# Chestnut Leaf DNA Extraction Protocol

**Introduction:** we will extract the nucleic acid from leaf tissue by grinding it in a 'reducing' medium (the beta-mercaptoethanol chemical is a reducing agent, it smells like rotten eggs), on ice to prevent enzymes that would degrade our DNA from acting. We will filter the leaf mush to remove the large bits that we could not grind fine enough. Then we will perform a CTAB incubation. CTAB is a detergent that grabs carbohydrates which plant leaves have a lot of and incubation means letting the materials sit or mix over a period of time, to allow the chemical reaction time to go to completion – in this case we also use heat, which speeds up chemical reactions. Then we will perform by 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. After centrifuging to separate the chloroform and aqueous (water-base) layers, we will keep the aqueous layer and add isopropanol, because nucleic acids are not soluble in 40% isopropanol, to precipitate (cause to become insoluble) the nucleic acid (right now we have both RNA and DNA and we will also carry along any proteins still attached to them). Once we pour off the isopropanol we will add some more buffer and get the nucleic acid re-dissolved. Then we will carry out enzymatic reactions that give more purification: the enzymes we will use 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 add isopropanol to precipitate our DNA, and we will re-solubilize the DNA and test it to see if it is of sufficient quality for our assays: PCR and restriction endonuclease digestions. Each team will extract one sample from the native trees collected at Crowders, and one from an orchard of related individuals (similar to those at the Pryor Farm).

## 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. Turn on the 60C water bath
   b. Identify location of samples in -80 freezer.
   c. **Per sample** to be processed
      i. One chilled mortar/pestle
      ii. One ice bucket with packed-down ice
      iii. One spatula
      iv. One 4" square of Miracloth (to remove large chunks that don't pulverize)
      v. One small weigh boat with 0.5gm of sorbitol and onew with 0.5 gm of polyvinylpyrrolidone (PVPP)
      vi. Two labeled weigh boats (label with the sample name)
      vii. One 1.5ml microfuge tube containing 200ul of beta-mercaptorethanol and 200ul of Tween-20 (per sample). You will add each to the sample in the mortar.
      viii. One 50ml Falcon tube.
      ix. One 15ml Falcon tube
      x. Grinding Buffer ~7ml per sample (recipe at end)
      xi. 2X CTAB Buffer ~ 7ml per sample (recipe at end)
      xii. TE buffer, 500ul per sample
      xiii. 5M NaCl
      xiv. Resolubilization buffer (1:1 Grinding Buffer and CTAB buffer, 0.5ml per sample)
      xv. 100% Isopropanol (4-5 ml)
      xvi. 70% isopropanol (10 ml to balance solutions)
      xvii. 70% ethanol (1-2 ml)
      xviii. 95-100% ethanol (1-2 ml)
      xix. Chloroform ($CHCl_3$) is in the fume hood

B. Stage 2 processing
      i. Set a heat block to 37C
      ii. RNAase A, 25ul per sample
      iii. Proteinase K, 25ul per sample
      iv. Chloroform, 250 ul per sample
      v. 3M NaOAc (sodium acetate) or 7.5M $NH_4OAc$ (ammonium acetate), 1ml
      vi. Isopropanol
      vii. 70% ethanol (1-2 ml)
      viii. 95-100% ethanol (1-2 ml)
      ix. TE Buffer, 100ul per sample

## Stage 1 Grinding Tissue

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

Experiment Type:
Experiment Goals:
Sample Label:
Scientist Name:
Date:

Wear Protective gear, including eye glasses, lab coats and gloves. 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 couple of Popsicle sticks available.
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 ~2-3g of plant material into a labeled weigh boat, removing stems and large veins.
    c. Return the sample baggie to the dry ice cooler.
    d. Transfer the sample to the chilled mortar.
D. To each sample add
    a. A few small chunks or powdered dry ice from the small cooler
    b. The sorbitol and PVP
    c. 2.5 ml of Grinding Buffer per gram of leaf tissue.
    d. 50ul of the beta-mercaptoethanol per gram of leaf tissue
    e. 50ul of the Tween per gram of leaf tissue (very thick, use the 1000ul micropipetter and cut the end off of the tip with some scissors)
E. Grind for 15 minutes until a smooth light-green paste is formed.
    a. Scrape down the mortar and the pestle occasionally with a wooden stick or spatula.
    b. Initially you will have a powder, since the dry ice keeps everything frozen. After that you should get a paste, and it may even become rather liquid toward the end.
F. Label a 50 ml tube 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 50ml tube with your finger.
H. Scrape the plant material from the mortar and pestle into the Miracloth funnel.
    a. Rinse the mortar, pestle and spatula/wooden sitck with 1 ml of Grinding Buffer and add to the funnel.
I. Squeeze the material through a Miracloth ‘funnel’ into the labeled 50 ml Falcon tube (label the cap as well)
    a. Discard the Miracloth in regular waste.

b. Note the volume of the solution in the tube: _____ml
c. Change your gloves!

J. Add an equal volume of CTAB Buffer (should be ~4-6 ml)
   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 for the last 10 minutes.
   a. Start time:_____
   b. End time:_____

L. Remove from the water bath
   a. Cool to room temperature (you can put in ice or run cool water over the tube to speed this up)

M. Take to the chemical hood and add an equal volume of chloroform (should be around 7-10ml).
   a. _____ ml sample
   b. _____ml Chloroform

N. Cap and shake thoroughly, continue to shake for 10 minutes.

O. If you have more than 15ml, balance your solution equally (adjust them to weigh within 0.5g of each other, don't just use an equal liquid level, and use chloroform to increase the mass of the lightest tube) between two 15ml tubes, spin at 6000 x g for 45 minutes
   a. If you have less than 15ml, balance with another group. Add Chloroform to increase the mass of the lightest tube.
   b. 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.

P. Using a 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) The original tube can go in regular trash once empty.

Q. 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 10ml serological pipette– layer this 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 15 ml tubes.
   b. If you have time to let this stand overnight you will be able to spool out the DNA. If you don't have time for that, mix the solutions thoroughly and let them stand 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 – store at 4C for up to month.

1. Label:_____
2. Storage location:_____

R. Balance two tubes within 0.5g of each other (using 70% isopropanol to adjust the mass) and spin tubes at 6000 x g for 60 minutes

S. Drain off the isopropanol by pouring it down the sink (be sure the pellet does not slide out).
   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 (pipette 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

T. Add 500ul of Resolubilization Buffer (1:1 mixed Grinding buffer/CTAB buffer), put on the cap and resuspend the pellet by using the Vortexer on low.
   a. Pipette up and down, then remove to a labeled 1.5ml or 2.0 ml microfuge tube.
   b. Put on a shaker 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.

U. 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 tube with the pellet.
   b. Add 300ul of Resolubilization Buffer to the tube and 500ul of CHCl3
   c. Vortex for 10 second.

V. Spin in the microfuge for 30 minutes at 14,000 x g, making sure there is a balance tube.

W. Remove the top layer to a fresh labeled 1.5 ml tube, avoiding anything in the middle layer.
   a. Dispose of the (bottom) chloroform layer in the labeled bottle in the hood.

X. Measure the volume of each sample: _____ul

1. Add 0.7 vol times your sample volume of 100% isopropanol
   a. Mix and let stand for 10 minutes.
   b. Spin in the microfuge for 30 minutes at 14,000 x g
   c. Pour off the liquid into the sink or a beaker
   d. Pulse-spin in the minifuge to collect the remaining liquid at the bottom of the tube.
   e. Pipette off the last of the liquid.
   f. Leave cap open and air dry for 10 minutes
2. Add 200ul of TE buffer
   a. Resuspend on the Vorexter (Low setting) for 60 minutes to overnight.
   b. Note – this time you should see much less undissolved material, but if you do it is OK, we are still doing one more extraction step.
      i. If you are not proceeding at once to the next step freeze the sample at -20.
      ii. Label:_____

iii. Storage location: _____

## Stage 2 – Removing Contaminants

*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 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 25 ul of the Proteinase K solution, mix by pipetting up and down 5-6 times, and incubate at 37C for 30 minutes
C. From the Chloroform tube, remove 500 ul using a 1000ul micropipetter for each sample, carefully cap the tube. Dispose of the tip in the solid BioHazard waste container in the hood.
    a. Vortex each sample for 10-15 sec
    b. Balancing two tubes opposite each other, spin in a microfuge at 12,000 x g for 45 minutes
D. Remove the top aqueous layer to a new labeled microfuge tube.
    a. Note the volume: _____
    b. Label on your tube:_____
    c. Pour the bottom CHCl3 layer into the Organic Waste labeled bottle in the chemical hood. Dispose of the tubes in the solid Biohazard Waste bag.
E. Divide the volume of your sample by 5 and add that amount of 3M NaOAc to the sample.
    a. Original volume:_____
    b. Added NaOAc:_____
    c. Mix thoroughly and let stand for 5 minutes
F. Add 0.7 volumes (of the new total) of isopropanol to each tube.
    a. Original volume + $1/5^{th}$ = new volume:_____
    b. 0.7 (new volume) = _____
    c. Cap and vortex to mix thoroughly.
    d. 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:_____
G. Spin at 12,000 x g for 60 minutes
H. Remove the isopropanol as completely as possible, let the pellet air dry for 10 minutes
I. Add 200ul of TE and 4ul of 5M NaCl to the tube, and put on a vortexer in gentle shaking mode overnight.
        i. If the pellet does not completely dissolve do another extraction with Phenol-Chloroform and then precipitate with $1/5^{th}$ volume of 3M NaOAc and 2.5 volumes of 95% ethanol.
        ii. If the pellet does completely dissolve
            1. Precipitate with $1/5^{th}$ volume of NaOAc and 2.5 volumes of 95% ethanol
            2. Let sit at Room temperature for 10 min (or longer, see above)
            3. Spin out of solution at 14,500 x g for 45 minutes
            4. Remove the liquid layer
            5. Air dry the tube for 10 minutes
            6. Resuspend in 100ul of TE buffer (no added salt).
J. 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

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

Grinding 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 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 water |
| TE Buffer | |
| 10mM Tris-HCl | pH 8.0 |
| 1mM EDTA | pH 8.0 |