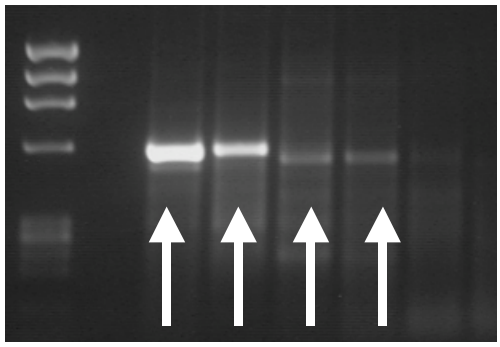
Cycle Sequencing Protocol

Cycle sequence is similar to PCR. It uses most of the same ingredients, follows the same basic procedure, and is done in a thermocycler 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 exponetial. Another key difference is that dideoxynucleotides are used which interrupts the extention of the DNA strand when incorporated. Remember that when doing cycle sequencing t**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 thermocycler 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. 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. If possible, use differently colored strip tubes for each primer. 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 tube. Keep all samples amplified with the same primer together (ie, all tubes with primer 1 in one strip and all with primer 2 in another strip).

3. Label two 1.5mL microcentrifuge tubes with the names of your two primers.

4. Make 2 master mixes with the following amounts for each sample, omitting the template:

Big Dye	1 µL
Primer (10 µM stock = 3.3 pmol)	0.33 µL
5x buffer	1.5 µL
Template (after SAP/EXOI)	1-3 µL
Water	up to 10 µL

One master mix will have primer 1, and the other will have primer 2. Do not put template directly into the master mix, and only put one primer in each master mix. **Calculate the volume of water** such that the reagents plus water plus template will yield a total volume 10 µL. Just like PCR, make sure that all reagents are thawed and mixed (by finger flicking) completely before use, and that you change tips for each reagent.

5. When all reagents have been added, pipette MM1 up and down several times. Aliquot 9uL the master mix into the tubes labeled MM1 (this volume assumes the use of 1ul of template). When finished, change tips and repeat with MM2. Leave tops open.

6. Add 1ul of each template to each tube, changing tips for each tube. Pipette up and down when adding each template. Close the top on each tube after you have added template to help keep track of where you are.

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

8. Place all tubes in thermocyler 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.

9. 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.