P/N 608118·AC
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GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit

Material Required

Materials provided by Beckman Coulter:

- Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (P/N 608120):
  - DTCS Quick Start Master Mix
  - pUC18 Control Template (0.25µg/µL)
  - M13 -47 Sequencing Primer (1.6pmol/µL or 1.6µM)
  - Glycogen (20mg/mL)
  - Mineral Oil
  - Sample Loading Solution (SLS; see Appendix C for storage conditions)

Required materials not provided by Beckman Coulter:

- Molecular Biology Grade: sterile dH_2O, 95% (v/v) ethanol/dH_2O, 70% (v/v) ethanol/dH_2O
- 3M Sodium Acetate pH5.2 - Sigma Cat # S 7899
- 100mM Na_2-EDTA pH8.0, prepared from 0.5 M Na_2-EDTA - Sigma Cat # E 7889)
- Sterile tubes - 0.5mL microfuge, 0.2mL thin wall thermal cycling tubes or plates
- Thermal cycler with heated lid

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

1. Preparation of the DNA sequencing reaction*:

Prepare sequencing reaction in a 0.2mL thin-wall tube or microplate well. All reagents should be kept on ice while preparing the sequencing reactions and should be added in the order listed below.

dH_2O (to adjust total volume to 20µL)	0 - 9.5µL
DNA Template† (See Template Preparation)	0.5 - 10.0µL
Customer supplied or -47 Sequencing Primer (1.6pmol/µL or 1.6µM)	2.0µL
DTCS Quick Start Master Mix	8.0µL
TOTAL	20.0µL

†Use 0.5µL for pUC18 control template.

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

2. Thermal cycling program:

96°C	20 sec.
50°C	20 sec.
60°C	4 min.

for 30 cycles followed by holding at 4°C

3. Ethanol precipitation:

Precipitation in Individual Tubes

a) Prepare a labeled, sterile 0.5mL microfuge tube for each sample.
b) Prepare fresh Stop Solution/Glycogen mixture as follows (per sequencing reaction): 2µL of 3M Sodium Acetate (pH 5.2), 2µL of 100mM Na_2-EDTA (pH 8.0) and 1µL of 20mg/mL of glycogen (supplied with the kit). To each of the labeled tubes, add 5µL of the Stop Solution/Glycogen mixture.
c) Transfer the sequencing reaction to the appropriately labeled 0.5mL microfuge tube and mix thoroughly.
d) Add 60µL cold 95% (v/v) ethanol/dH_2O 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_2O immediately before centrifugation.
e) Rinse the pellet 2 times with 200µL 70% (v/v) ethanol/ dH_2O 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.
f) Vacuum dry for 10 minutes (or until dry).
g) Resuspend the sample in 40µL of the Sample Loading Solution (provided in the kit).

Precipitation in the Samples Plates

The Thermal Cycling and Ethanol precipitation can be performed in the sample plate. For instructions, see pages 19-20 in the Dye Terminator Cycle Sequencing Chemistry Protocol (P/N 718119).

4. Sample preparation for loading into the instrument:

a) Transfer the resuspended samples to the appropriate wells of the sample plate (P/N 609801).
b) Overlay each of the resuspended samples with one drop of light mineral oil (provided in the kit or Sigma Cat # M 5904).
c) Load the sample plate into the instrument and start the desired method.

Template Preparation

1. DNA Template preparation:

Prepare sufficient template to allow for its accurate quantitation and purity verification. The 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.

2. 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 ≥ 40:1. Listed below are the recommended amounts of DNA:

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

The following table can be used to estimate DNA concentrations.

Table for estimating the dsDNA concentration.

Size (kilobase pairs)	ng for 25fmol	ng for 50fmol	ng for 100fmol
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.
*Use no more than 1.5µg of template DNA.

3. Template Pre-Heat Treatment

For certain plasmid DNA templates (not the included pUC18 control DNA), the following pre-heat treatment improves both signal strength and current stability.

- Dilute the template with water to the appropriate concentration.
- 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 performing this pre-heat treatment.
- If the raw data signal declines steeply when using this treatment, change the heating conditions to 86°C for 5 min. If the current is low or unstable following this treatment, increase the treatment to 96°C for 3 min.

*See the Detailed Dye Terminator Cycle Sequencing Chemistry Protocol (P/N 718119) for more information.

Appendix

Appendix A

Sequencing of PCR products

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

- Purified PCR products
  a) Remove unincorporated primers and dNTPs using QIAGEN QIAquick™ PCR purification system.
  b) Use 25-100fmol of PCR product and 3.2pmoles of primer.

Unpurified PCR products

a) For the original PCR amplification, the primer concentration should be 0.2µM or less, while the dNTP concentration should be 50µM or less.
b) The amplification should be sufficient to produce a concentration of amplified fragment that is ≥ 10fmol/µL
c) Dilute this amplified fragment approximately 10 fold to result in a concentration of ≥ 1fmol/µL.
d) Use 5-15fmol of this diluted, unpurified PCR product and 3.2 pmoles of primer.

Appendix B

Sequencing of Large Templates

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

1. Use 1.5µg of the template in 6µL of sterile, deionized water.
2. Pre-heat the template at 96°C for 1 minute.
3. Add the sequencing-reaction components as described in the standard protocol.
4. Cycle for 50 cycles using the appropriate cycling conditions for the primer being used.
5. Precipitate with ethanol, as previously described.

Appendix C

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

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