Purpose: optimize primers for gene family sequencing

Introduction: This week we have several things to work on. For most of the samples, the tubulin primers on Phaseolus did not give *strong* signal, although one group did get a detectable response. This may simply be due to the use of a number of cultivars – natural sequence differences. However, it seemed that perhaps the signal was faint rather than absent, so I want to spin out the PCR product, resuspend it in TE buffer, and run it on an agarose gel – that will be task 1.

The sheared DNA that we made was quite good, for the most part, but rather than getting 75-150bp we ended up in the 150-300bp range. Also, we are going to need a larger mass for the polishing/adaptor ligation step, so I want to repeat the Bioruptor step, using 100ul of 20ng/ul solution. We will carry this out for 6x 15-minute cycles rather than 4 cycles. If this does not increase the fragmentation we will proceed, since we will still obtain good sequence data, it just won't read all the way through our fragments.

The degenerate primers have only given product in 2 cases, under our current conditions: the FSH2 (~250) and FSH4 (~175), and possibly IFS7(~100). We used a step-up protocol, using a lower than predicted Tm and increasing every 3 cycles in order to get the higher yield. However, our lower Tm may actually still be too high, since it is the average of all the possible Tms. For these 3 primers pairs you will receive a Master Mix and we will carry out the PCR on soy and phaseolus, so we will have at least some of the products that we need for the sequencing.

Thus the activities for this lab include centrifuging ethanol-precipitated PCR product from solution and running a gel to test one set of PCR products, repeating the shearing on the DNA to make it the correct length for a library and testing the outcome, and performing step-up PCR using 3 primer pairs on soy and phaseolus (and a negative control). Make sure to keep checking the blossom color and time to blooming of your two varieties.

Protocol

1. Phenotype check: observe/record whether your soy and/or phaseolus is blooming, if they have not already. Record the colors and date (there is clearly a wide range in time to blooming with these varieties).

2. Prepare samples for the Bioruptor – the processing takes 90 minutes per set of 6 samples, and will be done in the cold room. Once the fragmentation is complete you will run a fraction of the sample on an agarose gel to determine whether you have approached the target length (100-150 bp is our goal). Measure out 2ug of each of your DNAs in a total volume of 100ul (use TE buffer if you need to dilute the volume, the final concentration will be 20ng/ul) and place each in a separate Bioruptor tube. Take these to the cold room (around the corner from the lab, turn left going out the classroom door, just around the corner of the corridor).
    a. 6 tubes can fit into the adaptor at one time. Chilled water is placed in the chamber and a bit of ice is on top. The tube holder is placed in the device.

b. The settings are the same as last time
c. Check every 15 minutes to make sure the chamber still contains a bit of ice – supplement as needed – repeat 5 times (5 cycles in all).
d. Remove the tube to the bench – put the sample in a regular 1.5 ml microfuge tube (labeled with P1 or S1 if you are group 1 and it is soy or Phaseolus, for example, and the date, and B-DNA for Bioruptor DNA).
e. Remove 10ul of each solution to the tubes set up by Dr. Weller, who will run the samples on agarose gels tonight and post the pictures.
f. Store the remainder of the fragmented sample in ethanol in the -20, in the designated box.

3. Transfer you Phaseolus-tubulin PCR products (ethanol slurry of negative, positive and unknown samples) to 0.5ml Eppendorf tubes (labeled). You will put these in the microfuge (using an adaptor) and spin at 13,000rpm for 30 minutes.
   a. Have the hinge on the outside rim
   b. When it is done spinning, remove the solution, avoiding the pellet, which should be slightly up on the side where the hinge is.
   c. Let air dry for 10 minutes.
   d. Add 10ul of 1X Tris-EDTA (TE) buffer. Note this is not the same as 1X TBE buffer!
   e. Pipette up and down gently 10 times. Close tube
   f. Put the tube in the designated rack, and note the position and label on the key so Dr. Weller can run the gel this evening.
4. Test the Soy and Phaseolus DNA with the FSH2, FSH4 and IFS7 primer pairs, using a step-up PCR protocol. You will set up 3 reactions: one with the Soy DNA, one with your Phaseolus DNA, and one with water. The primer sequences are on the posted spread sheet on the class Web page.
   a. Reaction components: you will be given a Master Mix of reaction components that includes the primers and enzyme – you will use 12ul of the Master Mix per reaction, allowing you to make up the remaining 3ul with template that is at 20ng/ul. We will only do step-up reactions, starting with 3 cycles at 50C (30 sec), then 3 cycles at 51C, up to 57C, and then another 12 cycles at 57C to make lots of product.
   b. Use barrier tips for pipetting. For 15ul final volume reactions add to 200ul thin-wall PCR tubes (on ice) , in this order:
      i. Master Mix……12ul Components are listed below.
         1. 10X buffer 1.5 ul 1X
         2. dNTP (10mM) 0.75ul 0.5mM
         3. $MgCl_2$(50mM) 0.9ul 3mM
         4. Primer 1 +2 (5uM) 1.5ul
         5. Water 6.35ul
         6. Taq enzyme (1.25U/ul) 1.0 ul
      ii. Add 60ng of DNA (except the no-template control) <u>in *3* ul of water</u>
      iii. Final volume 15ul.
      iv. Briefly perform a Quick-spin to collect the reagents at the bottom of the tube - then keep on ice until ready to put in thermocycler.

c. The following are the thermocycling conditions – use the Techne as it does step-up:
   i. Melt 95°C 5 minutes
   ii. Cycling x 3
      a. 94°C 30sec
      b. 50°C 30sec ** increment by 1 degree after 3 cycles, repeat to 57C
      c. 72°C 30sec
   iii. Cycling x 12
      a. 94°C 30sec
      b. 57°C 30sec
      c. 72°C 30sec
   iv. Finishing 72°C 7 minutes
   v. Hold 4°C indefinite

5. The PCR reactions products will be run on 2% agarose gels, to see if they worked in the different cultivars –those that did work will be polished and adapted, and included in the sequencing pool. Please fill in the key left by the PCR instrument so Dr. Weller knows whose samples are whose when running the gels.
6. AFTER removing aliquots for gels, PCR products and sheared DNA will be ethanol precipitated, as described last week.
7. Note: optimization of the degenerate primers is going to be somewhat more problematic than we hoped. Assignment for next week: determine the LOWEST and HIGHEST possible Tm for each primer pair. Assume the lowest-melting combination and the highest-melting combination of bases. Please email me your estimates, and the program you used to make them. I will use these to set up and optimization scheme for next week.