer is sufficient. Dissolve each primer in water and determine its optical density at 260 nm (OD_{260}). For primers containing N bases (measured in a cuvette with a 1 cm path length), the approximate concentration (pmol/µl) is given by the formula: Concentration (pmol/µl) = OD_{260}/ (0.01 x N) where N is the number of bases.
- **Ethanol (95% and 70%)**—For post-reaction cleanup. **Note:** Do not use denatured alcohol.
- **Gel reagents**—Prepare sequencing gels according to instructions provided by the manufacturer of the sequencing instrument. Use electrophoresisgrade reagents.

Equipment

- **Liquid handling supplies**—Vials, pipettes, microcentrifuge, and vacuum centrifuge. Perform all sequencing reactions in 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 use with the ABI 373 DNA Sequencer and the ABI Prism 377, ABI Prism 310, ABI 3100, ABI Prism 3700 and ABI 3730, ABI 3730XL DNA Analyzer instruments.
- **Thermal cycler**—For thermally cycled incubations between 50°C and 95°C (1–100 cycles).

5. Introduction

DYEnamic ET Terminator Cycle Sequencing Kit is designed for sensitive and robust sequencing using novel energy transfer dye-labelled terminators (1) and Thermo Sequenase™ II DNA polymerase. For sequencing with this product, a sequencing reagent premix is combined with template DNA and primer and thermally cycled. The reaction products are then precipitated with ethanol to remove unincorporated dye-labelled terminators. Samples are finally dissolved in an appropriate loading solution for separation and detection using the ABI 373, ABI 377, ABI 310, ABI 3100, ABI 3700 or the ABI 3730, ABI 3730XL sequencing instruments.

Dye terminator-based sequencing

DYEnamic ET Terminator Cycle Sequencing Kits are based on a modification of traditional dideoxynucleotide chain termination chemistry (2) in which terminators are labelled with fluorescent dyes for automated detection (3, 4). In this case, however, each of the four dideoxy terminators—ddG, ddA, ddT and ddC—is labelled with two dyes—fluorescein and one of four different rhodamine dyes—rather than a single dye. Fluorescein has a large extinction coefficient at the wavelength (488 nm) of the argon ion laser used in the sequencing instrument. Acting as the donor dye, fluorescein absorbs energy from incident laser light and transfers it to the rhodamine acceptor dye on the same terminator molecule. Each acceptor dye then emits light at its characteristic wavelength for detection that identifies the nucleotide that terminates extension of the DNA chain. This energy transfer format (1) is more efficient than direct excitation of the acceptor dye by the laser, and produces a sequencing method that is very sensitive and robust.

The acceptor dyes used in the kits are the same standard rhodamine dyes—rhodamine 110, rhodamine-6-G, tetramethyl rhodamine, and rhodamine X—used in earlier *Taq* DNA polymerase dye terminator

methodologies, so the DYEnamic ET reaction products can be detected on any instrument that can monitor the original *Taq* dye terminator chemistry.

The kit also features dITP, as well as Thermo Sequenase II DNA polymerase, a thermostable enzyme that efficiently incorporates dITP. By replacing dGTP with dITP, even very strong compression artifacts common to high GC-content DNA are resolved for more accurate data interpretation.

Thermo Sequenase II DNA polymerase

Thermo Sequenase II DNA polymerase is a thermostable DNA polymerase engineered by GE Healthcare specifically for cycle sequencing. The enzyme readily accepts dideoxynucleotide terminators (5) and generates bands of uniform intensity, much like T7 Sequenase™ DNA polymerase (6, 7). Its tolerance to high salt conditions, efficient utilization of dITP, high processivity, and excellent performance on GC-rich templates make it an efficient and robust sequencing enzyme.

Cycle sequencing

Thermostable DNA polymerases allow sequencing reactions to be cycled through alternating periods of thermal denaturation, primer annealing, and extension/termination, which increases the signal levels generated from template DNA (8–13). This amplification process employs a single primer so the amount of product increases linearly with the number of cycles. By using a thermostable enzyme, such as Thermo Sequenase II DNA polymerase, programmed cycling can proceed without multiple additions of enzyme. A cycling protocol is especially useful when the amount of template is limiting or the sensitivity of the detection system is low.

6. Protocols

6.1. Preliminary preparation and general handling instructions (Important)

Researchers who work with DYEnamic ET terminators for the first time should carefully read Appendix 7.1: "Setting up the instrument to run and analyze DYEnamic ET terminators" (page 19). A mobility file must be installed and a color correction matrix must to be created prior to their use on ABI sequencing instruments.

Thaw and maintain all kit reagents on ice prior to use. Whenever possible, cap the tubes to minimize evaporation of the small volumes of reagents used. Dispense reagents using disposable-tip micropipettes, and exercise caution to avoid contamination of stock solutions. Thoroughly mix reaction mixtures after each addition, typically by "pumping" the solution two or three times with a micropipettor without creating air bubbles. Centrifuge briefly tubes/plates to collect the reaction mixtures at the bottoms of the vessel. With care and experience, reactions can be completed in 15–20 min.

The protocol described below provides high-quality sequencing results using the control DNA and primer provided in the kit. However, regard this protocol only as a starting point. Optimization of protocols might be necessary to obtain best sequencing results for specific templates. For additional information on optimization and some important precautions, please refer to Appendixes 7.2–7.7 and the troubleshooting section.

For general information concerning templates, primers, and cycling conditions, see Appendixes 7.2–7.5 and 7.7.

6.2. Preparation of sequencing reactions

This section describes the procedure and important parameters for performing the sequencing reaction. The protocols in this section are the same for all current ABI and MegaBACE™ sequencing instruments.

1. Assemble each sequencing reaction as follows:

Template DNA (0.1–0.2 pmol)	__ µl
Primer (5 pmol)	__ µl
Water	__ µl
Sequencing reagent premix	8 µl
Total volume	20 µl

Note: 0.1–0.2 pmol of DNA and 5 pmol of primer are recommended for routine sequencing. The volumes of DNA and primer added to each reaction will depend on their concentrations. Adjust the amount of distilled water added so that the total volume of DNA, primer, and water is 12 µl. When combined with 8 µl of sequencing reagent premix, the total volume of the reaction mix should be 20 µl. For additional information concerning the amount of template and primer to use in the reaction, see Appendixes 7.2 and 7.3.

Note: The most consistent results are obtained with 8 µl of sequencing reagent premix in a reaction volume of 20 µl. However, some researchers might choose to economize by diluting the premix. GE Healthcare provides DYEnamic ET Terminator Dilution Buffer (US84002) for this purpose. Please see Appendix 7.4 for additional information concerning the use of dilution buffer.

2. Assemble the control reaction **exactly** as follows:

M13mp18 Control template (200 ng)	1 µl
Primer (2 pmol)	4 µl
Sequencing reagent premix	8 µl
Water	7 µl
Total volume	20 µl

Note: The sole purpose of the control reaction is to confirm the performance of the sequencing premix under specified and tested conditions. The reaction should therefore be assembled and performed **exactly** as described above. Customer data can then be compared with GE Healthcare quality control data should questionable performance of the sequencing premix occur.

3. Mix the contents of each tube thoroughly by gentle pipetting. Cap the tubes or seal the plates. Centrifuge briefly to bring contents to the bottom of the tubes or wells.

4. Place the tubes or plate into the thermal cycler. Run the following cycling program for 25 cycles:

95°C, 20 seconds
50°C, 15 seconds
60°C, 60 seconds
(Cycling is completed in approximatley 1 hour.)

Note: For additional information concerning cycling conditions, see Appendix 7.5.

5. When cycling is complete, add 2 µl (1/10 volume) of sodium acetate/EDTA buffer to each tube or well.

6. Add 80 µl of 95% ethanol to each reaction and mix well using a vortex mixer.

Note: When working with 384-well plates, scientists at GE Healthcare routinely use 30 µl of 95% ethanol. Please refer to

Appendix 7.8. for additional information regarding 384-well plates and small volume reactions.

Note: Post-reaction cleanup is most easily and inexpensively achieved by ethanol precipitation using the sodium acetate/EDTA buffer provided with the kit. This buffer contains 1.5 M sodium acetate (pH > 8.0) and 250 mM EDTA. EDTA dramatically reduces the amount of unincorporated terminators that precipitate with the DNA, virtually eliminating dye blobs that distort sequence data near the primer and in adjacent lanes.

Note: Do not combine the sodium acetate/EDTA buffer and ethanol prior to addition to the sequencing reaction. EDTA has very low solubility in ethanol and will precipitate.

7. Centrifuge the tubes at room temperature in a microcentrifuge for 15 minutes at ~ 12 000 rpm. Centrifuge 96-well or 384-well plates for at least 30 minutes at 2 500 x *g* or greater.

8. Remove the supernatant by aspiration from each microcentrifuge tube. For plates, a brief inverted spin (< 1 minute at 300 x *g*) is sufficient for supernatant removal. Remove as much liquid as possible at this step to prevent dye blobs.

9. Wash the DNA pellets with 70% ethanol. Use as large a volume of 70% ethanol as the tube or well can safely accommodate. Centrifuge briefly.

Note: Scientists at GE Healthcare routinely use 250–500 µl for 0.5 ml microcentrifuge tubes, 100 µl for 96-well plates, and 50 µl for 384-well plates.

10. Remove the supernatants by aspiration or by an inverted spin. Air-dry (preferably) or vacuum-dry (in a vacuum centrifuge) the pellets for 2–5 minutes. Do not overdry.

6.3. Resuspension of samples and electrophoresis

Protocols from this point on vary with the instrument being used. Be sure to follow the specific recommendations described below for the instrument that will be used to analyze the sequencing reactions.

General recommendations for resuspension of samples

a. 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.

b. It is not necessary to heat samples prior to injection. In fact, heating samples may cause excessive evaporation of the resuspension buffer and speed the breakdown of the dye-labelled sequencing products.

c. Two buffers are recommended for resuspension of the samples prior to electrophoresis. The pink formamide loading dye (US79448) supplied with the kit is recommended only for use on slab-gel instruments (ABI 373, ABI 377). MegaBACE loading solution (US79916) is recommended for capillary instruments (ABI 310, ABI 3100, ABI 3700, ABI 3730, ABI 3730XL).

If the same samples are to be analyzed on both slab-gel and capillary instruments, dissolve the samples in a small volume (4 µl) of MegaBACE loading solution. Remove enough volume of the samples for slab-gel analysis, then add MegaBACE loading solution (up to a final volume of 10 µl) to the remainder of the sample and mix well prior to injection on the capillary instrument.

ABI 3730/3730XL

1. Dissolve each pellet in 10–20 µl of MegaBACE loading solution and vortex vigorously for 10–20 seconds to ensure complete resuspension. Centrifuge briefly to collect the sample at the bottom of the tube/well.
2. Inject and electrophorese the samples according to the instructions provided with the ABI 3730/3730XL instrument. Use the appropriate run settings for DYEnamic ET terminators.

Note: For information on setting up the instrument to run and analyze DYEnamic ET terminators, see Appendix 7.1.

Note: Recommended injection parameters for MegaBACE loading solution with the control DNA is 1 kV for 20 seconds. Injection time and voltage parameters may require some adjustment for optimal results according to the instrument manufacturer's recommendations.

ABI 3700

1. Dissolve each pellet in 10–20 µl of MegaBACE loading solution and vortex vigorously for 10–20 seconds to ensure complete resuspension. Centrifuge briefly to collect the sample at the bottom of the tube/well.

Note: For additional information on the use of MegaBACE loading solution for samples analyzed on the ABI 3700, see Appendix 7.6.

2. Inject and electrophorese the samples according to the instructions provided with the ABI 3700 instrument. Use the appropriate run settings for DYEnamic ET terminators.

Note: For information on setting up the instrument to run and analyze DYEnamic ET terminators, see Appendix 7.1.

Note: Recommended injection parameters for MegaBACE loading solution with the control DNA is 1 kV for 30 seconds. Injection time and voltage parameters may require some adjustment

for optimal results according to the instrument manufacturer's recommendations.

ABI 3100

1. Dissolve each pellet in 20–30 µl of MegaBACE loading solution and vortex vigorously for 10–20 seconds to ensure complete resuspension. Briefly centrifuge to collect the sample at the bottom of the tube/well.
2. Inject and electrophorese the samples according to the instructions provided with the ABI 3100 instrument. Use the appropriate run settings for DYEnamic ET terminators.

Note: For information on setting up the instrument to run and analyze DYEnamic ET terminators, see Appendix 7.1.

Note: Recommended injection parameters for MegaBACE loading solution with the control DNA is 1.5 kV for 20 seconds. Injection time and voltage parameters may require some adjustment for optimal results according to the instrument manufacturer's recommendations.

ABI 310

1. Dissolve each pellet in 10 µl of MegaBACE loading solution (preferably) or in 10 µl of formamide loading dye and vortex vigorously for 10–20 seconds to ensure complete resuspension. Briefly centrifuge to collect the sample at the bottom of the tube/well.
2. Carry out injection and electrophoresis according to the instructions provided with the ABI 310 instrument.

Note: For information on setting up the instrument to run and analyze DYEnamic ET terminators, see Appendix 7.1.

ABI 377/373

1. Dissolve each pellet in 4 µl of formamide loading dye and vortex vigorously for 10–20 seconds to ensure complete resuspension. Briefly centrifuge to collect the sample at the bottom of the tube/well.
2. For the ABI 373, load as much sample as feasible into a single well. For the ABI 377, load half of the sample (2 µl) into a single well.
3. Perform electrophoresis according to the instructions provided with the sequencing instrument.

Note: For information on setting up the instruments to run and analyze DYEnamic ET terminators, see Appendix 7.1.

MegaBACE DNA Analysis System

1. Resuspend each pellet in 10–20 µl of MegaBACE loading solution. Vortex vigorously (10–20 seconds) to ensure complete resuspension. Briefly centrifuge to collect the sample at the bottom of the tube or plate and to remove bubbles.
2. The analysis of DYEnamic ET terminator sequences on MegaBACE DNA Analysis Systems does not require the installation of a mobility file or the creation of a color correction file. The MegaBACE analysis software automatically performs both of these functions.

7. Appendixes

7.1. Setting up the instrument to run and analyze DYEnamic ET Terminators

The analysis of DYEnamic ET terminator sequences on ABI instruments requires a mobility file and a color correction file (also known as a *spectral separation file, matrix file,* or *instrument file*). The procedures are different for **each** ABI instrument. Please proceed to the section below that pertains to the instrument being used.

Mobility file installation

- **General comments for all instruments**

Two functions are performed by the mobility file: 1) to correlate the specific dye with its corresponding base-call designation, and 2) to correct for differences in the electrophoretic mobility of sequencing products terminated with the four different terminators. The correct mobility file is supplied by GE Healthcare and need only be installed once on the computer used for analysis.

Note: The mobility file must be installed before analysis, but not necessarily before the run.

A compact disk containing the DYEnamic ET terminator mobility files for the ABI 373, ABI 377, ABI 310, ABI 3100, ABI 3700, ABI 3730 and ABI 3730XL instruments can be ordered separately (US84003). Mobility files are also available on the GE Healthcare web site. The DYEnamic ET terminator mobility files for the ABI 373, ABI 377, and ABI 310 are unchanged from those previously provided with DYEnamic ET Terminator Mobility Disk (US80872).

Note: The sequencing analysis software must be restarted after the mobility files are installed to properly recognize them.

- **ABI 3730**

The filename **DT3730POP-7{ET}.mob** refer to the mobility files

necessary to analyze runs accomplished on the ABI 3730 with POP-7™ polymer.

1. Quit the Sequencing Analysis software.
2. Copy the file from the disk to the folder:
   Copy the mobility files from the disk to the **E:\Applied Biosystems\SeqA 5.0\Shared\Analysis\Basecaller\Mobility** folder prior to launching the analysis program.

ET Terminator Data Analysis

Automatic data analysis is not yet available for DYEnamic ET Terminators on the ABI 3730. Therefore, one must load the completed sequencing run into the ABI Sequence Analysis software and select the correct DYEnamic ET Terminator mobility file prior to sequence analysis. The following protocol describes the necessary steps:

1. Select **Start>Programs>Applied Biosystems>Sequencing Analysis 5.0>Sequencing Analysis 5.0.**
2. With the **Sample Manager** window active, click either the **Add Samples** button or select **File > Add Sample(s).**
3. In the **Add Samples** dialog box, locate and open the folder that contains the files you want to add to the **Sample Manager** window.
4. Click on **Add Selected Samples**.
5. Click **OK**.
   The selected files are then loaded into the **Sample Manager**.
6. Click on the **Dye Set/Primer File** cell for the first sample in the Sample Manager.
7. From the drop-down menu, select the **DT3730POP7{ET}36cm.mob file.**

8. Fill down the **Dye Set/Primer File** cells so that the **DT3730POP7{ET}36cm.mob** file is applied to all samples.
9. Be sure that the Analysis check box (A) is checked for all samples.
10. Click on the **Start Analysis** icon in the Toolbar.
11. Click on the **Save All Samples** icon on the Toolbar.
12. The samples are now ready for viewing and further data analysis.

- **ABI 3700**

The filenames **DT3700POP-5{ET}.mob, DT3700POP-6{ET}.mob** and **DT3700POP37{ET}.mob** refer to the mobility files necessary to analyze runs accomplished on the ABI 3700 with POP-5 polymer, POP-6 polymer and POP-37 polymer, respectively.

1. Quit the Data Collection and the Sequencing Analysis software.
2. Copy the appropriate files from the disk to the folder:
   Copy the mobility files from the disk to the **Perkin-Elmer\Abi\Shared\Analysis\Basecaller\Mobility** folder prior to launching the analysis program. For newer version instruments, the folder path is: **appliedbio\Shared\Analysis\Basecaller\Mobility.**
3. Restart the Data Collection and the Sequencing Analysis software.

- **ABI 3100**

The filenames **DT3100POP-6{ET}36cm.mob, DT3100POP-6{ET}50cm.mob** and **DT3100POP4{ET}80cm.mob** refer to the mobility files necessary for analysis of runs accomplished on the ABI 3100 with POP-6 polymer and POP-4 polymer.

1. Quit the Data Collection and the Sequencing Analysis software.

* A Windows NT™ version of this mobility file can be downloaded from www.gehealthcare.com/sequencing_downloads

2. Copy the appropriate files from the disk to the folder:
   Copy the mobility files from the disk to the **Perkin-Elmer\Abi\Shared\Analysis\Basecaller\Mobility** folder prior to launching the analysis program. For newer version instruments, the folder path is:
   appliedbio\ Shared\Analysis\Basecaller\Mobility.
3. Restart the Data Collection and the Sequencing Analysis software.

- **ABI 310**

The filename **DYEnamic ET Term. POP-6*** refers to the mobility file necessary for analysis of runs accomplished on the ABI 310 with POP-6 polymer.

1. Quit the Sequencing Analysis software.
2. Copy the appropriate file from the disk to the ABI folder in the system folder.
3. Restart the Sequencing Analysis software.

- **ABI 377 and ABI 373**

The filename **DYEnamic ET Term**. {US80872}* refers to the mobility file necessary to analyze runs accomplished on the ABI 373 or ABI 377.

1. Quit the Sequencing Analysis software.
2. Copy the appropriate file from the disk to the ABI folder in the System folder.
3. Restart the Sequencing Analysis software.

Making a color-correction file

- **General comments for all instruments**

The color correction file allows the computer to identify a unique spectral signature for each of the four different dye terminators. A unique color correction file must be generated on each sequencing instrument that analyzes DYEnamic ET

terminator sequences. The color correction file is specific to the performance of each individual instrument so these files should not be copied from one instrument to another. A color correction matrix only need to be created once for the ABI 377 or 373 slab gel instruments. For the ABI 3100, ABI 3700, ABI 3730 and ABI 3730XL capillary instruments, preform a spectral calibration whenever a new capillary array is installed, or when switching from one separation medium to another.

GE Healthcare supplies matrix standards to produce color correction files for each type of ABI instrument as described below.

If a matrix file has been generated previously with any DYEnamic ET terminator kit, it is not necessary to generate a new one. Otherwise, prepare a new matrix specific for DYEnamic ET terminator chemistry for each instrument that will use the kit for each instrument.

- **ABI 310, ABI 377, and ABI 373**

Two methods are available to create a matrix on ABI 310, ABI 377, and ABI 373 instruments. The preferred method is to use the DYEnamic ET matrix standards, but the DYEnamic ET terminators can also be used.

DYEnamic ET terminators can be used on either the **run module A** setting or the **run module E** setting with equivalent performance. However, the module used to generate the matrix file must match the module used for sample files. If both types of runs are routinely performed, it is convenient to make a matrix for each run module, **A** and **E**, and use the corresponding matrix to analyze files.

a) Making a matrix from sequence data

A matrix can be created after DYEnamic ET terminator sequences have been electrophoresed, but it must be done prior to data analysis.

1. Copy the matrix file **DYEnamic ET Terminator** from the mobility file disk into the ABI folder found in the system folder. This is a template must be modified because it is not optimal for the instrument; it is simpler to modify an existing file than to create a new one.
2. Select a sequence run that appears successful based on the raw data view (i.e. good signal, well-resolved peaks throughout the run). Locate this file on the computer's hard drive. Make three copies of the file using the **Duplicate** command on the computer's file menu. This will produce four copies of the file with four distinct names.
3. Open the DataUtility program that is located within the ABI software folder.
4. Choose the menu function **Make Matrix...** under the **Utilities** menu. This function will display the window in Figure 1:

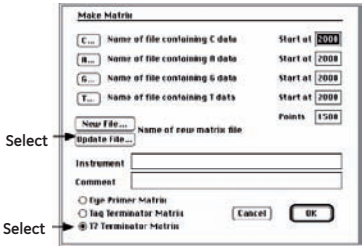

Fig 1. Make matrix window

5. Select the button **T7 Terminator Matrix**, and then select **Update File....** Choose the **DYEnamic ET Terminator** file that was copied into the ABI folder in step 1.
6. The software must now be told the names of the original sequence file and the copies of the files. To do this,

Click on **C...** then locate and open the original sequence file.
Click on **A...** then locate and open one of the copies generated in step 2 above.
Click on **G...** then locate and open a different copy.
Click on **T...** then locate and open the remaining copy.

Note: There must be four different copies of the same file.

7. Click **OK**. This generates the DYEnamic ET terminator matrix file. After a few seconds, the alert in Figure 2 is displayed:

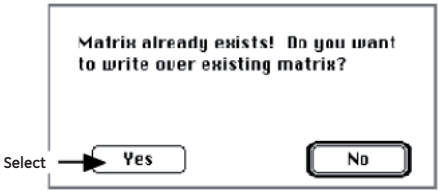

Fig 2.

8. Choose **Yes** to enter the new matrix data into the file.

Note: To avoid confusion, rename the matrix file to clearly identify the instrument, such as "ET terminator 377 serial number xxxx".

9. Steps 1–8 create a matrix file suitable for a specific instrument. This procedure must be repeated for each instrument that runs DYEnamic ET terminator sequences. A matrix only needs to be created once for each instrument, but this must be done before analysis.

Note: If any error messages are generated, check the raw data to confirm that the region set by the **Start at** and **Points** settings contains well-defined peaks.

b) Making a matrix from matrix standard data

The analysis of matrix standards is the preferred method to

create a matrix. DYEnamic ET Terminator Matrix Standard (US80860) is specifically formulated for creating a color-correction matrix file on the ABI 373, ABI 377, and ABI 310 instruments. Four matrix standards, consisting of purified single-color reaction products in 4 µl of TE buffer, are supplied in this product.

Important! The following instructions apply to DYEnamic ET Terminator Matrix Standards (US80860) with lot numbers 9614 and greater. If the lot number of your matrix standard preparation is less than 9614, please contact your local GE Healthcare office for an alternate procedure.

Note: You cannot use the DYEnamic ET Terminator Matrix Standard for the ABI 3700 (US84001) to make a matrix on the ABI 310, ABI 373, or ABI 377.

- **ABI 373 and ABI 377**

1. Add 4 µl of formamide loading dye to each matrix standard and mix well.

2. For ABI 373 instruments, load 2 µl of each matrix standard mixture into separate lanes of the gel and run the gel. For ABI 377 instruments, load 1 µl of each mixture into separate lanes and run the gel. These matrix standards can analyzed on the same gel as the sequencing reations.

Note: Be sure to record the lane order and file name of the standards because the colors displayed in the gel file will not be those commonly observed for these bases.

3. Follow steps 1–5 in the previous section "Making a matrix from sequence data."

4. The software must now be told the names of the four sample files created by the four standard lanes that were run in steps 1 and 2 above. Because the association of the dyes with bases

for the DYEnamic ET terminators is different from that for T7 terminators, different standard sample lanes from those called for in the software must be used.

As is illustrated in Figure 3:
Click on **C...** then locate and open the file containing the T matrix standard data.
Click on **A...** then locate and open the file containing the A matrix standard data.
Click on **G...** then locate and open the file containing the C matrix standard data.
Click on **T...** then locate and open the file containing the G matrix standard data.

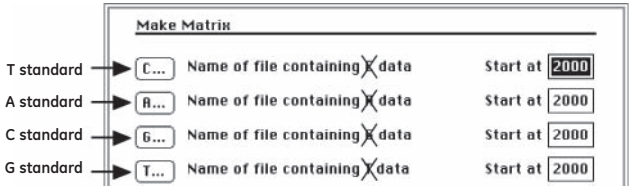

Fig 3.

5. Continue with steps 7–9 in the previous section "Making a matrix from sequence data" (page 23).

Note: If any error messages are generated, check the raw data to be sure that the region set by the Start at and Points settings contains well-defined peaks. More importantly, make absolutely sure that the order of the matrix standard data files is correct as shown above. Do not use the order suggested by the DataUtility software because errors will result, and no matrix will be created.

- **ABI 310**

1. Add 6 µl of MegaBACE loading solution to each of the four matrix standards and mix well.

2. For each of the four matrix standards, inject the entire 10 µl volume using standard conditions for injection.

Note: Each standard will require a separate run for the ABI 310 instrument.

Note: Be sure to record the lane order and file name of the standards because the colors displayed in the gel file will not be those commonly observed for these bases.

3. Follow steps 1–5 in the previous section "Making a matrix from sequence data" (page 23).

4. The software must now be told the names of the four sample files created by the four standard lanes that were run in steps 1 and 2 immediately above. Because the association of the dyes with bases for the DYEnamic ET terminators is different from that for T7 terminators, standard sample lanes different from those called for in the software must be selected.

Click on **C...** then locate and open the file containing the T matrix standard data.
Click on **A...** then locate and open the file containing the A matrix standard data.
Click on **G...** then locate and open the file containing the C matrix standard data.
Click on **T...** then locate and open the file containing the G matrix standard data.

5. Continue with steps 7–9 in the previous section "Making a matrix from sequence data" (page 23).

Note: If any error messages are generated, check the raw data to be sure that the region set by the Start at and Points settings contains

well-defined peaks. More importantly, make certain that the order of the matrix standard data files is correct as shown above. Do not use the order suggested by the DataUtility software because errors will result, and no matrix will be created.

- **ABI 3730**

DYEnamic ET Terminator Matrix Standard for the ABI 3730 (US84001) is formulated for performing a spectral calibration and creating a spectral matrix for the ABI 3730 sequencing instrument.

Preparing the matrix standard

1. Briefly centrifuge the tube containing the DYEnamic ET Terminator Matrix Standard to bring the contents (40 µl) to the bottom of the tube (**2 tubes for 3730XL**).
2. Add 460 µl of distilled water to the tube containing the matrix standard. Mix the contents of the tube thoroughly by vigorous vortexing. Briefly centrifuge the tube.

Note: Combine 2 tubes for 3730XL.

3. Dispense 10 µl of the DYEnamic ET terminator matrix standard into every other column of a 96 well plate (**all wells for 3730XL**): It is essential to centrifuge the plate to position the samples at the bottom of each well.

Creation of Spectral Protocol

1. Expand the view in the tree pane.
   a. Click the **+ box** next to the GA Instruments icon.
   b. Click the **+ box** next to the ga3730 icon.
2. Click the **Protocol Manager** icon.
3. In the Instrument Protocols section, click **New**.
   The Protocol Editor dialog box opens.
4. Create a spectral protocol.

a. Type **ET_Spectral** or a similar name in the Name field.
b. Select **Spectral** from the Type drop-down list.
c. Select the appropriate run module from the drop-down list:
i. Spect36_SeqStd_POP7_ET for 36-cm array with POP-7

Note: Prior to this step the **Spect36_SeqStd_POP7** module must be edited to include a run time of 2000 seconds. The module can be edited in the **Protocol Manager** by creating a new module based on the **Spect36_SeqStd_POP7** module.

ii. Spect50_SeqStd_POP7 for 50-cm array with POP-7

5. Select **E-BigDyeV1** or **Z-BigDyeV3** from the **Dye Set** drop-down list.

Note: Select a **Dye Set** not in use. An ET spectral calibration will overwrite any previous spectral calibrations performed on the **Dye Set** selected.

6. Select **SeqStd(Any4DyeSet).par** from the **Params** drop-down list.

Note: If the **SeqStd(Any4DyeSet).par** is not available an existing parameter file must be edited to be compatible with the **DYEnamic ET terminators**. See **Editing Spectral Calibration Section** at the end of this protocol.

7. Click **OK**.
The module is saved and displayed in the Instrument Protocols section of the Protocol Manager view.

Creating a Plate Record

1. Expand the view in the tree pane.
a. Click the + box next to the GA Instruments icon.
b. Click the + box next to the ga3730 icon.

2. Click the **Plate Manager** icon.
The Plate Manager view opens.

Terminator Matrix Standard to bring the contents (40 µl) to the bottom of the tube.

3. Click **NEW**.
   The New Plate Dialog opens.
4. Complete the plate information
   a. Type a name for the plate ID in the ID (barcode) field.
   b. Type a name for the plate in the Name field.
   c. Select **Spectral Calibration** from the application drop-down list.
   d. Select **96-Well** from the Plate Type drop-down list.
   e. Select Heat Sealing or Septa from the Plate Sealing drop-down list.
   f. Type a name for the owner and operator in the appropriate fields.
5. Click **OK**.
   A blank plate record opens.
6. Complete the plate record:
   a. In the sample name column, type a name.
   b. In the Instrument Protocol column, select the protocol created in the 'Creation of Spectral Protocol' section.
   c. Select the Sample Name and Instrument Protocol columns and fill down.
7. Click **OK**.

Adding a Plate to the Run Scheduler

1. Click the **Run Scheduler** icon.
   The Run scheduler view opens.
2. In the Input Stack section, click Search.
   A search dialog box opens.
3. Search for the spectral calibration record.
4. Add a plate record.
   a. Select the plate you want to use in the name column.
   b. Click **Add**.

c. Click **Done**.

The plate is added to the Run Scheduler view.

5. Click on the **Run Instrument** button.

Evaluating Spectral Calibration results

To view pass/fail status of each capillary:

1. Locate the log file at the following location:.

E:\AppliedBiosystems\UDC\Data Collection\Data\ga3730\instrument name\SpectralCalMclFiles\ E-BigDyeV1

2. Open the file in Notepad.

3. View the results.

The condition number (c) is a measure of the spectral overlap of the dyes. As the spectral overlap of the dye decreases, so does the condition number. a condition number of 1.0 would indicate no spectral overlap for a particular dye set. The expected condition number for ET dyes on the ABI 3730 is 6.7 ± 0.5. The Q-value is a measure of how well the spectralcalibration fits the data it was created from. A Q-value (q) of 1.0 represents a perfect fit. Any spectral calibration with a Q-value less than 0.92 will automatically fail the calibration and be replaced with spectral calibration data from an adjacent capillary.

Editing Spectral Calibration Files

If the **SeqStd{Any4DyeSet}.par** is not available when setting up the ET terminator spectral calibration protocol on the insrument, an existing parameter file must be edited to be compatible with the DYEnamic ET teminators.

1. Locate the Spectral Calibration ParamFiles folder in the following file path: E:/Appliedbiosystems/UDC/ga3730/CalibrationData/ SupportFiles/ SpectralCalibration/ParamFiles

Note: Search for files with a '**.par**' extension if the **ParamFiles** folder cannot be located.

2. Edit the **SeqStd{E}.par** if you are performing the ET spectral calibration on Dye Set E, and edit the **SeqStd{Z}.par** if the callibration is to be performed on Dye Set Z.

3. Rename the existing SeqStd{E or Z}.par file OrgSeqStd{E or Z}.par

4. Make a copy of the file to be edited by copying and pasting the file into the ParamFiles folder.

5. Edit the copied parameter file to include the following:
minQ = 0.92
conditionBounds = [1.0, 10.0]
maxScansAnalyzed = 5000

6. Save the file.

7. Rename the edited file, **SeqStd{E}.par** or **SeqStd{Z}.par** as appropriate.

Note: The instrument will only recognize the files if named exactly as described above.

8. Proceed with the ET spectral calibration protocol.

9. If at a later date a calibration must be performed with a sequencing chemistry other than the DYEnamic ET terminators, the edited parameter file must be renamed and the **OrgSeqStd {E or Z}.par** file must be renamed to its original file name.

- **ABI 3700**

DYEnamic ET Terminator Matrix Standard for the ABI 3700 (US84001) is specifically formulated for performing a spectral calibration and creating a spectral matrix for the ABI 3700 sequencing instrument.

Preparing the matrix standard

1. Briefly centrifuge the tube containing the DYEnamic ET

2. Add 360 µl of distilled water to the tube containing the matrix standard. Mix the contents of the tube thoroughly by vigorous vortexing. Briefly centrifuge the tube.
3. Transfer 200 µl of the matrix solution to each of two 200 µl microtubes as recommended by the instrument manufacturer.

Performing a spectral calibration run for POP™-5

For general guidelines on performing a default calibration, see page 6–25 of the ABI 3700 user's manual. To create a spectral matrix for DYEnamic ET terminator chemistry, follow the protocol below.

1. Firmly place the two tubes containing the DYEnamic ET Terminator Matrix Standard into the right bar in slot positions 9 and 10 as shown in the Figure 4 below and on page 6–25 in the ABI 3700 user's manual.
2. Open the ABI 3700 Data Collection software. Initiate a spectral calibration run by selecting the **Spectral Run** command under the **Run Setup** page. This opens up the **Calibration Module and Dye**

Fig 4.

Set dialog window. From here, the appropriate type of calibration module, dye set and parameter files are selected.

Note: For a schematic of the procedure, see page 6–26 in the ABI 3700 user's manual.

3. Using the pull-down menu under **Calibration Module**, choose the **SpecSQ1_1POP-5DefaultModule** file for spectral calibration for POP-5.

Note: For a schematic of the procedure, see page 6–26 in the ABI 3700 user's manual.

4. (**Important**) Under **Dye Set**, choose **F** from the pull-down menu.
5. (**Important**) Under **Parameter File**, choose **SeqStd(AnyDyeSet).par** from the pull-down menu.
6. Click **OK** to accept these chosen fields.
7. The spectral run will be displayed in the run queue. This action will simultaneously engage the **Start Run** button. Activate the run by clicking on **Start Run**.
8. After electrophoresis is completed (< 3 hours), a dialog window will display **Spectral Calibration Result** as shown on page 6–29 in the ABI 3700 user's manual. This display indicates the number of capillaries (caps) that passed spectral calibration. For a schematic, see page 6–29 in the user's manual. Accept the result by clicking **OK**. The software will automatically assign proper calibration values to failed caps from adjoining successful caps. The capillary status bar will indicate the passed caps in black and questionable caps in yellow.

Note: For further information on the significance of color-coding in the capillary status bar, see pages 6–29 and 5–68 in the ABI 3700 user's manual.

9. Upon completion of the spectral run and data processing, the quality of the spectral profile (the emission spectra for all four dyes) for each capillary must be examined. The condition number is a measure of the spectral overlap of the dyes. As the spectral overlap of the dyes decreases, so does the condition number. A condition number of 1.0 would indicate no spectral overlap for a particular dye set. The expected condition number for ET dyes on the ABI 3700 is 7.3 ± 0.5. The *Q-value* is a measure of how well the spectral calibration fits the data it was created from. A Q-value

of 1.0 represents a perfect fit. Any spectral calibration with a Q-value less than 0.92 will automatically fail the calibration and be replaced with spectral calibration data from an adjacent capillary. Those caps that have a questionable matrix should be replaced with data from a successful matrix.

Note: For further information and a schematic, see "*Reviewing and overriding the spectral calibration profiles*" on page 6–34 of the ABI 3700 user's manual.

To review the calibration profile, open the Data Collection software. Go to the **Data Acquisition** menu and open the **Override Spectral Calibration** function. A dialog window titled **Select the dye set to display** will appear.

10. Select **F** from the pull-down menu (**Important**). Click **OK**. This action opens a dialog window title **Spectral Calibration Profile for F**. The fluorescence emission spectra for all four dyes in a particular capillary will be displayed as shown in Figure 1.

11. Examine the results for each cap and verify that the profile is similar to that shown in Figure 5.

Note: For additional schematic illustrations, see page 6–35 of the user's manual.

12. Although, the software assigns values from successful caps to failed caps, in some instances the profile may not be similar to the one shown in the illustration. When this occurs, replace the values manually by using the values from a successful adjacent matrix. At the bottom left of the dialog box, under **Override matrix from another source**, click on the button **From capillary**. This allows the selection of a value from any capillary. Choose any acceptable capillary.

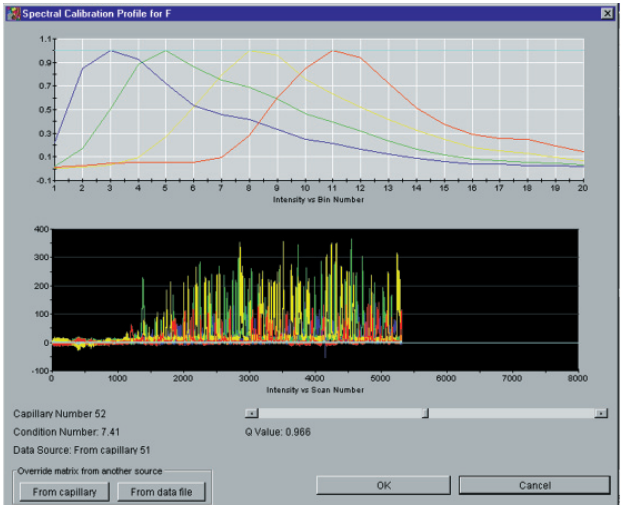

Fig 5. Spectral calibration profile for F using POP-5.

Performing a spectral calibration run for POP-37

For general guidelines on performing a default calibration, see page 6–25 of the ABI 3700 user's manual. To create a spectral matrix for DYEnamic ET terminator chemistry, follow the protocol on page 38.

Fig 6.

1. Firmly place the two tubes containing the DYEnamic ET Terminator Matrix Standard into the right bar in slot positions 9 and 10 as shown in Figure 6 below and on page 6–25 in the ABI 3700 user's manual.
2. Open the ABI 3700 Data Collection software. Initiate a spectral calibration run by selecting the **Spectral Run** command under the **Run Setup** page. This command opens the **Calibration Module and Dye Set** dialog window from which the appropriate calibration module, dye set, and parameter files are selected.

Note: For a schematic of the procedure, see page 6–26 in the ABI 3700 user's manual.

3. Use the pull-down menu under **Calibration Module** and choose the **SpecSQ1_2POP37DefaultModule** file for spectral calibration for POP™-37.

Note: For a schematic of the procedure, see page 6-26 in the ABI 3700 user's manual.

4. **(Important)** Under **Dye Set**, choose **F** from the pull-down menu.
5. **(Important)** Under **Parameter File**, choose **SeqStd(AnyDyeSet).par** from the pull-down menu.
6. Click **OK** to accept these chosen fields.
7. The spectral run will be displayed in the run queue. This action will simultaneously engage the **Start Run** button. Activate the run by clicking on **Start Run**.
8. After electrophoresis is complete (< 3 hours), a dialog window, **Spectral Calibration Result**, appears as shown on page 6–29 in the ABI 3700 user's manual. This display indicates the number of capillaries (caps) that passed spectral calibration. For a schematic, see page 6–29 in the user's manual. Accept the result by clicking **OK**. The software will automatically assign proper calibration values to failed caps from adjoining successful caps. The capillary

status bar will indicate passed caps in black and questionable caps in yellow.

Note: For further information on the significance of color coding in the capillary status bar, see pages 6–29 and 5–68 in the ABI 3700 user's manual.

9. Upon completion of the spectral run and data processing, the quality of the spectral profile (the emission spectra for all four dyes) for each capillary must be examined. The *condition number* is a measure of the spectral overlap of the dyes. As the spectral overlap of a dye set decreases, so does the condition number. A condition number of 1.0 indicates no spectral overlap for a particular dye set. The expected condition number for ET dyes analyzed on the ABI 3700 is 7.3 ± 0.5. The Q-value is a measure of how well the spectral calibration fits the data it was created from. A Q-value of 1.0 represents a perfect fit. Any spectral calibration with a Q-value less than 0.92 will automatically fail the calibration and be replaced with spectral calibration data from an adjacent capillary. Those caps that have a questionable matrix should be replaced with data from a successful matrix.

Note: For further information and a schematic, see "Reviewing and Overriding the Spectral Calibration Profiles" on page 6–34 of the ABI 3700 user's manual.

10. To review the calibration profile, open the Data Collection software. Go to the **Data Acquisition** menu and open the **Override Spectral Calibration** function. A dialog window **Select the dye set to display** will appear.

11. Select **F** from the pull-down menu (**Important**). Click **OK**. This action opens the dialog window **Spectral Calibration Profile for F**. The fluorescence emission spectra for all four dyes in a particular capillary will be displayed as shown in Figure 5.

12. Examine the results for each cap and verify that the profile is similar to that shown in Figure 5.

Note: For additional schematic illustrations, see page 6–35 of the ABI 3700 user's manual.

Although the software assigns values from successful caps to failed caps, in some instances the profile may not be similar to the one shown in the illustration. When this occurs, replace the values manually by using the values from a successful adjacent matrix. At the bottom left of the dialog box, under **Override matrix from another source**, click on the button **From capillary**, which allows the choice of values from any capillary. Choose any acceptable capillary.

Performing a spectral calibration run for POP-6

For general guidelines on performing a default calibration, see page 6–25 of the ABI 3700 user's manual. To create a spectral matrix for DYEnamic ET terminator chemistry, follow the protocol below.

Fig 7. Loading the matrix standard

1. Firmly place the two tubes containing the DYEnamic ET Terminator Matrix Standard into the right bar in slot positions 9 and 10 as shown in Figure 7 and on page 6–25 in the ABI 3700 user's manual.

2. Within the ABI 3700 Data Collection software, open the **Module Editor** listed under the **Instrument Utilities** menu. Select the **Others** tab under modules menu within the **Module Editor**. Load

the **SpectSQ1_1POP-6DefaultModule** parameters into the **Module Editor** by clicking on its listing in the **Others** tab.

3. Scroll down to the **Run Time** parameter (number 15) listed in the **Module Parameters** window. Edit the **Run Time** by clicking on the default value of 2 700 seconds, and replace that with 4 000 seconds.
4. Click the **Save As** button and enter **SpecSQ1_1POP-6Extended** as the new module file name. Click **OK** to save the new parameters. Click **Done** to exit the **Module Editor**.
5. In the Data Collection software, initiate a spectral calibration run by selecting the **Spectral Run** command within the **Run Setup** page. This opens up the **Calibration Module and Dye Set** dialog window. From here, the appropriate type of calibration module, dye set, and parameter files are selected.

Note: For a schematic of the procedure, see page 6–26 in the ABI 3700 user's manual.

6. Using the pull-down menu under **Calibration Module**, choose the **SpecSQ1_1POP-6Extended** File for spectral calibration for POP-6.

Note: For a schematic of the procedure, see page 6–26 in the user's manual.

7. Continue the spectral calibration run by following steps 4–12 in the previous section, "Performing a spectral calibration run for POP-5" (page 34).

- **ABI 3100**

DYEnamic ET Terminator Matrix Standard for the ABI 3700 (US84001) is formulated for performing a spectral calibration and creating a spectral matrix for the ABI 3100 sequencing instrument.

Preparation of DYEnamic ET Terminator Matrix Standard

1. Briefly centrifuge the tube containing the DYEnamic ET Terminator

Matrix Standard to bring the contents (40 µl) to the bottom of the tube.

2. Transfer 5 ml of the DYEnamic ET terminator matrix standard to a tube containing 195 ml of distilled water.
3. Mix the contents of the tube thoroughly by vortexing vigorously.
4. Briefly centrifuge the tube and dispense 10 ml of the DYEnamic ET Terminator Matrix Standard into a 96 well plate as shown in Figure 8. It is essential to centrifuge the plate to position the samples at the bottom of each well.
5. Assemble the plate for loading on the ABI 3100 (See diagram on page 3–9 of the ABI 3100 user's manual).

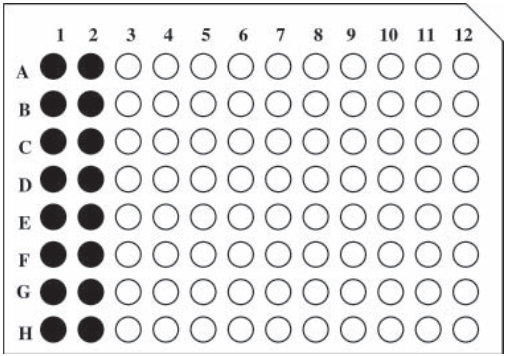

Fig 8.

Performing a spectral calibration

For general guidelines on performing a default calibration, see page 4–17 of the ABI 3100 user's manual. To create a spectral matrix for DYEnamic ET terminator chemistry, follow the protocol below.

1. Place the plate on the autosampler.

Note: For a schematic of the procedure, see page 4–24 in the ABI 3100 user's manual.

2. Open the ABI 3100 Data Collection software and initiate a spectral calibration run by selecting **New** within the **Plate View** page. This opens up the **Plate Editor** dialog box. Name the plate, select **Spectral Calibration**, and select **96-Well plate** type. Click Finish. This opens the **Plate Editor** spreadsheet.
3. Within the **Plate Editor** spreadsheet, complete the following for the 16 samples in the sample plate:
   - Name the samples.
   - Select **Dye Set F. (Important)**
   - Select the run module appropriate for you capillary array size:
     36-cm: **Spect36_POP-6DefaultModule**
     50-cm: **Spect50_POP-6DefaultModule**
     80-cm: **Spect80_POP-4DefaultModule**
4. Select the spectral calibration parameters, **SeqStd{AnyDyeSet}. par. (Important)**
5. Click **OK**.
6. The newly created plate record then appears in the **Pending Plate Records** table of the **Plate Setup** page. In the **Plate Setup** page, select the plate you just created from the **Pending Plate Records** table.
7. Click on the graphic that corresponds to the plate on the autosampler. The plate then moves from the **Pending Plate Records** table to the **Linked Plate Records** table. For a pictorial representation see page 3–39 in the ABI 3100 user's manual.
8. Click the **Run Instrument** button on the toolbar to begin the run.
9. After the run is completed (40–150 minutes) a dialog window displays "Spectral Calibration Result" as shown on page 4–23

in ABI 3100 User's Manual, indicating the number of caps that passed spectral calibration. Accept the result by clicking **OK**. The software then assigns proper calibration values to failed caps automatically from the adjoining successful caps.

10. Upon completion of the spectral run and data processing, the quality of the spectral profile (the emission spectra for all 4 dyes) for each capillary must be examined. Any capillary that generated a questionable matrix should be carefully examined and replaced with data from a successful matrix. Instructions for matrix replacement are provided in the ABI 3100 user's manual on page 4–27, "Overriding a Spectral Calibration Profile."

11. The *condition number* is a measure of the spectral overlap of the dyes. As the spectral overlap of the dyes decreases, so does the condition number. A condition number of 1.0 would indicate no spectral overlap for a particular dye set. The expected condition number for ET dyes on the ABI 3100 is 7.3 ± 0.5. The *Q-value* is a measure of how well the spectral calibration fits the data it was created from. A Q-value of 1.0 represents a perfect fit. Any spectral calibration with a Q-value less than 0.92 will automatically fail the calibration and be replaced with spectral calibration data from an adjacent capability.

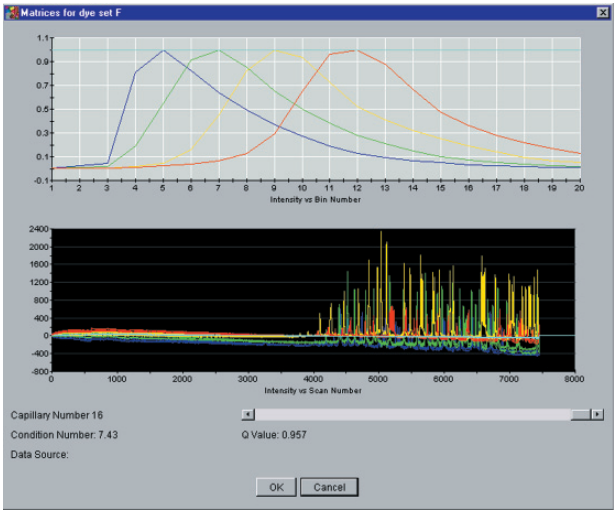

Fig 9. Spectral calibration profile for F using POP-6 with the ABI 3100.

12. Review the calibration profile using the following steps. Open the Data Collection software; select **Override Spectral Calibration** from the **File** menu. A dialog window titled "Select the dye set to display" appears. Select F from the pull down menu (Important). Click **OK**. This action opens the dialog window **Spectral Calibration Profile for F**. The fluorescence emission spectra for all four dyes in a particular capillary is displayed as shown in Figure 9. Examine the results for each cap and verify that it is similar to the one shown in this example and as illustrated on page 4–29 of the ABI 3100 user's manual. Although, the software assigns values from successful caps to failed caps in some instances, the profile may not be similar to the one shown in the illustration. When this happens, manually replace the values with the data from a successful adjacent matrix.

At the bottom left of the dialog box, under **Override matrix from another source**, click on the button **From capillary**. This will allow you to choose values from any capillary. Choose any acceptable capillary.

13. Examine the results for each cap and verify that the profile is similar to that shown in Figure 9.

Note: For additional schematic illustrations, see page 4–29 of the ABI 3100 user's manual.

7.2. Template DNA—general considerations

Template amount

This protocol usually produces optimal results using 0.1–0.2 pmol of template DNA but these numbers should be considered as guidelines. In some cases, more or less template can be used due to the sensitivity and robustness of DYEnamic ET terminators. For example, scientists at GE Healthcare have obtained good results on an ABI 377 using 10–10 000 ng (4 fmol/4 pmol) of pure M13mp18 DNA. For routine sequencing, however, follow the guidelines described above.

The following formula is useful for calculating the optimal mass (0.15 pmol) of double-stranded template to include in the sequencing reaction:

Mass of template (ng) = Total length of DNA (in base pairs) x 0.1

For example, a typical plasmid that is 3 800 base pairs in length (vector plus insert) should produce optimal data using ~ 380 ng in these protocols. The recommended range for template amount is 250–500 ng (0.1–0.2 pmol).

These relationships are shown graphically in the figures 10 and 11:

These relationships are shown graphically in the figures 10 and 11:

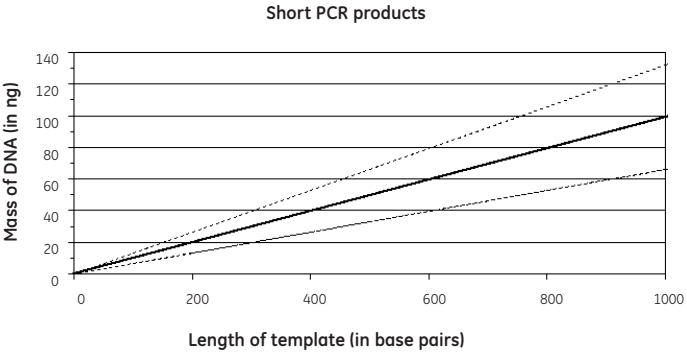

Fig 10. The recommended amount of template DNA to use in a sequencing reaction (PCR products)

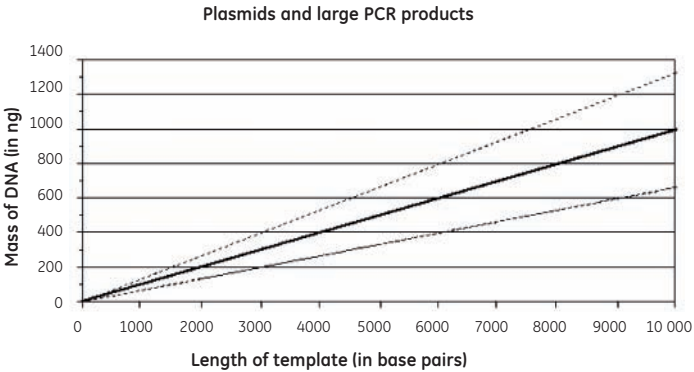

Fig 11. The recommended amount of template DNA to use in a sequencing reaction (Plasmids)

Insufficient template DNA in the sequencing reaction can produce low signal strengths (< 50) that can cause poor basecalling and short reads. In contrast, too much template can yield very high signal strengths (> 1500), that might mask software miscalls. Excessive template DNA can also deplete the supply of nucleotides in the sequencing premix and lead to short sequence reads. This is especially problematic with Polymerase Chain Reaction (PCR) products where small mass amounts of DNA are required to provide the optimal picomole amount of template.

Recommended buffer for dilution of DNA template

Dilute the DNA template in water (preferably) or in a weakly buffered solution containing no more than 0.1 mM EDTA. A suitable buffer is 10 mM Tris-HCl (pH 8.5), 0.1 mM EDTA. This concentration of EDTA is lower than in typical TE buffers because excess EDTA in the template or primer resuspension buffercan inhibit sequencing reactions by reducing the effective magnesium concentration.

Preparation of template DNA

Template of suitable quality for use with the DYEnamic ET Terminator Cycle Sequencing Kit can be prepared using a variety of procedures and commercially available products.

Single-stranded plasmid DNA

Several published methods are available for preparing single-stranded DNA from clones generated in M13 vectors and hybrid plasmid-phage (phagemid) vectors (14, 15).

Preparation of double-stranded plasmid DNA

Sequencing double-stranded templates with the DYEnamic ET Terminator Cycle Sequencing Kit requires no changes in the protocol. Alkaline denaturation is not required for these templates. For optimal results, use plasmid DNA purified by cesium chloride gradients, polyethylene glycol (PEG) precipitation, adsorption to glass, columns, and other common DNA purification methods. However, because

such small quantities of DNA are used in the reactions, even impure DNA samples can yield acceptable sequence data. Although there are many popular protocols for purifying plasmid DNA from 2 ml to 10 ml cultures, scientists at GE Healthcare achieve consistent success using boiling (16, 17) and alkaline (18) miniprep methods.

Templates for sequencing can also be prepared using TempliPhi DNA sequencing template amplification kits manufactured by GE Healthcare (for more details contact your local GE Healthcare office or visit us at www.gehealthcare.com/lifesciences and search with the keyword, "TempliPhi"). A variety of templates can be amplified by rolling circle amplification using Phi29 DNA polymerase and sequencing-quality DNA can be prepared within 4–6 hours directly from bacterial colonies (19). Microgram quantities of template DNA can be prepared at isothermal conditions from picogram amounts of starting material. Amplified DNA can be used directly for cycle sequencing without purification.

7.3. Primers—general considerations

Primer amount

The optimal amount of primer for sequencing with these protocols is 5 pmol. If not enough primer is used, signals may be weak. If too much primer is present, nonspecific priming can occur, resulting in "noisy" sequences characterized by high background or double (superimposed) sequences. Excess primer also can contribute to an artifact known as the "cliff effect" that typically appears as a short region (50–200 bases) of strong sequence abruptly followed by weak sequence. The likely cause of this artifact is the inadvertent generation of PCR products during cycle sequencing that accumulate rapidly and deplete the nucleotide supply in the sequencing premix.

Determine the concentration of primer and use 5 pmol for each sequencing reaction (2 pmol for the control primer in the kit).

The following method is useful for measuring the concentration of sequencing primer:

Dissolve the primer in water (preferably) or in buffer containing **no** more than 0.1 mM EDTA, and determine its optical density at 260 nm (OD_{260}). For primers containing N bases (measured in a cuvette with a 1 cm path lingth), the approximate concentration (pmol/µl) is given by the formula:

Concentration (pmol/µl) =
OD_{260}/(0.01 x N) where N is the number of bases.

Designing a sequencing primer

The length and sequence of a primer determines its melting temperature and specificity. For cycling temperatures recommended in this protocol, the primer should be ~ 18–25 bases in length. Check the sequence of the primer for potential self-annealing or hairpin formation, especially at its 3'-end. Possible sites of false priming in the vector or other known sequences should also be identified, again stressing matches involving the 3'-end of the primer.

7.4. Dilution and reduced reaction volumes

DYEnamic ET terminator sequencing products are designed to provide robust performance over a wide range of conditions. The DYEnamic ET terminator sequencing reagent premix is carefully formulated for use with Thermo Sequenase II DNA polymerase, and the conditions that yield the best sequencing results have been determined empirically during product development. Optimal results are achieved when the following conditions are observed: 1) the concentration of the buffer components is maintained to protect the stability and activity of the DNA polymerase; 2) the enzyme, cofactors, and substrates are present in excess such that the reaction is efficient; and 3) the volume of the sequencing reaction is sufficient to withstand evaporation. For the DYEnamic ET terminator

sequencing products, the most consistent results are obtained using 8 µl of sequencing reagent premix in a reaction volume of 20 µl, as described previously in the protocols. These are the recommended conditions for routine use and should be the starting point for any optimization experiments.

Some researchers might prefer to dilute the sequencing reaction mix to achieve greater economy, albeit at the expense of data consistency. The DYEnamic ET Terminator Dilution Buffer (US84002) provides the appropriate buffer conditions, salts, metal cofactors, and stabilizing agents necessary for optimal enzyme activity. In any case, the level of dilution that produces acceptable data must be determined empirically by the researcher.

DYEnamic ET Terminator Dilution Buffer is not included with this kit, but can be ordered separately (US84002). Dilution buffers provided by other manufacturers should **not** be used with DYEnamic ET terminator products.

Note: GE Healthcare makes every effort to develop products that are consistent in performance over a broad range of conditions. Dilution of this kit's components is not recommended or supported, and no guarantee is provided concerning the extent to which a product can be diluted. DYEnamic ET Terminator Dilution Buffer is provided as a courtesy for those researchers who choose to economize, with the understanding that the researcher must accept the risk of reduced performance.

How to use the dilution buffer

For optimal sequencing results, it is important to maintain the appropriate concentration of buffer components in the sequencing reaction. To achieve this, use 8 µl of DYEnamic ET Terminator sequencing reagent premix in a 20 µl reaction volume such that 40% of the final reaction mixture is sequencing premix. If the total

volume of the sequencing reaction mixture changes, then scale all of the components of the reaction appropriately. For example, if 10 µl reactions are assembled in a 384-well microplate, then 4 µl of sequencing premix would be required in each reaction to achieve a 40% concentration of premix in the final reaction volume.

If the researcher chooses to use less sequencing premix, then DYEnamic ET Terminator Dilution Buffer can be used to maintain the appropriate concentration of buffer components. Use the dilution buffer according to the following formula:

Volume of sequencing premix + dilution buffer =
40% of total reaction volume

For example, a 20 µl reaction (total volume) could be assembled using 8 µl of sequencing premix and no dilution buffer according to the recommended protocol. Alternatively, the 20 µl reaction could be assembled with 6 µl of sequencing premix and 2 µl of dilution buffer. This ratio maintains the appropriate concentration of buffer components and could be acceptable depending on the template being sequenced.

7.5. Cycling conditions

Of the three temperature incubation steps that comprise the cycling program (denaturation, annealing, and extension), the denaturation step is the most critical. While Thermo Sequenase II DNA polymerase has significant advantages over other DNA polymerases used for cycle sequencing, it is not as stable against thermal inactivation. The reaction buffer in the sequencing premix has been specially formulated to protect the stability of the enzyme and, with proper precautions, Thermo Sequenase II DNA polymerase has ample stability for robust sequencing.

Denaturation step

(**Important**) Do **not** use a denature at temperatures > 95°C or for

longer than 30 seconds. The inclusion of a long denaturation step prior to cycling is a common PCR strategy, but is unnecessary and not recommended for cycle sequencing reactions. Extended denaturing can prematurely in-activate Thermo Sequenase II DNA polymeraseand ultimately produce weak signals.

Annealing step

The appropriate annealing temperature varies with the length and sequence of the primer. In general, temperatures from 45–55°C work well. An annealing step is usually required only with primers < 20 bases in length. Optimal annealing temperatures are up to 5°C higher in DYEnamic ET terminator reactions than with other dye terminator sequencing products. For primers with sufficiently high melting temperatures, the annealing step can be omitted, and a two-step cycling program, alternating between denaturation and extension temperatures, can be used.

Extension step

Extension at 60°C for 60 seconds is optimal. Because Thermo Sequenase II DNA polymerase incorporates dITP more rapidly than other DNA sequencing enzymes, there is no apparent advantage to increase

the time or temperature of the extension step.

Number of cycles

Twenty five to thirty cycles are sufficient to sequence the recommended amounts of plasmids or PCR products. More cycles are usually not necessary and may lead to artifacts. Increasing the number of cycles might be appropriate when sequencing extremely large DNAs such as BACs. See appendix 7.7. for more details and recommendations.

7.6. Optimal solutions for sample resuspension and injection for the ABI 3730, ABI 3730XL, ABI 3700 and ABI 3100

Several solutions were tested for their ability to protect sequenced samples from breakdown prior to injection. These solutions included water, the ABI HiDi™ formamide, and several concentrations of formamide (deionized and nondeionized) in aqueous solutions at different pH in the presence and absence of EDTA.

In a comparison experiment, the purified sequencing reaction products were dissolved in various solutions, placed onto the ABI 3700 loading deck and incubated at ambient temperature for 24 hours. During this period, samples were loaded and run every 3 hours to simulate unattended operation. Each run was analyzed for the appearance of breakdown products (G blobs appear first with a concurrent decrease in G signal strength) and for the loss of resolution in the sequence ladder (C peaks show this first).

The MegaBACE loading solution (low-conductivity 70% formamide in water, 1 mM EDTA, pH 8) protected the sequenced samples from decomposition better than formamide solutions without EDTA, and much better than water. Samples resuspended in MegaBACE loading solution generated high-quality sequence data with minimal breakdown after 24 hours. Sequencing products resuspended in MegaBACE loading solution do not have to be covered with foil. Other loading solutions might also be appropriate, but must be tested by the investigator.

7.7. Sequencing BACs and other large templates

Bacterial Artificial Chromosomes (BACs) and other large templates > 10 kb in length can be difficult to sequence reliably. A large mass

of template DNA is required to provide enough sequencing targets (picomoles) for efficient sequencing. For example, 10 µg of a BAC 100 kb long would be required to supply the 0.15 pmol recommended for a sequencing reaction. Additionally, with a large mass of DNA in the sequencing reaction, specific and efficient priming becomes difficult.

The following suggestions are recommended for sequencing large templates:

1. Start with the purest template possible. Clean BAC preparations yield much more consistent sequencing results than "quick and dirty" preparations.
2. Include at least 1 µg of template DNA in the reaction. Although purified BACs are typically difficult to acquire in large amounts, ap-proximately 1 µg is a good general rule for minimum template mass.
3. Increase the amount of primer. Greater primer amounts (20–50 pmol per reaction) can improve priming efficiency.
4. Linearize the template. Linearizing or fragmenting the template by digestion with restriction enzymes, mechanical shearing, or sonication can improve sequencing results.
5. Denature the template. Before adding the sequencing premix, denature the template by heating for 5 minutes at 95–98°C. This denatures the template more completely and helps priming. Do **not** perform this heating step after the sequencing premix has been added to the reaction, because these temperatures inactivate the polymerase and result in failed or weak sequences.
6. Increase the number of cycles. Increasing the number of cycles to 50–100 may improve signal strengths.
7. Use full-strength or double-volume reactions. Use the recommended 20 µl reaction volume, or alternatively, perform

double-scale reactions (16 µl of Sequencing reagent premix in a 40 µl total reaction volume). This increases the final yield of sequencing products.

7.8. Considerations for post-reaction cleanup

The standard cleanup protocol is designed for 20 ml reactions carried out in 0.5 ml microcentrifuge tubes or 96-well microplates. This appendix provides a summary of the considerations for post-reaction cleanup. These recommendations are starting points for optimization. The duration of precipitation, length and speed of centrifugation, geometry of centrifuge rotor, and other parameters might need adjusting.

AutoSeq96 plates

Dye terminator is removed efficiently using AutoSeq96 filtration plates (27-5340-10). AutoSeq™96 is a 96-well spin plate containing prehydrated G-50 Sephadex™. Follow the instructions that accompany the plates. The purified sequencing product is recovered in approximately one reaction volume of water. The sample should then be dried down in *vaccuo* and resuspended in loading buffer for the ABI 310 and ABI 373/377 instruments. The eluate of the AutoSeq96 plate can be used directly for the ABI 3730, ABI 3730XL, ABI 3100, ABI 3700 instrument, following the instrument manufacturer's recommendations for injection from water.

384-well plates and reduced volume reactions

The standard ethanol precipitation protocol requires volumes that might not fit into all 384-well microplates, and many researchers choose reaction volumes smaller than 20 µl. In these instances, the standard ethanol precipitation protocol can be scaled to match the desired reaction volume. For instance, if total reaction volume is one quarter the recommended volume (5 µl instead of 20 µl), use 1/4 the recommended volume of sodium acetate/EDTA (0.5 µl instead of 2 µl) and 1/4 the recommended volume of 95% ethanol (20 µl instead of

80 µl). Success rates may be unacceptable with such small reactions. Alternately, isopropanol mediated precipitation can be used.

Isopropanol precipitation in 384-well plates

Isopropanol has two advantages over ethanol: 1) Lower concentrations are required for precipitation, hence smaller total volumes are involved during cleanup and 2) It is unnecessary to add salt to the reaction. After cycling, add 1–1.5 volumes of 80% isopropanol. Spin and wash as outlined in the standard ethanol precipitation. Good results are obtained using a final concentration of 40–65% isopropanol in the precipitation mix; 50–60% isopropanol is optimal. The disadvantage with isopropanol precipitation is that the pelleted DNA is less firm than with ethanol precipitation, and may be lost during wash and inverted spins. It is important to add an isopropanol solution that is less than 100% isopropanol because addition of pure isopropanol, even to the same final concentration, causes dye blobs. These blobs are caused by very high local concentrations of isopropanol before and during mixing.

Alternate salts for ethanol precipitation

The sodium acetate/EDTA buffer included with the kit produces the most efficient precipitation over the widest range of ethanol terminators. The disadvantage to this salt is that it cannot be mixed with ethanol prior to addition to the sequencing reaction. Alternate salt solutions have been investigated and the results are presented in Table 1.

To generate this table, control reactions were purified by ethanol precipitation with various salts and ethanol concentrations. To each 20 µl reaction, 1/10 volume (2 µl) of the given salt was added, the solution was mixed, and 95% ethanol was added to the tabulated final ethanol concentration. The mixture was centrifuged and washed as described in the standard protocol. “Blobs” denotes that unincorporated terminators were precipitated and not removed by the 70% ethanol wash. “Good” denotes no terminator blobs observed

and the sequence was strong and started at the expected position. "Delayed" denotes that the precipitation of sequencing fragments was inefficient and the trace was weak early in the sequence.

	Final ethanol concentration					
Salt added	85%	80%	75%	70%	65%	60%
1.5M NaOAc/250mM EDTA	Blobs	Good	Good	Good	Delayed	Delayed
7.5M ammonium acetate	Blobs	Blobs	Blobs	Good	Delayed	Delayed
7.5M NH40Ac/60mM EDTA	Blobs	Blobs	Good	Good	Delayed	Delayed
1.5M NaOAc	Blobs	Good	Good	Delayed	Delayed	Delayed
No Salt	Blobs	Blobs	Blobs	Blobs	Blobs	Blobs

Table 1. Precipitation efficiency

8. Troubleshooting (all instruments)

Under ideal conditions, the strengths of the G, A, and T signal values reported by the analysis software should be roughly equal and between 200 and 1 000. The C signal will be lower but should be at least 50% of the G, A, and T values. Signal strengths < 50 or > 1500 for ABI 373, ABI 377, and ABI 310 or < 10 or > 1500 for ABI 3700, 3100, 3730, 3730XL may indicate problems.

Note: Always run the kit control reagents in parallel with test samples during optimization.

Problem	Possible causes/solutions
1. Sequence data have high background.	**1. The matrix was bad.** Confirm that a matrix file has been made from the standards on each machine on which the kit will be used.
	2. The wrong matrix was used. Confirm that the matrix file being used was generated with the same run module as the samples on the specific instrument being used.
	3. Sequences were run with the wrong run module. Confirm that the matrix used for analysis matches the run module used for collection of the sequencing data. Either Run Module A or E can be used, but the module used to make the matrix must match the module used for the run.
	4. The sequence was not correct, even though the data looked good. Verify that the correct mobility file is being

Problem	Possible causes/solutions
1. Sequence data have high background *continued.*	**4.** *Continued.* used. Bases are correlated with dyes via the mobility file. **5. Nonspecific binding of the primer occurred.** Due to the formulation of the sequencing reagent premix, primer annealing may be more efficient with this terminator product. Increase the annealing temperature or eliminate the annealing step in the cycling program.
2. Sequencing signals are weak.	**1. The Thermo Sequenase II DNA polymerase was denatured.** Do not include an initial denaturation step in the cycling program. Do not incubate above 95°C. **2. Samples were not centrifuged sufficiently following salt/ethanol precipitation.** Centrifuge the samples for a longer period or at a higher speed. **3. The amount of ethanol used for precipitation was insufficient.** Verify that the final ethanol concentration is exactly 75% during precipitation of the DNA. **4. The primer or template contained excess EDTA.** Resuspend both primer and template in water or in dilute buffer containing < 0.1 mM EDTA.

Problem	Possible causes/solutions
2. Sequencing signals are weak *continued.*	**5. The DNA preparation was faulty.** Repeat the protocol using the control DNA supplied in the kit. **6. Either the quantity of template DNA or the number of cycles used for amplification was insufficient.** Increase either the amount of DNA used in the reaction or the number of cycles. **7. The annealing temperature was too high for the primer being used.** Use a lower annealing temperature for cycling. **8. Insufficient primer was used.** The recommended amount of primer is 5 pmol per reaction. **9. The primer was faulty.** Some primers form dimers or hairpins that can interfere with annealing. Use a different preparation of the primer or a primer of different sequence. **10. The wrong volume of sequencing reagent premix was used.** The reagents are carefully formulated to work optimally with 8 µl of premix in a 20 µl reaction volume. No other configuration is recommended or supported. Check the volumes of reagents added. **11. Primer and template DNA were used in excess.** Check the quantities added to the sequencing reaction mix.

Problem	Possible causes/solutions
2. Sequencing signals are weak *continued.*	**12. The sequencing reagent premix was diluted.** Repeat the experiment using the recommended amounts of reaction components. **13. The sequencing reagent premix was diluted in the wrong dilution buffer.** DYEnamic ET Terminator Dilution Buffer is specially formulated for use with this product. Dilution buffers from other manufacturers are not optimized for use with DYEnamic ET terminators. **14. For capillary instruments, the sample contained too much salt.** Salt can interfere with electrokinetic injection used on capillary instruments. Perform a 70% ethanol wash of the pellet after precipitation or use a gel filtration medium to remove salt.
3. Extensions appear short with read-length limited to < 350 bases.	**1. Excess template DNA was present in the reaction.** In some cases, the use of too much template, especially PCR product DNA, can exhaust the supply of ddNTPs in the reaction. If this occurs, the sequence will suddenly fade before reaching 350 bases. This problem is especially prevalent if excess primer is also used. Use < 1 pmol of template DNA and 5 pmol of primer for each reaction. By using less template,

Problem	Possible causes/solutions
3. Extensions appear short with read-length limited to < 350 bases. *Continued.*	**1.** *Continued.* the concentration of any potential contaminant is also reduced. **2. The extension step incubation was too brief.** Increase the duration of the extension step in the cycling program to 2–4 minutes.
4. Peaks in the sequence are broad and poorly resolved.	**1. The DNA preparation was contaminated.** Repeat the sequencing protocol using the control DNA supplied in the kit. **2. For the ABI 373 and ABI 377 instruments, the sequencing gel was faulty.** Repeat the sequencing protocol using a new gel. **3. Too much template DNA was used in the sequencing reaction.** Use less template.
5. Localized broad peaks—terminator blobs—are prevalent in the sequences	**1. Residual terminators were not removed from the samples.** Carefully follow the protocol for post-reaction cleanup using a final concentration of exactly 75% ethanol and the sodium acetate/EDTA buffer supplied with the kit. Alternatively, residual terminators can be removed using spin columns. If problems persist, please contact GE Healthcare Technical Service for assistance.

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