**DNA Barcoding PCR of plants**

*DNA extraction from plant tissue*

You will need to obtain a small piece of tissue for this extraction, if using a leaf, your tissue should be approximately the size of a hole punch piece. If you use a hole punch, be sure to clean it off with Ethanol.



Be careful to not use too much tissue, if you use too much tissue, your PCR reaction will not be as efficient, therefore it is crucial to only use a small piece of tissue. Take extreme care to not contaminate your sample with another tissue, wear gloves and use only clean and sterile materials.

1. Macerate tissue using a blue pestle and hand homogenizer for 30-60 seconds (may take longer if you are hand homogenizing.) Be careful to not flick your tissue out of the tube with the homogenization. You will still have some solid pieces, but you should have a slurry. Be sure to write down the label on your tube.
2. Add 400 µl of Edward’s extraction buffer to your plant slurry.
3. Vortex for 5 seconds to mix thoroughly.
4. Centrifuge the extract at 13,000 rpm for 3 minutes to separate the solids from the liquid, your DNA will be in the liquid.
5. While your sample is centrifuging, add 300µl of isopropyl alcohol (IPA) to a fresh tube and label it. As soon as the centrifuge has finished spinning, pipet 300µl of the supernatant (the liquid) the IPA tube, take care to not bring any solid material, you may need to add less than 300µl. Mix by inverting twice
6. Leave the mixture at room temperature for 2 minutes.
7. Centrifuge at 13,000 rpm for 5 minutes.
8. Quickly pour off the liquid, making sure not to dislodge the pellet (the pellet may not be visible, but something is there).
9. Centrifuge for 10 sec to pool the remaining IPA, and remove all of this liquid with your p200 pipet leaving the pelleted solids in the tube.
10. Add 1 ml of 70%-75% ethanol to the Eppendorf tube.
11. Centrifuge at 13,000 rpm for 2 minutes.
12. Quickly pour off the ethanol making sure not to dislodge the pellet.
13. Centrifuge for 10 sec to pool the ethanol and remove all of the ethanol with your p200 pipet.
14. Leave your tube open on your lab bench upside-down for approximately 30 minutes. Your sample needs to be dry and should not smell of ethanol.
15. After your pellet has dried for 30 min, add 100µl sterile water to the Eppendorf tube to dissolve the pellet. You may need to pipette the liquid up and down vigorously several times to resuspend the pellet. It is highly likely that much of the pellet will not dissolve. If everything dissolved then the DNA should be dissolved in this water and can now be used for PCR amplification or stored at -20°C. Be sure your tube is labeled. If the pellet is not completely dissolved, proceed to #16.
16. If there is material that did not dissolve, then centrifuge at 13,000 rpm for 3 minutes to pellet the debris that did not dissolve in water and pipette the liquid into a clean sterile tube. The DNA should be dissolved in this water and can now be used for PCR amplification. Be sure your tube is labeled.

*PCR amplification of the Rubisco gene*

You will use **polymerase chain reaction (PCR)** to amplify (make copies of) a small piece of DNA, which is used for DNA barcoding, using primers specific to a Chlorplast gene called Rubisco Large Subunit. Then you will analyze the PCR products using gel electrophoresis to determine that your amplified DNA is the correct size. This DNA will be submitted for DNA sequencing. The sequenced DNA can be compared with previously sequenced DNA to determine the species of that organism. PCR is similar to DNA replication in your cells.

**What proteins and small molecules are involved in DNA replication inside a cell?**

You will use some of these same ingredients and steps to replicate DNA in a test tube instead of a cell. The piece of DNA you will replicate will be in the Rubisco gene. Based on sequence information of Rubisco genes, universal primers have been designed. You will be using two primers to amplify the DNA information between the primers in the PCR reaction. The reaction happens over and over again and grows exponentially due to an increase in the number of available pieces of DNA to react off of with each round of PCR, we call this amplification.

**What ingredients are needed for PCR?**

After you have added the 100 l of sterile water to your extracted DNA (see #15 and 16 in the DNA extraction protocol above) you will be ready to set up the PCR reaction.

**Conducting PCR**

*For each PCR reaction:*

* 0.2 ml PCR tube
* 25 l of GoTaq
* 3 µl of nuclease free sterile water
* 10 µl of the 2M Rubisco Forward primer
* 10 µl of the 2M Rubisco Reverse primer
* 2 µl of extracted DNA

***Procedure***

1. Label the top of the PCR tube on the top with information that will allow you to know this is your tube. Change tips with every pipetting!
2. The GoTaq is already added to your PCR tube.
3. Add 3 l of nuclease free sterile water.
4. Add 10 µl of each primer (why do you need two primers?).
5. Add 2 l of DNA.
6. Place your tube into the thermocycler to run the 'barcode' program. This program is:
   * 94°C for 5 minutes to completely denature all DNA in the tube

30 cycles of:

* + 94°C for 30 seconds (denature)
  + 52°C for 40 seconds (anneal)
  + 72°C for 1 minute (extension)

Then a final extension

* + 72°C for 10 minutes
  + 4°C hold

**Electrophoresis of your PCR reactions**

*Pouring an agarose gel*

1. Get your electrophoresis apparatus and seal both ends of the gel tray with tape or stoppers.
2. Keeping the bottle lid loose, melt the 1.2% agarose in the microwave until it looks like water, and pour into the gel space until the gel is about 5 mm deep (this is about 25-30 ml). Put the comb next to the black electrode. **Why the black?** Let the agarose harden, which should take 5-10 minutes. Don’t touch/move your gel until it’s hard.
3. Remove the stoppers and pour Running Buffer over the gel so that it is completely submerged.
4. Carefully remove the comb allow you access to the wells.

*Electrophoresis of your PCR reactions*

You will load 15 l of your PCR reaction into one of the wells of the gel. One of the wells will be loaded with 10 l of standard DNA marker. A standard DNA marker has a bunch of different pieces of DNA of known sizes that are used to compare to the DNA from your PCR reaction to determine the approximate size of your DNA.

1. Draw a picture of your gel and label which wells have which samples (PCR reaction(s), DNA marker). Be certain to have the information of where the other groups added their samples.
2. Load your samples into the wells - be sure you keep track of which samples you're loading into which wells.
3. Plug the electrodes into your electrophoresis apparatus (**red to red, black to black**), being careful not to bump your gel too much. Plug the power source into an outlet and set the voltage to about 125V. Run that gel!
4. Let the gel run until the dye migrates about 5-6 cm from the wells (about 20-30 minutes).
5. Turn off the power supply, disconnect the electrodes, and remove the top of the electrophoresis apparatus. Recycle the running buffer for future use. After you have gloved hands, carefully remove the gel.

*Staining gels to examine PCR reactions*

1. ****Put on gloves and a lab coat- you will be using Ethidium Bromide and it is important to avoid *any* *and all* contact with this chemical. **Ethidium Bromide is a known mutagen** and a possible carcinogen.
2. Place gel in staining tray, add a few ml of buffer to the top of the gel so the top is wet.
3. Remove the plastic from the ethidium bromide sheet and place the ethidium bromide paper on the gel. Gently rub the paper with your gloved fingers to make sure it is contacting the gel all over, the paper should be completely moist, if not add more buffer.
4. Stain for about 10 minutes.
5. Put the gel on the UV light box and, with the UV shield down, view your gel.
6. Photodocument your gel and save the picture for your lab book. **Be sure to label the picture.**
7. If you have a PCR product, your sample will be sent to be sequenced. **How do you know that you have a PCR product? What is the size of your PCR product?** Refer to the image of 1Kb + ladder to determine the size of you PCR product.