Molecular Ecology Lab DNA Sequencing

Overview: This protocol covers the steps necessary to obtain DNA sequence on the ABI 3730xl capillary sequencer after PCR products have been amplified and EXO-SAP purified (extraneous primer has been removed). It includes steps involved in the cycle sequencing reaction, but does not cover cycle-sequencing clean-up, for which many options exist (Ethanol precipitation, Sephadex column centrifugation, and magnetic bead separation).

The Cycle Sequencing Reaction:

1. Make master mixes with **Big Dye 3.1**, 5x Sequencing Buffer, 10uM primer and ddH20. A "1/16 reaction" is our lab standard.

	1/16 RXN
ddH20 (vol.)	**7.5 ul**
10 uM Primer (vol.)	**0.33 ul**
Big Dye 3.1 (vol.)	**0.5 ul**
5x Buffer (vol.)	**0.5 ul**
*DNA (vol.)**	**2.0 ul**
	10.83 ul

*Note: DNA concentrations should be quantified to 100-1200ng/ul. Increasing template concentration seems to have little effect, but sticking closer to 100-300ng/ul (especially at the 1/32 dilution) is recommended. Using a standard (e.g. 100bp ladder) at different concentrations and running it on an agarose gel next to your samples is a good method of quantification. When sequencing longer fragments (>850bp), you should first try less DNA (1.0-1.2) so that you don't saturate the reaction.

2. Fill a 96-well PCR (thin-walled) plate or strip tubes with the master mix. Label the plate so you know its orientation. Name the plate with a unique name (your name plus the date, e.g. Schoville_04112015).
3. Add Exo-SAP-cleaned PCR products to appropriate wells.
4. Be sure to cover the plate or tubes securely—use heat-seal film for plates.
5. Optional: Spin-down briefly in the plate centrifuge.
6. Run Cycle Sequencing program in thermocycler. This takes 2.5+ hours. We use the following protocol:

2 min. @ 95 deg. (Initial Denaturation) → [*15 sec. @ 95 deg.* → *15 sec. @ 50 deg.* → *4 min. @ 60 deg.*] X 25 cycles → *10 deg. hold forever*.

7. Prepare your sample sheet, relaying the order of your sequences. The first column (rows 1-96) should be the sample names, in this order relative to the sequencing plate columns: 1A-1H, 2A-2H, 3A-3H, etc. The second column in the sample sheet should be an underscore and the primer name. Email this sample sheet so it can get uploaded to the sequencing center.
8. Transport plate to sequencing facility or store reactions at 4 deg. (at most 2 days), covered with aluminum foil. Make sure it is labeled on the aluminum foil with the plate name.

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