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Dye-terminator DNA sequencing V.5

Diep R Ganguly[1]

[1]The Australian National University

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Pogson Genomics Group

Diep Ganguly
The Australian National University

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

BigDye Terminator v3.1.pdf

wizard-sv-gel-and-pcr-clean-up-system-protocol.pdf

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PROTOCOL CITATION

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KEYWORDS

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MATERIALS

NAME	CATALOG #	VENDOR
XRN-1 - 100 units	M0338L	New England Biolabs
Antarctic Phosphatase - 1,000 units	M0289S	New England Biolabs
96 well PCR Plate Non-skirted	MPS-499	Phenix Research
Wizard SV Gel and PCR Clean-Up System	A9281	Promega
Nuclease-free water (e.g. MilliQ or HPLC grade water)		
primers		
EDTA		

NAME	CATALOG #	VENDOR
10 mM dNTPs	10297-018	Life Technologies
Ethanol	100983	Merck Millipore
BigDye™ Terminator v3.1 Cycle Sequencing Kit	4337454	Thermo Fisher
Exonuclease I (E. coli)	M0293S	NEB
Hi-Di™ Formamide	4311320	Thermo Fisher Scientific

BEFORE STARTING

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 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: 1h

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 Perform sequencing PCR in PCR tubes (or 96-well plate) with BigDye Terminator cycling kit and forward or reverse primers. 30m

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).

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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.

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