

Biology 362, Principles of Genetics

LAB #5

This week's lab exercises consist of:

- (1) Dye-terminator cycle-sequencing of your purified PCR product.
- (2) Tour of the IAB Core Facility

Dye-Terminator Cycle-Sequencing

The object of this lab component is to sequence the DNA that you extracted, amplified with PCR, and purified using agarose electrophoresis. To sequence the DNA, you will use dye-terminator cycle-sequencing. In the cycle-sequencing process, the template DNA is subjected to PCR with:

- (1) *Taq* DNA polymerase
- (2) dNTPs (deoxy nucleotides) in large concentration
- (3) ddNTPs (dideoxy nucleotides) with fluorescent dyes in low concentration
- (4) DNA primer

The primer may either be a primer used in PCR or a primer that matches the DNA sequence somewhere internally. Each primer is used in a separate reaction, and the DNA can be sequenced in either direction from anywhere on the sequence depending which primer is selected. In this lab exercise, we will sequence with the same two end primers (L78 & H774) that you used to generate the PCR product two weeks ago. Good primer sites typically are GC-rich (>50% so they have higher melting temperatures than AT-rich primers) and lack hairpins and other secondary structures that can interfere with primer binding. Sequencing primers also should not bind more than one part of the sequence.

We will sequence only those individual PCR products that produced bands. Negative controls that produced bands also can be sequenced. If your PCR reaction did not result in a band, please obtain a subsample of a PCR product from a student that got a band.

After you complete the cycle sequencing reaction set-up and thermalcycler steps, the labeled sequencing products will be purified (excess dye removed) by your TAs and sequenced on the Applied Biosystems 3100 Genetic Analyzer in the Core Facility for Nucleic Acid Analysis (<http://mercury.bio.uaf.edu/core/index.html>). Your results will be available for analysis in two weeks.

How does the dye-terminator cycle-sequencing protocol work?

During DNA replication, the DNA polymer is lengthened at the 3' end. This is because there is an -OH group at the 3' carbon, which is used to attach additional nucleotides. Nucleotides that lack a 3' -OH group are called dideoxy nucleotides, and these nucleotides result the termination of the extension process each time they are encountered. Each dideoxy nucleotide, ddCTP, ddATP, ddTTP, or ddGTP, has a different fluorescent dye attached to it, one unique color for each ddNTP. When exposed to a UV laser, the four different ddNTPs fluoresce with four different wavelengths and are detected and recorded by a sensor on the DNA sequencer.

In each reaction tube, you will combine: **(1) BigDye** (which contains *Taq* DNA polymerase, standard dNTPS, and labeled ddNTPs), **(2) one primer**, **(3) DNA** (purified PCR product), and **(4) water**. There will be a much higher concentration of dNTPs in your tube than ddNTPs. If you had 100% ddNTPs, the *Taq* polymerase would add one ddNTP to the primer and it would no longer be able to lengthen the DNA polymer! On the other hand, with 100% dNTPs, you would be unable to sequence the template DNA because all extension products would be complete and no dye would be attached to any nucleotides. Thus, the Big Dye contains dNTPs and ddNTPs in a mixture of approximately 100:1. Thus, each time the polymerase adds a nucleotide to the DNA polymer, there is a small chance that it will add a ddNTP. For instance, where the polymerase might add a dCTP, it occasionally adds a dye-labeled ddCTP instead, thereby ending the extension of that particular DNA fragment. Repeating the PCR-cycle many times using a thermalcycler results in a population of fragments ranging from the length of the primer plus one nucleotide to the length of the primer plus all nucleotides in the PCR product! Since these extension products are labeled with different fluorescent dyes depending on the base composition, the composition of the 3' nucleotide base can be identified by separating the fragments on a gel and scanning the fluorescence pattern with a UV laser that energizes the dyes attached to each ddNTP. Relatively little dye is required because the dyes fluoresce brightly when illuminated by the laser, and the detector is very sensitive.

MATERIALS

0.2 mL thin-walled PCR tubes
BigDye (contains dNTPs, ddNTPs, Taq Polymerase)
Primers (1 μ M) – L78 & H774
Template DNA (this is your purified PCR product)
De-ionized water
Pipettes and tips

METHODS

Those of you who did not get a product from the electrophoresis lab, please use a friend's PCR product. The products that fluoresced brighter in last week's lab will likely produce the best sequences.

1. Add **2.0 uL** of **L78** to a 0.2 mL PCR tube.
2. Add **2.0 uL** of **H774** to a second 0.2 mL PCR tube.
3. Add **3.0 uL** of template DNA to each tube.
4. Add **3.0 uL** of de-ionized water to each tube.
5. Add **2.0 uL** of BigDye to each tube. There should be a total of 10 uL of solution in each tube, one for each primer.
6. Cap the PCR tubes and label them on the cap. Place your tubes on ice.

The samples will be run on the thermalcycler as follows:

1. 96 C for 1 second
2. 96 C for 10 seconds (denature)
3. 50 C for 5 seconds (anneal)
4. 60 C for 4 minutes (extension)
5. Repeat steps 2–4 24 times
6. 4C for an infinite hold

Please note that this is a PCR reaction with 25 cycles of denature, anneal, & extension.