Lab: Chloroplast (cp) PCR test and Agarose Gel

Introduction: With our DNA we may have co-purified some small organic molecules that will inhibit enzymatic reactions like PCR, restriction digestion and DNA ligation, the assays used to genetically type the DNA. We will do a PCR test using primers that pick up chloroplast DNA – there are hundreds of copies of the chloroplast DNA in most leaf cells, so we don't have to use very much of the sample to carry out this test. We will run an agarose gel again, to test whether the reaction worked. If it is successful then we can be pretty confident that the genotyping reactions we want to run will also work.

Dilute your DNA to a standard concentration

1. From your Nanodrop spectrophotometer data you should have a value for the concentration of your DNA in nanograms per microliter. We will add 100ng of DNA to each PCR reaction, and it is convenient if we have a standard DNA stock to use for this - 50ng/ul is a useful stock concentration, and we will end up wanting about 100ul of that stock. 50ng/ul *100ul = 5000ng
2. Divide 5000ng by your concentration of DNA
    a. For example, if I had 231ng/ul, I will take 5000/231 = 21.6ul
    b. I will use 21.6 ul of my purified DNA. I need a total of 100ul so I subtract 100-21.6 = 78.4ul, which is the amount of 1X TE buffer I need to add to make my DNA for PCR stock.
    c. Vortex it, spin it down and label it!!!
    d. Write out your calculations in your notebook.

PCR test with Chloroplast primers

A. For each sample you will combine the following to make a 50ul reaction volume on ice for each sample. The Master Mix combines 50mM Tris buffer, 100mM KCl, 4mM $MgCl_2$, 10ug BSA, 2U Taq polymerase, 0.8mM dNTPs and the primers at 1uM each.
    a. Include one 'positive control' reaction that uses the control DNA you are provided.
    b. Include one 'negative control' reaction that uses MB-H_2O in place of DNA
    c. Your team should end up with 4 PCR reactions: one for each sample you have purified, and one positive control and one negative control.
        1. Genomic Sample DNA 100ng/ul 3 ul
        (use the same volume for positive control or water for the negative control)
        2. Master Mix 45ul
        3. 0.2uM f/r primers from 10uM stock 2ul
        4. Place tubes in PCR rack in ice bucket next to the PCR machine.
            a. Be sure your tubes are labeled so you can tell them apart from everyone else's!

Cycling conditions (should be pre-set)
95C 4 min
35 cycles of
92C 20 sec
48 C 20 sec
72X 20 sec
72C 7 min
4C Hold

Primer sequences
Forward: CAT TAC AAA TGC GAT GCT CT
Reverse: TCT ACC GAT TTC GCC ATA TC

Products should be 800-950bp in length.

Agarose Gel Electrophoresis

Your PCR products should be ~1kb in length. Thus we want to run a 1.2% agarose gel, and use DNA markers that go up to 1000bp (1kb). You will load 7ul of each of your PCR products. If you incorporate ethidium bromide in the gel you can check the migration of the DNA on a trans-illuminator and return it to the electrophoresis chamber to run longer if necessary. Most of the directions for running this gel are the same as before, except the amount of agarose you dissolve in 1X TBE buffer is different, and the amount of sample you use is different.

Select your gel tray depending on the number of samples: you will want 4 lanes per team and 2 lanes for the DNA standards. The mini gels require ~40ml of gel solution, the midi-gels about 70 ml and the large-format gels about 130ml.

1.2% agarose gel with ethidium Bromide

Note: (wear gloves, eye protection and lab coats and dispose of the gel in the chemical hood – let it try out and then place in BioHazard waste).

1. Prepare the gel tray by putting the dams on the ends (or taping up the two open ends if dams are not available).
   i. Make sure the casting tray and comb are clean – do a final rinse with distilled water and wipe dry with a Kimwipe.
   ii. Fit dams on each end of the casting tray
   iii. Place on a flat surface and insert a comb near the top (this will form wells).

2. Pour a 1.2% agarose gel.
   i. Weigh out 1.2gm of agarose into a weigh boat (don't forget to tare it) and transfer to a 250 ml Erlenmeyer flask
   ii. Measure 10ml of 10X buffer and add to the flask, swirling to avoid lumps in the powder
   iii. Measure 90ml of ddH2O and add to the flask, swirl again.
   iv. In order to hydrate the agarose so it goes into solution you have to heat it – for this we use the microwave, and because the agarose is granular, you do have to worry about superheating – when you remove the flask to swirl the solution be sure to wear a protective orange glove, glasses and to point the neck of the flask away from your face.
   v. Swirl, then place in the microwave for 30 sec on maximum power.
   vi. Wearing a protective glove, swirl the solution, pointing the neck of the flask away from your face.
   vii. Repeat until the solution is perfectly clear – it will be bubbling, but you don't want it to boil over.
   viii. Cover the neck of the flask with foil, and let the mixture cool to about 60C – this usually takes ~ 20 minutes or so- swirling occasionally
      1. You can put it in the water bath so it will stay liquid while you do other things but it will slow down the cooling
      2. You can swirl it under cool water in the sink to speed up the cooling.
      3. If you pour it when it is too hot it will crack the gel tray. (BAD)
   ix. Add 10ul of the 10ug/ml *ethidium bromide* and swirl to mix. *Dispose of the tip in Biohazard waste*.
3. Pour ~40 ml into the prepared apparatus (more if you are using a larger format gel).
      1. Cover the remainder with Parafilm and label it ('1.2% agarose in 1X TBE with EtBr')– you will be able to use this later.
4. Let stand 20- 30 minutes. The gel is set when it is no longer clear – it will look like fogged glass and the casting tray will be cool to the touch.
      1. If not running today, wrap in plastic wrap or a Baggie, label, and store at 4C.

Note: while waiting for the gel to set you can make the 1X TBE running buffer and prepare the samples for loading on the gel. You can also start preparing the acrylamide gel apparatus, putting together the plates and sealing them.

5. To set up the gel for electrophoresis
      1. Remove the tape or dams from the edges
         a. Note: if you use a spatula to hold the end of the gel nearest to the comb away from the dam it is less likely to tear.
      2. Remove the comb.
      3. Place the gel on the tray in the electrophoresis chamber.
      4. Make 1L of 1X TBE running buffer by adding 100ml of 10X TBE to a graduated cylinder and filling to the 1L line with Nanopure water from the carboy. Stretch a piece of Parafilm over the end, then use your palm to form a pressure seal – invert 5-6 times to mix.
      5. Pour 1X TBE buffer into the chamber, until you just barely cover the gel.
         a. You will need ~300 ml
         b. Pour the rest of the buffer in a 1L reagent bottle and cap it, then label it (you will need it for the acrylamide gel).

c. Place the graduated cylinder in the sink, after rinsing with Nanopure water.

6. Now you are ready to load the gel – these wells generally hold 15-20ul but this depends on the thickness of the gel. You don't want the sample to overflow the well, so you might want to check your maximum volume with some water with LB in it (you can rinse it out once you are sure of the volume). Make sure the wells are at the end of the rig with the black electrode so samples run towards the red electrode. Regular micropipetter tips will work fine for loading samples, but you will need a steady hand since you need to layer the sample into the bottom of each well without stabbing through it.
   i. Samples
      1. 2ul of Bionexus Hi-Low ladder, 2 ul of water
      2. 6ul of PCR Sample 1 + 2ul of 4X Gel Loading Buffer G(LB)
      3. 6ul of PCR Sample 2 + 2ul of 4X loading Buffer (GLB)
      4. 6ul of PCR Positive Control + 2ul of 4X loading Buffer (LB)
      5. 6ul of PCR Negative Control + 2ul of 4X loading Buffer (LB)
      6. 2ul of Bionexus Hi-Low ladder, 2 ul of water

   ii. Put the lid on the apparatus, plug the electrodes into the power supply (red electrode on the lid should be at the bottom, the direction the samples are heading towards),
      1. Turn on constant voltage, set at 50V. You will run this until the lower of the blue dyes is about 75% of the way down.
      2. This takes 2-3 hours, so you will have to come back to turn off the gel and take a picture on the GelDoc (5 minutes).

b. Wearing gloves, place the gel on the GelDoc trans-illuminator surface. Close the door, turn on the UV light. Look through the observation window. You should see glowing bands in the gel – if you do, start the imaging software and take a picture. If you do not, make a note in your notebook that the gel failed. If you see bands, save the image to the class Desktop folder, print out a copy for you and your partner for your notebook (reverse the image to save ink, please).
   i. Dispose of the gel in the labeled box in the small sink by the lab door - we will let the gels dry out and then put them in the waste to be auatoclaved.
   ii. Spray the transilluminator surface with 70% ethanol and then wipe the surface dry with a Kimwipe.
   iii. Make sure the transilluminator position is 'off' before leaving.

c. Wearing gloves, using a funnel, put a 1L reagent bottle in the sink, pour the used electrophoresis buffer into the bottle, cap it. Label this as '1X TBE +EtBr, used' and put the date. The buffer can be re-used 4-5 times.
   i. Rinse the funnel, casting tray and the gel rig in Nanopure water.