THE NIGERIAN INSTITUTE OF MEDICAL RESEARCH
CENTRAL RESEARCH LABORATORY
CYCLE SEQUENCING WORKFLOW

ABSTRACT

This protocol is a gel-based/ExoSAP IT method of purifying template amplicon for cycle sequencing. This protocol also exhibits flexibility of using either the BigDye Terminator v3.1 system, ethanol/bead or alcohol/EDTA - HiDye/Formamide approach for cycle sequencing and cycle sequenced product purification before capillary electrophoresis using a SeqStudio genetic analyzer.

MATERIALS

1. ExoSAP IT Kit
2. Gel purification kit
3. Bright Dye or BigDye Terminator v.3.1 kit
4. X-terminator/Sam solution
5. Absolute Ethanol
6. 0.5M EDTA
7. Hi Dye
8. Formamide
9. Seq Studio Genetic Analyzer
10. Adhesive film
11. 96 well plate
12. Cathode buffer
13. Sequencing cartridge

PROTOCOL

1. Run 5 µl of amplified DNA on 1.5% agarose gel to confirm the presence of the gene of interest, to ascertain its band size and purity.
2. Proceed to cycle sequencing if amplicons are pure and band size falls within the range of interest. Otherwise, clean up amplicons directly with SAP-EXO method or excise the bands of interest from the agarose gel and clean up with gel purification method.

SAP-EXO method: using SAP/EXO KIT, cat. Pp 218S. (Jena Bioscience)

1. Add 2 µl of SAP-EXO mix directly into 5 µl PCR product.
2. Incubate for 10 minutes at 37 °C
3. Inactivate for 10 minutes at 80 °C

GEL PURIFICATION METHOD: using DNA CLEAN AND CONCENTRATOR, CAT. D4013. (ZYMO RESEARCH)

1. Add 24 ml 100% ethanol to the 6 ml DNA Wash Buffer concentrate.
2. Add 250 µl of DNA binding buffer to 50 µl DNA fragment (5:1)
3. Transfer the mixture into the ZymoSpin™ VI Column in a collection tube and centrifuge for 30 seconds at 3,000 x g and discard the flow-through
4. Add 200 µl DNA Wash Buffer to the Column. Centrifuge the column for 30 seconds at 3, 000 x g.
5. Discard the flow-through and repeat the wash step.
6. Transfer the column to a 1.5 ml micro-centrifuge tube and add ≥ 6 µl DNA elution buffer directly to the column matrix and incubate at room temperature for 1 minute
7. Centrifuge the column to elute the DNA at 3000 x g for 30 seconds.
8. Store at -20^{0}C.

CYCLE SEQUENCING: Using BrightDye Terminator Cycle Sequencing Kit, cat. BDT3-24. (MCLAB)

Template DNA quantity per reaction;

PCR product: 100-200bp	1-3 ng
PCR product: 200-500bp	3-10 ng
PCR product: 500-1,000bp	5-20 ng
PCR product: 1,000-2,000bp	10-40 ng
PCR product: > 2,000bp	20-80 ng

1. Using the appropriate template concentration, prepare a forward or reverse sequencing reaction mix in a PCR tube/PCR plate.
2. Seal the reaction plate with adhesive film or caps, then centrifuge briefly.
3. Run the reactions in a thermal cycler with the following cycling conditions: 96°C 3 minutes, 30 cycles of (96°C 10 seconds, 50°C 5 seconds, 60°C 150 seconds) and 4°C hold.
4. After the cycle sequencing reactions are complete, spin the plate briefly. (Optional) Store the reaction plate at 4°C overnight or at –15°C or –25°C for long-term storage.

Component	**Volume**	**Volume (recommended)**
BrightDye Terminator	0.25 µl	0.5 µl
5X Sequencing buffer	1.875 µl	1.75 µl
Template		
Primer	3.2pmol	3.2pmol
Add water to 10 µl		

CLEANUP OF SEQUENCING PRODUCTS; Using BigDye Sequencing Clean Up Kit, Cat. BCB-100. (MCLAB)

1. Add 10 µl beads to each 10 µl sample.
2. Add 40 µl of 80% ethanol to mixture.
3. Pipette the system 3-5 times for homogenous mixture
4. Load the reaction plate to a magnet plate and keep it for 1 minute.
5. Keep the reaction plate on the magnet plate and aspirate the cleared solution (supernatant) from the plate and discard it.

NOTE: To avoid disturbing the beads, place the pipette tip at the bottom of the well when aspirating and remove as much supernatant as possible because it contains excess fluorescent dye and contaminants.

6. Add 80 µl of 70% ethanol to each well.
7. Pipette the system 3-5 times and hold the washing system for 1 minute.
8. Keep the reaction plate on the magnet plate and aspirate the cleared solution (supernatant) from the plate and discard it.
9. Add 40 µl of elution buffer to each well.
10. Remove the reaction plate from the magnet plate and pipette the system 3-5 times.
11. Re-load the reaction plate to a magnet plate and hold for 1 minute.
12. Aspirate 25 µl of the supernatant (pure sequence product) into a collection plate and spin slightly.
13. Store at -4^{0}C or proceed immediately to capillary electrophoresis.

Note :

CAPILLARY ELECTROPHORESIS

You can store dried, cleaned up, and sealed sequencing reactions at –20°C for many days.

References

1. Blakesley RW. Cycle sequencing. Methods Mol Biol. 1993;23:209-17

2. Mardis E, McCombie WR. Cycle-Sequencing Reactions. Cold Spring Harb Protoc. 2017;2017(7):pdb.prot094607