

Sequencing Protocol for ABI 3730 Capillary Sequencer:

1ul Big Dye terminator V3.1
100ng -500ng template (plasmid)*
3.2pmol primer
3.5ul 5x buffer
dH2O to 20ul

- for pcr products 20-50ng depending on size.
- **note: ABI supply enough buffer (free) for 1.5ul of buffer per reaction only.**

PCR as follows:

Repeat the following for 25 cycles:

Rapid thermal ramp to 96°C

96°C for 10 secs

Rapid thermal ramp to 50°C

50°C for 5 secs

Rapid thermal ramp to 60°C

60°C for 4mins

Rapid thermal ramp 4°C and hold until ready to purify.

Purification Methods:

Use **Columns** to remove excess Big Dye terminators followed by an ETOH precipitation (best method)

or

Ethanol/EDTA Precipitation

To precipitate 20uL sequencing reactions in a 96 well reaction plate

Remove the 96 well reaction plate from the thermal cycler and spin briefly

Add the following:

5ul of 125mM EDTA

60µl of 100% ETOH to each well (make sure EDTA reaches the bottom of the wells)

Seal the plate with aluminium tape and mix by inverting 4 times.

Incubate at room temperature for 15 mins to precipitate the extension products.

NB: Precipitation times shorter than 15 mins will result in loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.

If you are using:

(a) Beckman Allegra 6A centrifuge with a GH-3.8A rotor, set at 4°C and spin the plate at 1650 x g for 45 min.

(b) Any other centrifuge: use a plate adapter and spin plate at the maximum speed as follows:

- 1400-2000 x g for 45 min

or

- 2000-3000 x g for 30min

Important: proceed to the next step immediately (if not possible, spin for an extra 2 mins before performing next step).

Invert the plate and spin up to 185 x g, then remove from the centrifuge.

Important: The supernatants must be removed completely or else unincorporated dye terminators will remain in the samples.

Add 60µl of freshly made 70% ethanol to each well.

With the centrifuge set at 4°C, spin at 1650 x g for 15 min.

Invert the plate and spin up to 1 min, then remove from the centrifuge.

Note: the timing starts when the rotor starts moving.

To store, cover with aluminium foil, and store at 4°C.

IMPORTANT! Make sure wells are dry. You may use a speed-vac for 15 mins to dry. Make sure samples are protected from light while drying.

Note: The method is particularly good for removal of excess dye terminators (unincorporated dye labelled terminators). While this method produces the cleanest signal, it may cause loss of small molecular weight fragments) so chose the cleanup method that works best for your needs.

Ethanol/EDTA Cleanup (1.5ml tubes)

Pipette entire contents (20µl) of the extension reaction into a 1.5ml microfuge tube

Add the following:

5ul of 125mM EDTA

60µl of 100% ETOH to each

Close the tubes and vortex briefly.

Leave the tubes at room temperature for 15 mins to precipitate the extension products.

NB: Precipitation times shorter than 15 mins will result in loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.

Place tubes in microcentrifuge and spin for 20 mins at Max speed.

Important: proceed to the next step immediately (if not possible, spin for an extra 2 mins before performing next step).

Carefully aspirate the supernatants with a separate pipette tip for each sample and discard. Pellets may or may not be visible.

Important: The supernatants must be removed completely or else unincorporated dye terminators will remain in the samples.

Add 250µl of freshly made 70% ethanol to the tubes and vortex briefly.

Place the tubes in the microcentrifuge in the same orientation as in the previous spin step and spin for 10 mins at max speed (set the centrifuge temp at 4°C for this step).

Aspirate the supernatants carefully as previously.

Dry the samples in a vacuum centrifuge for 10 -15 mins or to dryness (alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90°C for 1 minute.