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Dye-terminator DNA sequencing V.5

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Diep Ganguly[1]

[1]University of Pennsylvania

Pogson Group

Diep R R Ganguly
The Australian National University

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Protocol status: In development
Works well for plasmid DNA, optimising sequencing of amplified PCR amplicons.

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Abstract

This protocol (based on the BigDye® Terminator v3.1 Cycle Sequencing Kit) is for performing terminator cycling sequencing reactions for Sanger sequencing of amplified PCR products or plasmid DNA on the 3130X genetic analyser (Applied Biosystems).

Attachments

PDF BigDye Terminator v3... 350KB

PDF wizard-sv-gel-and-pc... 426KB

Materials

MATERIALS

- XRN-1 - 100 units **New England Biolabs Catalog #**M0338L
- Antarctic Phosphatase - 1,000 units **New England Biolabs Catalog #**M0289S
- 96 well PCR Plate Non-skirted **Phenix Research Catalog #**MPS-499
- Wizard SV Gel and PCR Clean-Up System **Promega Catalog #**A9281
- Nuclease-free water (e.g. MilliQ or HPLC grade water)
- primers
- EDTA
- 10 mM dNTPs **Life Technologies Catalog #**10297-018
- Ethanol **Merck Millipore (EMD Millipore) Catalog #**100983
- BigDye™ Terminator v3.1 Cycle Sequencing Kit **Thermo Fisher Catalog #**4337454
- Exonuclease I (E. coli) **NEB Catalog #**M0293S
- Hi-Di™ Formamide **Thermo Fisher Scientific Catalog #**4311320

Before start

Optimize PCR cycling (if sequencing amplified PCR products) to ensure your reaction produces a single product. If needed, perform gel excision and clean-up to purify the target DNA fragment. Incubate with Antarctic phosphatase (SAP, AP, or CIP) and Exonuclease 1 to dephosporylate and degrade unincorporated dNTPs prior to incorporating fluorescent nucleotides in the sequencing PCR (BigDye reaction).

Enzymatic PCR clean-up

1h

1 1h

If sequencing a PCR amplified DNA fragment, gel purify target DNA band based on expected fragment size (if multiple bands present). Perform gel purification with Wizard SV Gel and PCR Clean-Up System (Promega, as per attached Manufacterer's instructions) followed by enzymatic clean-up (hydrolyze excess primers and nucleotides) with the following reaction:

	Component	Volume (µl)
	10X Antarctic phosphatase reaction buffer	1
	Antarctic phosphatase	0.5
	XRN-1	0.5
	Purified DNA fragment	50-150 ng DNA
	Nuclease-free water	to 10 µl

Enzymatic clean-up of PCR products

Incubate the above in a thermal cycle for:

1. 37 °C for 30 minutes
2. 80 °C for 15 minutes.

Terminator cycling reaction

2 30m

Perform sequencing PCR in PCR tubes (or 96-well plate) with BigDye Terminator cycling kit and forward or reverse primers.

	Component	Volume (µl)
	v3.1 Ready reaction mix	1
	5X Sequencing buffer	1.5
	20 µM F/R Primer	0.5

	Template (plasmid or cleaned PCR product)	50-150 ng DNA
	Nuclease-free water	to 10 µl

BigDye Terminator Cycling reaction

5x reaction buffer=400 mM TRIS, 10 mM $MgCl_2$

3 Run the following thermal cycling protocol: 4h
1. 1 min at 96 °C
2. 30-40 cycles: 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 min.
3. Hold at 4-12 °C.

Purification 1h 30m

4 Transfer PCR reaction to nuclease-free eppendorf tube. To the reaction, add 2.5 µL of 125 mM EDTA (make sure it touches bottom of tube).

5 Add 30 µl of 100% ethanol, *mix well* (inversion).

6 Incubate at room temperature for 15 minutes.

7 Centrifuge at 4 °C at max speed for 30 minutes.

8 Discard supernatant and add 50 µl of ice-cold 70% ethanol.

9 Centrifuge at 4 °C at max speed for 5 minutes.

10 Discard supernatant and allow to air-dry in the dark for >15 minutes.

Prepare for sequencing

11 Resuspend the pellet (likely transparent) in 7.5 µL HiDi Formamide (add to any empty wells). Incubate at RT for 5 minutes then transfer to plate. Spin down briefly.

12 Incubate plate at 95 °C for 3 minutes (denature) then place immediately on ice. Spin down briefly.

13 Submit for sequencing on 3130X genetic analyser (Applied Biosystems). Keep samples on ice.