VERSION 5

JUL 12, 2020

OPEN ACCESS

DOI:

dx.doi.org/10.17504/protocols.io.bhxmj7k6

Protocol Citation: Diep R Ganguly 2020. Dye-terminator DNA sequencing. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.bhxmj7k6>

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

Created: Jun 26, 2020

Last Modified: Jul 12, 2020

PROTOCOL integer ID:
38605

Keywords: Sanger sequencing, Dye-terminator sequencing

Dye-terminator DNA sequencing V.5

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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

[BigDye Terminator v3.1.pdf](#)

[wizard-sv-gel-and-pcr-clean-up-system-protocol.pdf](#)

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) **Contributed by users**
- primers **Contributed by users**
- EDTA **Contributed by users**
- 10 mM dNTPs **Life Technologies Catalog #10297-018**
- Ethanol **Merck 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 INSTRUCTIONS

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 dephosphorylate 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 Manufacturer'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 μ M F/R Primer	0.5
Template (plasmid or cleaned PCR product)	50-150 ng DNA
Nuclease-free water	to 10 μ l

BigDye Terminator Cycling reaction

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

- 3** Run the following thermal cycling protocol:
 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.

4h

Purification

1h 30m

- 4** Transfer PCR reaction to nuclease-free eppendorf tube. To the reaction, add 2.5 μ L of 125 mM EDTA (make sure it touches bottom of tube).
- 5** Add 30 μ l 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 μ l 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.