Lab 4 part 3: DNA Quality check for inhibitors using PCR against a chloroplast region.

1 day

Introduction: As discussed previously, with our DNA we may have co-purified some small organic molecules that will inhibit the PCR reactions we are using to obtain the material to genetically type the samples. To check for this, we will perform a PCR test using primers that pick up a short region of chloroplast DNA. Because there are hundreds of copies of the chloroplast DNA in most leaf cells, we don't have to use very much of the sample to carry out this test. Once the PCR reaction is complete we will run an agarose gel again, to visualize the product and see whether the reaction worked. If it is successful, then we can be pretty confident that the long PCR reactions we are using to amplify the entire chloroplast for sequencing 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 (concentration units will be nanograms per microliter which is 10^{-9} grams per 10^{-6} liters).
    a. We will add 50ng of DNA to each PCR reaction.
    b. We will make a 10ng/ul stock of your sample DNA, for a final volume of the diluted DNA of 50ul.
2. Since 50ul*10ng/ul = 500 ng, figure out how much of your DNA is needed to make 500ng.
    a. For example, if I had 25ng/ul, I will take 500/25 = 20ul
        i. I will use 20 ul of my purified DNA sample solution.
    b. I need a total final volume of 50ul so I subtract the amount og my sample I used from the total: 50-20 = 30ul
    c. To make the final volume work I have to add 30ul of something, it could be water but we are going to use a buffer called TE (Tris-EDTA, which has 10mM Tris and 1mM EDTA at pH 8.0).
    d. Combine your sample and the 1X TE in a 0.5ml tube, cap, label, vortex the two liquids, spin it down in the minifuge and it store on ice until ready to use.

PCR test with Chloroplast primers

PCR requires a lot of components: a buffer and some salt to keep the enzyme working (Tris and KCl), magnesium because the enzyme is not active without it, some stabilizing protein (BSA), the nucleotide building blocks (dNTPs), the short pieces of DNA that direct the polymerase to the part of the chloroplast you want to copy (primers) the the enzyme itself, Taq DNA polymerase. And your sample DNA!

There is a lot of fiddly pipetting of small volumes, and it is easy to make a mistake, or just not do the same thing multiple times. In order to fix this, most scientists will make what is called a Master Mix – combine everything in one tube except the sample DNA (or perhaps the primers if they are testing those and keeping the same DNA). Then you can divide the Master Mix up into tubes and add the one thing that varies across the experiment. The amounts of the individual components for the Master Mix are given at the end of this protocol, but for this lab we are going to provide you with the Master Mix. You will still have to combine the Master Mix and your sample correctly.

For each sample you will combine the following to make a 50ul final reaction volume. Keep everything on ice so no mis-priming occurs. Your team will make 4 PCR reactions: one with each of your samples, one negative control (which means you just add water – you should not see anything from this tube unless you contaminated the Master Mix) and one positive control (we will give you some DNA that we have already tested, so if you have done the pipetting correctly you will see a product from this tube.

Component	Volume to use per tube
Genomic Sample DNA 10ng/ul	5 ul
(same 5ul volume for positive control or use water for the negative control)	
PCR Master Mix	45ul

Place in PCR rack in ice bucket next to the PCR machine. Be sure your tubes are labeled so you can tell them apart from the other groups' samples.

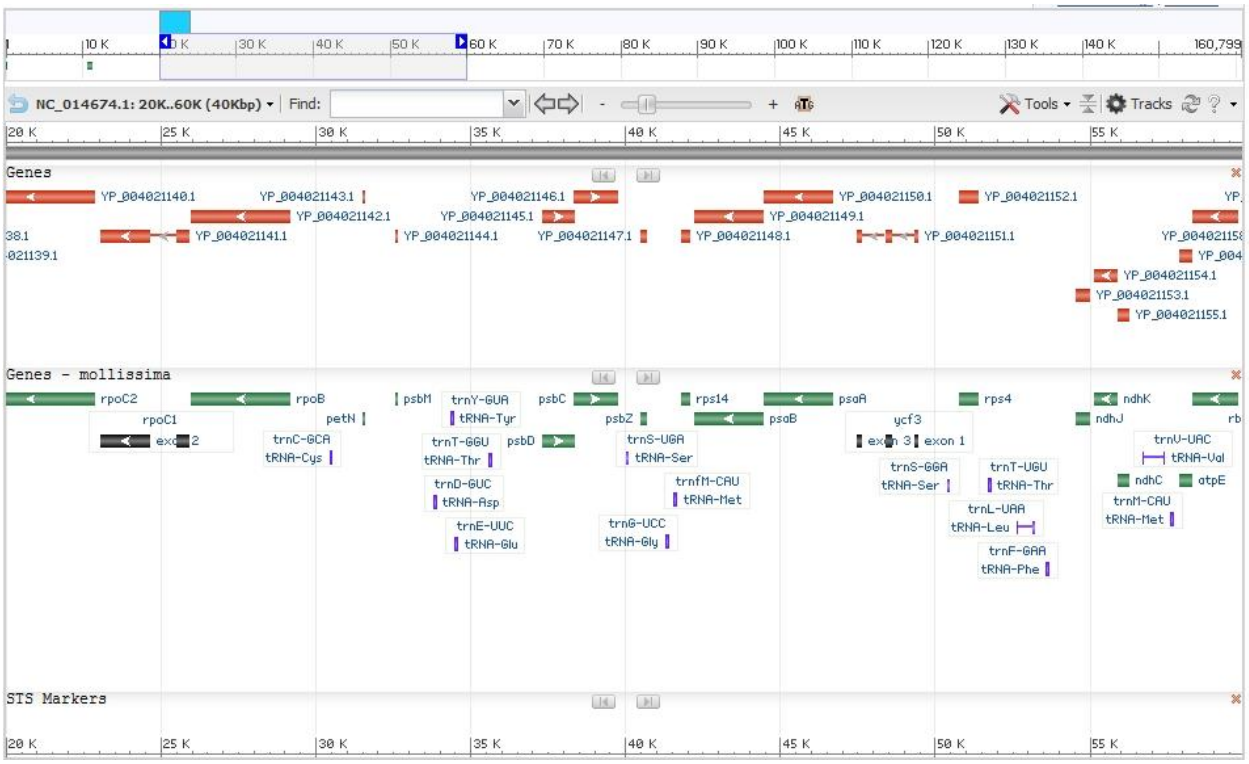
Cycling conditions (should be pre-set)
95C 4 min
35 cycles of
92C 20 sec
48 C 20 sec
72X 20 sec
72C 7 min
4C Hold

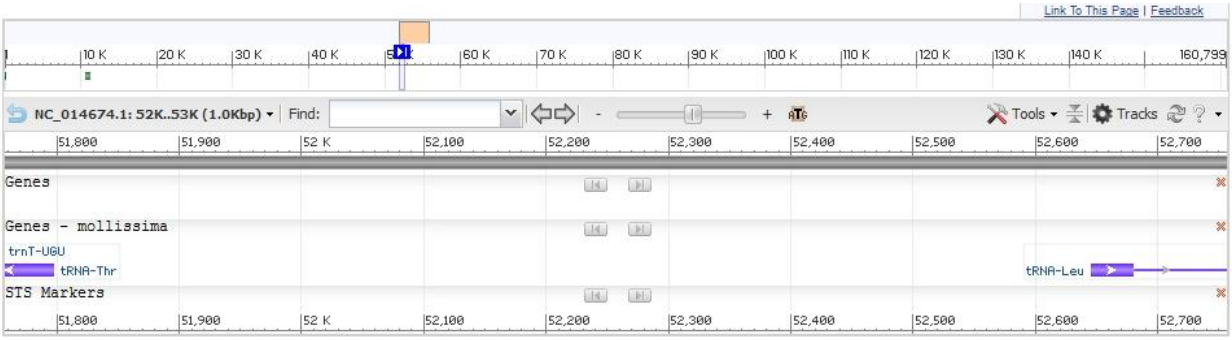
PCR Master Mix – to make 1.045 ml, enough for 20 reactions with a bit extra for pipetting errors.
Add in the components in the following order, mixing after each addition by pipetting up and down with a 1000ul micropipettor set on 700ul – use the blue tips)

1. 605ul molecular-biology grade water
2. 125ul 10X PCR buffer (that has no Mg in it – some do, so check)
3. 100ul of the four dNTPs (a 10mM stock, each at 2.5mM)
4. 100ul of MgSO4 (from a 50mM stock)
5. 50ul of BSA (a protein, from a 10mg/ml stock)
6. 20ul of Taq polymerase (from a 5Units/ul stock – this contains glycerol so it will need to be pipetted slowly, and you should 'rinse' the tip by pipetting the solution up and down 7-8 times)
7. 45ul of mixed primers (from a 10uM stock containing 5uM of each).
8. If each team is to set up 4 PCR reactions, they will need 45ul of this mixture for each reaction, or 180 total. We should give them about 10% extra for pipetting errors, so each team should receive 200ul of this solution in a 0.5ml tube. This is enough for 5 teams, so make as many batches as there are groups.

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.
This is at position 51758 and includes the intergenic regions between 2 tRNA genes: trnT (UGU, Thr) and trnL (UAA, Leu).





Agarose Gel Electrophoresis for PCR product

Your PCR products should be almost 1000bp, or 1kb, in length. We will pour and run a 1.2% agarose gel, and use DNA markers that go up to 1000bp (1kb). You will load 7ul of each of each PCR product in a lane of the gel. 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 (1.2g instead of 1gm), the amount of sample you load in a well is a volume rather than a mass (ul instead of ug), and the DNA ladder is different (up to 1kbp instead of up to 15kbp).

Select your gel tray depending on the number of samples: you will want 4 lanes per team PCR reactions and 1-2 lanes for the DNA standards or sizing ladder. The mini gels require ~40ml of gel solution, the midi-gels about 70 ml and the large-format gels about 130ml.

Pour, load and run a 1.2% agarose gel

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. Weigh out 1.2g of agarose and place in a 250ml Erlenmeyer flask.
2. Add 10ml of 10X TBE buffer, swirl to mix
3. Add 90ml of Nanopure water, swirl again.
4. Wearing safety glasses as well as your labcoat and gloves, microwave on high for 30 second intervals, swirling the solution each time (pointed away from your face and not at anyone else) until the solution is bubbling and completely clear.
5. Cool the solution by swirling in ice water for 30 seconds.
6. Add 10ul of 10mg/ml ethidium bromide, swirl to mix into the agarose and immediately pour into the prepared casting tray (~40 ml for a mini-gel).
7. Put the tip used with the ethidium bromide into the BioHazard waste.
8. Put Parafilm over the Erlenmeyer flask and label it.
9. Once the gel has set, remove the comb, place in an electrophoresis unit and pour 1X TBE buffer into the tanks until the gel is just barely covered.
10. Add 3ul of 4X Loading Dye to 7ul of your PCR product, mix by pipetting up and down 3-4 times, then load the whole amount into a lane of the gel.
11. Use 10ul of LadderB (it already has loading dye) in one lane of your gel
12. Set the electrophoresis voltage to 75V if the power supply will go that high, and run the gel until the slower of the two blue dyes is about half way down the gel (60-90 minutes at 75V, 2-3 hours at 50V).
13. Turn off the electrophoresis unit and unplug the electrodes.
14. Wearing gloves, remove the gel from the tank
15. Place the gel on the UV transilluminator, with the shield in place
16. Turn on the transilluminator, determine whether you have a PCR product and its approximate length.
17. You can put the gel back in the electrophoresis tank and continue to separate the products as needed.

18. Once the products have migrated where you want them in the gel, turn off the electrophoresis unit, remove the gel and place in a labeled Baggie. Give to Dr. Weller to make a digital record at UNCC.
19. Wearing gloves and using the provided funnel, pour the 1X TBE buffer back into the carboy.
20. Rinse the electrophoresis tank, casting tray and comb with Nanopure water, set on paper towels at your bench to dry.