

## Material Required

### Materials provided by Beckman Coulter:

CEQ Dye Terminator Cycle Sequencing Kit (P/N 608000):  
 DNA polymerase\*\*  
 CEQ Dye Terminators (ddUTP, ddGTP, ddCTP, ddATP)  
 dNTP Mix Solution  
 Sequencing Reaction Buffer  
 pUC18 Control Template (0.25 µg/µL)  
 M13 -47 Sequencing Primer (1.6 pmol/µL or 1.6µM)  
 Glycogen (20 mg/mL)  
 Mineral Oil  
 Sample Loading Solution (SLS)

### Required materials not provided by Beckman Coulter:

Molecular Biology Grade:  
 sterile dH<sub>2</sub>O, 95% (v/v) ethanol/dH<sub>2</sub>O, 70% (v/v) ethanol/ dH<sub>2</sub>O  
 3M Sodium Acetate pH5.2 - Sigma, Cat # 430771  
 100mM Na<sub>2</sub>-EDTA pH8.0  
 Sterile tubes - 0.5 mL microfuge, 0.2 mL thin wall thermal cycling tubes  
 or plates  
 Thermal cycler with heated lid

### NOTICE TO PURCHASER: LIMITED LICENSE

The purchase price of this product includes a limited, non-transferable license under U.S. Patent 5,332,666; and claims in its foreign counterparts that correspond to processes for DNA sequence and fragment analysis, to use this product in DNA sequence and fragment analysis and related processes described in said patents for the internal research and development activities of the purchaser when this product is used in conjunction with an authorized DNA sequence analysis instrument for detection sequence fragments. No right to perform or offer commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted, either by implication or estoppel. No other patents are licensed by purchase of this product, either by implication or estoppel. Further information relating to the purchase of licenses for DNA sequence and fragment analysis and other applications may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, Applied Biosystems Division, 850 Lincoln Centre Drive, Foster City, CA 94404.

### \*\*CAUTION\*\*

The enzyme is in a 50% glycerol solution. Pipet this solution slowly and carefully. The viscosity of the glycerol in the enzyme solution can lead to pipetting errors.

## CEQ 2000 Dye Terminator Cycle Sequencing

Storage of the CEQ Cycle Sequencing kit must be in a -20° C non-frost free freezer.

### Preparation and Storage of the DTCS Premix:

1. Prepare DTCS Premix in a sterile 1.5 microfuge tube:

Component	Volume
10X Sequencing Reaction Buffer	200 µL
dNTP Mix	100 µL
ddUTP Dye Terminator	200 µL
ddGTP Dye Terminator	200 µL
ddCTP Dye Terminator	200 µL
ddATP Dye Terminator	200 µL
Polymerase Enzyme	100 µL
Total Volume	1200 µL

2. Mix and aliquot 200 µL into sterile 0.5 mL microfuge tubes. Each 200 µL aliquot is enough for 16 samples.
3. Store the aliquots in a -20° C non-frost free freezer. Minimize freezing and thawing of the aliquoted DTCS Premix.

### Preparation of the DNA sequencing reaction\*:

Prepare the 20 µL sequencing reaction in a 0.2 mL thin-wall tube or microplate well. Keep all reagents on ice while preparing the sequencing reactions and add components in the order listed below.

Component	Volume
H <sub>2</sub> O (to adjust total volume to 20 µL)	x.x µL
DNA Template† (See Template Preparation)	0.5 - 6.0 µL
Customer supplied or -47 Sequencing Primer (1.6 pmol/µL or 1.6µM)	2.0 µL
DTCS Premix	12.0 µL
Total Volume	20.0 µL

†Use 0.5 µL for pUC18 control template.

\*Note: Mix reaction components thoroughly. Consolidate the liquid to the bottom of the tube or well by briefly centrifuging before thermal cycling.

### Thermal cycling program:

96°C            20 sec.  
 50°C            20 sec.  
 60°C            4 min.  
 for 30 cycles followed by holding at 4°C

### Ethanol precipitation:

1. Prepare a labeled, sterile 0.5 mL microfuge tube for each sample.
2. To each labeled tube, add 4 µL Stop Solution (1.5 M NaOAc + 50 mM EDTA prepared fresh daily by mixing equal volumes of the 3M NaOAc and 100mM EDTA listed in "Required materials not provided by Beckman Coulter") and 1 µL of 20 mg/mL of glycogen (supplied with the kit). Transfer the sequencing reaction to the appropriate labeled 0.5 mL tube and mix thoroughly.
3. Add 60 µL cold 95% (v/v) ethanol/dH<sub>2</sub>O from -20°C freezer and mix thoroughly. Immediately centrifuge at 14,000 rpm at 4°C for 15 minutes. Carefully remove the supernatant with a micropipette (the pellet should be visible).

Note: For multiple samples, always add the cold ethanol/dH<sub>2</sub>O immediately before centrifugation.

- Rinse the pellet 2 times with 200  $\mu$ L 70% (v/v) ethanol/ dH<sub>2</sub>O from -20°C freezer. For each rinse, centrifuge immediately at 14,000 rpm at 4°C for a minimum of 2 minutes. After centrifugation carefully remove all of the supernatant with a micropipette.
- Vacuum dry for 40 minutes.
- Resuspend the sample in 40  $\mu$ L of the Sample Loading Solution (provided in the kit. See Appendix C for handling and storage of the Sample Loading Solution).

#### Sample preparation for loading into the CEQ:

- Transfer the resuspended samples to the appropriate wells of the polypropylene sample plate recommended for the CEQ.
- Overlay each of the resuspended samples with one drop of light mineral oil (provided in the kit).
- Load the sample plate into the CEQ and start the desired method.

## Appendix

### Appendix A

#### Sequencing of PCR products

All PCR products must be homogeneous in size as judged by gel electrophoresis.

#### Purified PCR products

- Remove unincorporated primers and dNTP's using QIAGEN QIAquick™ PCR purification system.
- Use 25-100 fmoles of PCR and 3.2 pmoles of primer.

#### Unpurified PCR products

- For the original PCR amplification, the primer concentration should be 0.2  $\mu$ M or less, while the dNTP concentration should be 50  $\mu$ M or less.
- The amplification should be sufficient to produce a concentration of amplified fragment that is > 10 fmoles/ $\mu$ L.
- Dilute this amplified fragment approximately 10 fold to result in a concentration of > 1 fmol/ $\mu$ L.
- Use 5-15 fmoles of this diluted, unpurified PCR product and 3.2 pmoles of primer.

### Appendix B

#### Sequencing of Large Templates

Adding 50-100 fmol for large templates such as BACs, cosmids and PACs is impractical. The following procedure should be used when sequencing large templates.

- Use 1.5  $\mu$ g of the template in 6 $\mu$ L of deionized water.
- Pre-heat the template at 96°C for 1 minute. (see Template Pre-Heat Treatment for details)
- Add the sequencing reaction components as described in the standard protocol.
- Cycle for 50 cycles using the appropriate cycling conditions for the primer being used.
- Ethanol precipitate, as normal.

### Appendix C

- Store the Sample Loading Solution in 350  $\mu$ L aliquots at -20°C in a non frost-free freezer.
- Use each aliquot only once - do not freeze/thaw the Sample Loading Solution.

## Template Preparation

### DNA Template Preparation:

Prepare sufficient template to allow for accurate quantitation and purity testing. Quality of the DNA template will depend upon the procedure and the source of the DNA used. The following are the recommended protocols:

- QIAGEN QIAwell™ and QIAprep™ DNA isolation protocols (dsDNA and ssDNA)
- QIAGEN QIAquick™ PCR purification protocol (PCR products) \*

\*Note: Determine the quality and quantity of template DNA by agarose gel electrophoresis.

### DNA Template Amount:

The amount of template DNA to use in the sequencing reaction depends on the form of the DNA (dsDNA plasmid, ssDNA, M13, PCR product, etc.). It is important to accurately quantitate the amount (moles) of DNA when performing the DNA sequencing reaction (see formula and table below for details). The molar ratio of primer to template must be  $\geq$  40:1. Listed below are the recommended amounts of DNA:

dsDNA	50-100 fmol
ssDNA	25-50 fmol
Purified PCR products	25-100 fmol

The following table can be used to estimate DNA concentrations.

Table for estimating the dsDNA\*\* concentration.

Size (kilobase pairs)	ng for 25 fmol	ng for 50 fmol	ng for 100 fmol
0.2	3.3	6.5	13
0.3	4.9	9.8	20
0.4	6.5	13	26
0.5	8.1	16	33
1.0	16	33	65
2.0	33	65	130
3.0	50	100	195
4.0	65	130	260
5.0	80	165	325
6.0	100	195	390
8.0	130	260	520
10.0	165	325	650
12.0	195	390	780
14.0	230	455	910
16.0	260	520	1040
18.0	295	585	1170
20.0	325	650	1300
48.5	790	1500†	1500†

\*\*For ssDNA, the values (ng) should be divided by 2.  
† Do not use more than 1.5  $\mu$ g of template DNA.

### Template Pre-Heat Treatment

For certain plasmid DNA templates, the following pre-heat treatment improves both signal strength and current stability.

Dilute the template to the appropriate concentration in water. Heat the template at 96°C for 1 minute in a thermal cycler and then cool to room temperature before adding the remainder of the sequencing-reaction components. Do not add any other sequencing-reaction components to the plasmid template before carrying out this pre-heat treatment. If the raw data signal declines steeply when using this treatment, change the heating conditions to 86°C for 5 minutes. If the current is low or unstable following this treatment, increase the treatment to 96°C for 3 minutes.

See the detailed "CEQ™2000 Dye Terminator Cycle Sequencing Chemistry Protocol" (P/N 718119) for more information.