Chestnut Leaf DNA Extraction Protocol – this will take 2-3 days

Introduction/Overview of the Chemistry in each step: we will extract the nucleic acid from leaf tissue by first breaking open the cells: we will grind the leaves in a very cold 'reducing' medium (dry ice has a temperature of ~-20°C, and the beta-mercaptoethanol chemical is a reducing agent, it smells like rotten eggs), with the mortar sitting in wet ice. Keeping the samples cold prevents enzymes that would degrade our DNA from acting, the reducing agent inhibits random chemical reactions that happen when cell contents mix as you crush them, some of which would chemically alter the DNA. We will filter the leaf mush to remove the large bits that we could not grind fine enough (first level of purification). Then we will add a solution that contains CTAB and let the solutions sit for 45 minutes (also called incubation). CTAB is a detergent that grabs carbohydrates, which plant leaves have a lot of, and incubation means letting the materials mix with the chemicals long enough for the chemical reaction to go to completion – in this case we also use heat, which speeds up chemical reactions. Then we will perform a chloroform extraction, which helps denature and remove proteins and will also extract the chlorophyll and some of the other colored compounds that might contaminate our DNA. The chloroform does not mix with the aqueous (water) layer so things that separate into the chloroform will be separated from the DNA and RNA that stay in the aqueous layer, and the CTAB-carbohydrate is not soluble in either solvent, so it ends up in between as a third layer (some proteins are in this layer too)- giving more purification. After centrifuging to separate the chloroform and CTAB and aqueous solutions into separate layers, we will remove the top, aqueous, layer to a fresh tube, measure its volume, and add 2/3 volume of isopropanol. This makes the final concentration 40% isopropanol, and nucleic acids (we still have both RNA and DNA and some proteins that stick to them) are not soluble in 40% isopropanol, so they will precipitate (become insoluble). We can collect the insoluble material in the bottom of the tube by centrifugation. Once we pour off the isopropanol we will add some more buffer to the pellet in the bottom of the tube, and get the nucleic acid re-dissolved. Then we will carry out enzymatic reactions that give more purification: the enzymes we will add break down RNA and protein so we will be left with only DNA. A phenol-chloroform extraction is used to denature and remove any final protein bits, which is combined with centrifugation to separate out the DNA-containing aqueous layer. Once again we will remove the top layer to a fresh tube, add 2.5 volumes of ethanol to precipitate our DNA (70% final concentration of ethanol which DNA is not soluble in). After pelleting the DNA in a microcentrifuge and removing the solution, we will re-solubilize (dissolve) the DNA in buffer. The next steps will be to test it to see if it is of sufficient quality for our assays: PCR reactions and restriction endonuclease digestions. Each team will extract one sample from the native trees collected at Crowder's Mt State Park, and one from an orchard of related individuals (similar to those at the Pryor Farm). Thus you should always have tubes that balance in the centrifuges!

Prep – make sure you can identify where these things are, on your bench and in the lab.

A. Stage 1 sample grinding and nucleic acid purification.
   a. Read over the protocol.
   b. Check that the 65C water bath has been turned on.
   c. Set up your station by putting a mortar on ice (described below) and making sure you know where all of the reagents are, and that you and your partner have a plan for who will do what.
      i. Equipment/supplies
         1. Collect one chilled mortar/pestle when you are ready to start – collect from one of the large coolers
         2. One ice bucket with packed-down ice, with foil to keep the mortar from sticking – can be used for both samples, but refresh the ice in between.
         3. Two spatulas (wrapped in foil)
         4. Two 4" squares of Miracloth (to remove large chunks that don't pulverize) – in a Baggie
         5. One 15ml Falcon tube with 1gm of sorbitol
         6. One 15ml Falcon tube with 1 gm of polyvinylpyrrolidone (PVPP)
         7. Two weigh boats (label with the sample name) for determining the mass of the leaf
         8. Four 15ml Falcon tubes

      ii. Reagents
         1. One 1.5ml microfuge tube containing 400ul of beta-mercaptoethanol
         2. One 1.5ml tube containing 200ul of Tween-20.
         3. 2X Grinding Buffer 15ml (recipe at end)
         4. 2X CTAB Buffer 15ml (recipe at end)
         5. TE buffer, 1.5ml
         6. 5M NaCl, 1 ml
         7. Resolubilization buffer (1:1 Grinding Buffer and CTAB buffer) – 5ml
         8. 100% Isopropanol (10 ml)– bench top
         9. 70% isopropanol (10 ml to balance solutions) – bench top
         10. 70% ethanol (5 ml) – bench top
         11. 95-100% ethanol (5 ml) – bench top
         12. Chloroform ($CHCl_3$) is in the fume hood
      iii. Samples
         1. Locate your sample in cooler that has dry ice (you will process two but only process one at a time, each partner taking a turn to carry out the grinding).

B. Stage 2 processing – before starting make sure there is a heating block set to 37C
    i. Reagents
        1. RNAase A, 50ul (25ul per sample) – small tube (RNA-A), keep on ice
        2. Proteinase K, 50ul (25ul per sample) – small tube (ProtK), keep on ice
        3. Chloroform ($CHCl_3$), 800 ul (400ul per sample) – in hood (small tubes labeled $CHCl_3$)
        4. Phenol-Chloroform (P-C), 800 ul (400ul per sample) – in hood (small tube, P-C)
        5. 3M NaOAc (sodium acetate) or 7.5M NH_4OAc (ammonium acetate), 1ml – small tube on bench top
        6. 100% Isopropanol (10ml) – bench top, 15 ml Falcon tube
        7. 40% isopropanol (10ml) – bench top, 15 ml Falcon tube
        8. 70% ethanol (5 ml) – bench top, 15 ml Falcon tube
        9. 95-100% ethanol (5 ml) – bench top, 15 ml Falcon tube
        10. TE Buffer, 1.5ml– small tube, bench top

Stage 1 Grinding Tissue

In your notebook, start the following information and make notes about what you observe as you follow the steps.

Preparation Note: Before the lab starts make sure that the water bath is turned on, has tube racks in it, and is close to 65C. There will be two ice chests at the front of class, one will have dry ice (where your samples are – use gloves to handle the dry ice), and crushed ice which has the chilled mortars and pestles. The leaf samples will be in your collection Baggies on the dry ice – collect them when you have the ice bucket set up with the mortar and pestle and also place some powdered dry ice in the mortar.

In your notebook, start a heading with the following:
Experiment Type:
Experiment Goals:
Sample Label:
Scientist Name:
Date:

Note: Wear Protective gear, including eye glasses, lab coats and gloves, hair tied back, closed-toe shoes. Process one sample until you get it to the CTAB incubation step (M) and then process the second sample. Each team will process two different samples.

A. Label a weigh boat so you know which sample you are handling.
B. Put a mortar and pestle on ice, setting the mortar on a piece of foil, then making sure it is well packed in.
    a. Make sure you have a spatula for scraping the mortar and pestle.
C. Collect a Baggie with your leaves in it from the dry ice cooler. Be careful not to burn your fingers on the dry ice.
    a. Tare the weigh boat.
    b. Weigh out ~1-2g of plant material into a labeled weigh boat, removing stems and large veins.
    c. If there is extra leaf material, return the sample baggie to the dry ice cooler.
    d. Transfer the sample to the chilled mortar.
D. To the sample in the mortar add
    a. Powdered dry ice from the small cooler (small pellets are OK).
    b. About 0.25gm of sorbitol and PVP per gram of leaf tissue – 0.25gm is about ¼ of what you have been given, and you may approximate it based on the level in the Falcon tube, or you can weigh it out.
    c. 2.5 ml of Grinding Buffer per gram of leaf tissue.
    d. 100ul of the beta-mercaptoethanol per gram of leaf tissue
    e. 50ul of the Tween per gram of leaf tissue (very thick solution, use the 1000ul micropipettor and cut the end off of the tip with some scissors, then be patient while you pull up and deliver the solution)

E. Grind with fair pressure for 15 minutes until a smooth light-green paste is formed. Then let the paste warm up until it is fairly liquid (this may take 5-10 minutes)
    a. As you are grinding, scrape down the mortar and the pestle occasionally with a spatula.
    b. Initially you will have a powder, since the dry ice keeps everything frozen. After that you should get a paste, and finally you want it to be somewhat liquid.
F. Label a 15 ml tube (and its cap) and set it upright in a tube rack.
G. Make a 'funnel' of the Miracloth with your hand, poking it into the top of the 15ml tube with your finger.
H. Scrape the plant material from the mortar and pestle into the Miracloth funnel – your partner can help keep it steady so it does not flop over and spill your sample.
    a. Rinse the mortar, pestle and spatula/wooden stick with 1 ml of Grinding Buffer and add to the funnel.
I. Gently push down on the sides of the Miracloth 'funnel' using a spatula or popsicle stick, to push the liquid down into the labeled 15 ml Falcon tube. You may want to squeeze the last bit out with your fingers.
    a. Discard the Miracloth in the hood (it is going to smell of beta-mercaptoethano).
    b. Note the volume of the solution in the tube: _____ml
    c. Note – this should be ~2-3 ml, if it less than 2 ml add more 2X Grinding buffer
    d. Change your gloves!
J. Add an equal volume of 2XCTAB Buffer
    a. ____ml added
    b. Cap carefully and mix thoroughly by inverting
K. Put in the water bath at 65C for 45 minutes, inverting every 5 minutes for the first 15 minutes and then again after 35 and 40 minutes.
    a. Start time:_____
    b. End time:_____
    c. Mixing steps (should be 5): _____
    d. Note: one of your two samples will incubate longer than 45 minutes, that is OK, longer is actually better.
L. Remove from the water bath
    a. Cool to room temperature (you can put in an ice bath or run cool water over the tube to speed this up). Why? Chloroform will 'boil' at 65C, so if you add it to your hot solution your material will boil out of your tube, very dismaying to have to start over now!
M. Take to the chemical hood and add an equal volume of chloroform (should be around 4-8ml).
    a. _____ ml sample
    b. _____ml Chloroform
N. Cap and shake thoroughly, continue to shake for 10 minutes.
O. If you have more than 15ml, split between two tubes: balance them to weigh within 0.5g of each other. Don't just use an equal liquid level, use the balance. You can use chloroform to increase the mass of the lightest tube if it is easier.
P. Making sure you have balanced your two sample tubes between two 15ml tubes, place opposite each other in the centrifuge and spin at 6000 x g for 45 minutes

a. This will separate the chloroform layer from the aqueous (water) layer that contains the DNA (at room temperature is fine). Note – the chloroform is denser than water so it will be on the bottom. It will also be bright green because it will extract the chlorophyll from the plant material. The DNA will be in the aqueous layer on top, but it is dissolved at this point so you won't see it. The will be a greyish mid-layer that is made up of proteins and carbohydrates complexed with CTAB.

Q. Using a 5 or 10ml serological pipette, remove the (top) aqueous layer to a fresh labeled 15 ml tube.
   i) _____Label
   ii) Dispose of the organic layer (bottom layer) by pouring it into the Organic Waste bottle in the chemical hood.
   iii) Dispose of the pipette in the orange Biohazard bag in the same hood.
   iv) The tube should also go in the orange biohazard bag.

R. Determine the volume of your sample. Then multiply this times 0.7 and this is the volume of 100% isopropanol you will add to your sample, using a 5 or 10ml serological pipette– layer the isopropanol over the aqueous layer carefully.
   a. For example, if I have 8ml of solution, 8*7=5.6ml, so I will add 5.6ml of 100% isopropanol. If the volume is going to end up being >15ml, we will split it in half again and use 2 15ml tubes.
   b. If you have time to let this stand over-night you will be able to spool out the DNA. If you don't have time for that, mix the solutions thoroughly and you can centrifuge them right away. Note: stopping point→ the samples are safe to sit at room temperature overnight.
      i. 0.7(____ml your aqueous solution) = _____ml isopropanol to add
      ii. Let stand at least one hour
         1. Start time_____
         2. End time _____
      iii. This is a good stopping point. If it is for longer than overnight, store at 4C for up to 1 month.
         1. Label:_____
         2. Storage location:_____

S. Balance two tubes within 0.5g of each other (using 70% isopropanol to adjust the mass), place opposite each other and centrifuge tubes at 6000 x g for 60 minutes.

T. Drain off the isopropanol by pouring it down the sink (be sure the pellet does not slide out – if it starts to slide use a serological pipette to pull off the solution).
   a. Wipe out the sides of the tube with a Kimwipe (don't get too near the pellet)
   b. Rinse the pellet with 1 ml of 70% ethanol – add to cover pellet, roll it around the sides of the tube, let sit 5 minute, then pour off carefully (or pipette off if it seems to slide around).
   c. Let the tube drain inverted on a Kimwipe for 5 minutes, then air dry right side up for 10 minutes

U. Add 1ml of Resolubilization Buffer (1:1 mixed Grinding buffer/CTAB buffer), put on the cap and loosen the pellet by using the Vortexer on low.

a. Pipette up and down, then, using a 1ml micropipetter with the tip cut off to make the opening wider, remove the sample to a labeled 1.5ml or 2.0 ml microfuge tube.
b. Put on a shaker/vortexer for at least 60 minutes (up to overnight) to thoroughly solubilize the nucleic acid.
    i. Note that there may be un-dissolved translucent bits floating around – this is carbohydrate that will not re-dissolve under these conditions. Our main goal in this step is to break it up enough to release any trapped DNA.

V. Spin the tube in the microfuge at 14,000x g for 15 minutes, making sure there is a balance tube with the same volume of liquid in it (you don't need to weigh these).
    a. Remove the upper liquid part to a fresh tube.
        i. Discard the old tube with the clear gelatinous pellet.
        ii. Make sure you have at least 400ul of sample – if it is low add enough Resolubilization buffer to make 400ul total (for each sample).

Stage 2 – Removing Contaminants

Safety Note: Very Important!!! Wear protective clothing: eye glasses, lab coat and gloves. Phenol can cause chemical burns – if you get any on your skin use lots of water to rinse the area and inform one of the instructors immediately.

A. To each of your two samples, add 25ul of the RNAase solution, mix thoroughly and incubate at 37C for 30 minutes
B. To each of your samples add 25ul of the Proteinase K solution, mix by pipetting up and down 5-6 times, and incubate at 37C for 30 minutes.
C. Remove 450ul of the sample to a fresh 1.5ml tube (labeled). If there is any left, store the remainder of the sample (labeled) in the -20 freezer at the front of the class.
D. Take your tubes to the hood, from the Phenol-Chloroform (PC) tube, remove 450 ul using the 1000ul micropipetter that is there, using a fresh tip for each sample.
    a. Once the PC is added, carefully cap the tube. Dispose of the tip in the BioHazard waste bag in the hood.
    b. Vortex each sample for 10-15 sec in the hood.
    c. Balancing two tubes opposite each other, spin in a microfuge at 12,000 x g for 45 minutes
E. In the hood, carefully open the cap (you don't want to spray any droplets around)
    a. Using a 1000ul micropipetter set on 300,
    b. Put on a fresh tip,
    c. Remove 300ul of the top aqueous layer to a new labeled microfuge tube.
    d. If you don't get 300ul (that is if you have to stop pipetting because you will pull up the bottom layer, so you end up with air at the bottom of the tip), put the solution you recover in the target tube, then measure how much you do get and add enough TE buffer to get 300ul volume.
    e. Note the volume: _____
    f. Label on your tube:_____
    g. Pour the bottom P-C layer into the Organic Waste labeled bottle in the chemical hood.
    h. Dispose of the tubes in the solid Biohazard Waste bag in the hood.

F. Add 300ul of chloroform to each sample. Cap the tube.
    a. Vortex for 60 seconds.
    b. Spin in a microfuge at 12,000 x g for 5 minutes
    c. (You can do this in the hood or at your bench – the volume of chloroform is quite small) Using a 1000ul micropipetter set on 250ul, put on a fresh tip, remove the top (aqueous) layer to a new (labeled) tube.
    d. Dispose of the used tip and the chloroform in the tube in the organic waste bottle, and the empty tube in the orange biohazard bag.

G. Add 50ul of Resolubilization buffer to each sample so you have a final volume of 300ul
    a. You can check the volume by setting the 1000ul micropipetter to 300 and pulling up the sample – if you have an air bubble at the bottom of the tip you don't have quite enough sample, if there is still liquid in the tube then you have too much sample.

H. Add 750ul (2.5 volumes = 2.5*300ul) of 100% ethanol to each tube.
    a. Cap and vortex to mix thoroughly.
    b. Let sit at room temperature for 10 minutes. If you do not see a precipitate let it sit at -20C for 60 minutes, or at 4C overnight (or longer).
        i. Storage location and label:_____

I. Spin at 12,000 x g for 60 minutes

J. Remove the ethanol as completely as possible, let the pellet air dry for 10 minutes

K. Add 200ul of TE and 4ul of 5M NaCl to the tube, and put on a vortexer in gentle shaking mode - this can go overnight.
        i. If the pellet does not completely dissolve, add 300ul of Resolubilization buffer, cap and vortex for 15 seconds, and then do another extraction with Chloroform (from Step F above) followed by another ethanol precipitation step.
        ii. If the pellet does completely dissolve store it at -20, we will measure the concentration and do other tests.

L. Store at 4C for 1-2 weeks. If storing longer, store at -20. Note the storage location in your notebook.

Stage 3 - Quality control steps (described in the next lab)

b) Check the concentration with a spectrophotometer
c) Check the average length of the DNA by running a 0.8% agarose gel in 1X TBE buffer with ethidium bromide. Use ~500ng (based on the spectrophotometer value) of the sample material and use a DNA ladder that has one marker band of at least 10,000bp.
d) Run a PCR test using chloroplast DNA primers

Buffers

2XGrinding Buffer:

0.04M Tris-HCl pH 8	4 ml 1M stock
5mM EDTA	1ml of 0.5M stock
200mM NaCl	4ml of 5M stock
	Bring to 100 ml volume with MilliQ or Nanopure water

2X CTAB Buffer

200mM Tris-HCl pH 8	20 ml 1M stock for 100 ml total
40mM EDTA pH 8	8 ml of 0.5M stock
2M NaCl	40ml of 5M NaCl
2% CTAB	2g of CTAB (Dissolve in Tris-EDTA, then add NaCl)
	Bring to 100 ml with MilliQ or Nanopure water

Resuspension Buffer is a 1:1 mixture of grinding buffer and CTAB buffer.

TE Buffer

10mM Tris-HCl	pH 8.0
1mM EDTA	pH 8.0