

### DNA sequencing

Today we will begin to sequence the PCR product that you purified last week. For DNA sequencing, you need to have a large number of identical DNA strands. The PCR reaction gave us this. Now we will carry out what is called "cycle sequencing." This is where we replicate DNA in the presence of regular dextroynucleotides (dNTPs) and dye-labeled dideoxynucleotides (ddNTPs). After you set up the reaction, it will go on the thermocycler to cycle through temperatures, allowing the DNA to be replicated many times. This will take a few hours. Then your teacher will clean up the reaction (remove extra nucleotides and primer) and run the reaction through a capillary that separates fragments by size. Next week, you should get the results.

#### Dilution and calculation:

Before you set up the cycle sequencing reaction, you need to dilute your cleaned up PCR product and calculate how much to add to your reaction. The concentration of DNA in your tube should be written on your tube. The concentration of DNA was determined using a spectrophotometer.

#### Obtain:

Your purified PCR product  
A tube of sterile ddH<sub>2</sub>O  
3 small (0.2ml) PCR tubes

1) You want to add 5ng of DNA to your sequencing reaction, so we will dilute your samples to 5ng/μl. Everyone is starting with their DNA at a different concentration, so you need to calculate the dilution volumes. We will make a 100μl solution with a concentration of 5ng/μl. This means we need 500ng DNA, which we will dilute to 100ul.

a) What is the current concentration of your DNA? \_\_\_\_\_ ng/μl = X ng/μl

Yul = the amount of solution containing 500ng

b) Here's the calculation

$X \text{ ng/ul} \times Y \text{ } \mu\text{l} = 500\text{ng}$

Now solve for Y

$500\text{ng} / X \text{ ng/ul} = Y \text{ } \mu\text{l}$

fill in your value for X and calculate Y.

$500\text{ng} / \text{_____ ng/}\mu\text{l} = \text{_____ } \mu\text{l}$  This is how much PCR product you will add to the dilution.

Now calculate how much water to add to the dilution

$100\mu\text{l} - Y\mu\text{l PCR product} = Z\mu\text{l water}$

$100\mu\text{l} - \text{_____ } \mu\text{l PCR product} = \text{_____ } \mu\text{l water}$

Add Zμl of water to the empty PCR tube

Add Yμl of your purified PCR product. Add it directly to the water already in the tube, not to the air or the side of the tube. **Mix well** by stirring with the pipette tip.

### Cycle sequencing:

You will set up two reactions – one for each primer. The reactions will be in small PCR tubes.

- Label the tubes with your initials or number and primer.** The little lip on the front of the cap is the best place to label it. If you are not sharing your PCR product with anyone, you will put one primer in one tube, and the other primer in the other tube.
  
- Add 5 $\mu$ l sterile water** to each PCR tube. Touch the tip to the side or bottom of the PCR tube to make sure it all goes into the tube.
  
- Add 2 $\mu$ l big dye buffer** (clear solution) to the water in each PCR tube. When adding the buffer, touch the tip of the pipettor to the top of the water already in the tube. The buffer ensures that the reaction has the right pH and salt concentration.
  
- Add 1 $\mu$ l of one primer (GL1-petF1 or GL1-REV).** If you and your lab partner are sharing a DNA extraction, one of you can use one primer, and the other person should use the other primer. If you are not sharing your PCR product with anyone, put one primer in one tube, and the other primer in the other tube.
  
- Add 1 $\mu$ l of your diluted PCR product (from step 1 above).** Again, put the tip of the pipettor into the liquid already in the PCR tube before pushing the liquid out of the pipettor.
  
- If you are way ahead of all the other students, **stop and wait.** Your reaction may fail if it sits around for too long with big dye in it before starting the reaction on the thermocycler. **Add 1  $\mu$ l of big dye** (the pink stuff) to the liquid in the PCR tube. Again, make sure the tip touches the top of the liquid in the tube. **Big dye is very expensive** (one tube costs \$300). If you use too much there won't be enough for the other students. Be sure your pipettor is set to the correct volume and you are only using 1 $\mu$ l. Ask your TA if you aren't certain. The big dye solution contains DNA polymerase and nucleotides (dNTPs and ddNTPs with fluorescent dyes)
  
- Place your tube in the thermocycler.** You are finished. Your TA will start the reaction when all of the students have their samples ready.

#### Thermocycling steps:

**96C 10 sec** denature DNA

**50C 5 sec** allow primer to anneal (stick)

**72C 4 min** strand extension

**repeat 25 times** to replicate DNA many times so we can get a few fragments terminating at each nucleotide site.

**4C forever** (to refrigerate samples)

