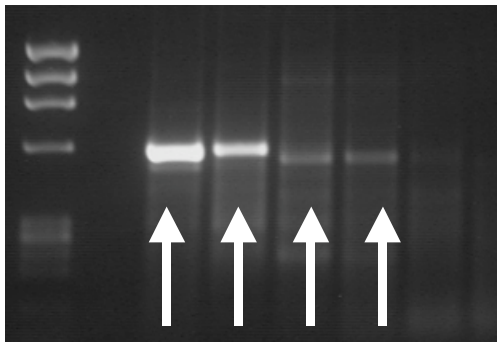
Cycle Sequencing Protocol (updated 10/30/05)

Cycle sequence is similar to PCR. It uses most of the same ingredients, follows the same basic procedure, and is done in a thermal cycler as well. One key difference is that only one primer is used in each cycle sequencing reaction so that the amplification of product is linear, not exponential. Another key difference is that dideoxynucleotides are used which interrupts the extension of the DNA strand when incorporated. Remember that when doing cycle sequencing **you will have 2 master mixes**, and that **only one primer goes in each master mix**. Because cycle sequencing is a linear amplification process, it is less susceptible to contamination, but maintain good sterile technique anyway. Cycle sequencing must only be done in the **HIGH DNA** area of the lab. Use thermal cycler specified for cycle sequencing.

1. Look at your PCR gel and determine how much template you may need for each reaction. Doing SAP/EXO cleanups you can use up to 3 uL per sequencing reaction if your template is weak. However, sequencing will work best with 1uL. For very strong reactions you may need to dilute your SAP/EXO reaction as too much template may inhibit the cycle sequencing reaction.



Yes Yes No No

2. Determine how many samples you will be sequencing.

3. Using freshly gloved hands, label 2 sets of 200 µL strip tubes. You will need one set for your forward primer, and one set for your reverse primer. Use differently colored strip tubes for each primer (Blue for HCO or CRA, clear for LCO or CRE). Put labels on side of tube, not on cap (heated lid will remove labels on cap), and remember to write which primer is in which set of tubes. Keep all samples amplified with the same primer together (i.e., all tubes with primer 1 in one strip and all with primer 2 in another strip).

4. Obtain one aliquot each of the forward and reverse primer sequencing master mixes (e.g. HCO and LCO for inverts, CRA and CRE for fish) from the freezer. These master-mixes have everything except for BigDye.

5. Thaw tubes completely and ensure contents are well mixed by finger flicking.

6. Label 2 new 0.5mL or 1.5mL tubes for each of the primers (e.g. HCO and LCO for inverts, CRA and CRE for fish).

7. Calculate the amount of master mix you need for the number of samples you have. You will require 10.5 µL per sample, plus one extra to compensate for pipetting error. For example, if you have 10 samples you will require (10+1) x 10.5µL or 115.5µL total of each master mix.

8. Pipette 10.5 µL of master mix into each of the prelabeled tubes. Remember, **only one master mix per tube.**

9. To each of the master mixes, add 0.5 µL of BigDye per sample, plus 0.5 µL extra for pipetting error. For example, if you have 10 samples you will add (10+1) x 0.5µL or 5.5µL total of BigDye to each master mix.

10. Add BigDye to your first master mix. Pipette up and down ~25times to mix in the BigDye. Change tips then add BigDye to your second master mix. Pipette up and down ~25times to mix in the BigDye.

11. When the BigDye has been added and mixed into each of the master mixes, set a pipette to 11uL and using one tip per master mix, aliquot 11uL the master mix into the strip tubes labeled MM1. When finished, **change tips** and repeat with MM2. Leave tops open.

12. Add 1ul of each template (your PCR reaction cleaned with SAP/EXO) to each tube**, changing tips between each sample**. Pipette up and down when adding each template. Close tubes with caps and press down firmly when completed. Remember that you will add each successfully amplified PCR product to both the strips labeled MM1 and to MM2.

13. Visually inspect tubes for bubbles. If bubbles are present, briefly centrifuge.

14. Place all tubes in thermal cycler and run cyc-seq program.
    Program runs for: (96˚C for 10 sec, 50˚C for 5 sec, 60˚C for 4 min) X 25 cycles.

15. When finished, store reactions at room temperature until ready to precipitate.

Tips:

1. Good sequencing comes from good PCR, so make sure you have good templates.
2. Always sequence in the **HIGH DNA** area.
3. Label carefully. There is so much handling of the samples that clear and consistent labeling is critical.
4. Although sequencing is less susceptible to contamination than PCR, still maintain good sterile technique. In particular, ensure that you clean up very well so as not to introduce PCR products into other parts of the lab.
5. Remember, nothing from the **HIGH DNA** area ever goes back to the low DNA area. This includes gloves. If you will be working in the **LOW DNA** area after being in the **HIGH DNA** area, dispose of your gloves, then wash your hands well prior to moving areas.