

Chestnut DNA extraction B3 Summer Science Camp 2014

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

General Idea: extract the nucleic acid from leaf tissue by grinding it in a reducing medium (the beta-mercaptoethanol, which smells like rotten eggs), on ice. Then perform a CTAB incubation (CTAB is a detergent that grabs carbohydrates), followed by a chloroform extraction (this help denature and remove proteins and will also etract the chlorophyll and some of the other colored compounds). After centrifuging to separate the chloroform and aqueous layers, use isopropanol to precipitate the nucleic acid (both RNA and DNA and any proteins still attached to them). Further purification (with enzymes that break down RNA and protein) removes the RNA and the last of the protein. A second phenol-chloroform extraction (to denature protein bits) and centrifugation, followed by isopropanol precipitation (to remove long-strands of DNA) should give DNA of sufficient quality for PCR and restriction endonuclease digestion, needed for doing the rest of our assays. 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

- 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 0.5 gm of polyvinylpyrrolidone
 - vi. Two labeled weigh boats (label with the sample name)
 - vii. One 1.5ml microfuge tube containing 200ul of beta-mercaptoethanol and 200ul of Tween-20 (per sample) or add them separately to the mortar.
 - viii. One 50ml -Falcon tube.
 - ix. One 15-ml Falcon tube
 - x. Grinding Buffer ~7ml per sample (recipe at end)
 - xi. CTAB Buffer ~ 7 ml 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)

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- B. Stage 2 processing
 - i. Set a heat block to 37C
 - ii. RNAase A, 25ul per sample
 - iii. Proteinase K, 25ul per sample
 - iv. Phenol-Chloroform, 250 ul per sample
 - v. 3M NaOAc or 7.5M NH₄OAc (1-2 ml)
 - 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

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.

- C. Label a weigh boat so you know which sample you are handling.
- D. 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.
- E. 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.
- F. To each sample add
 - a. A few small chunks or powdered dry ice from the small cooler
 - b. The sorbitol and PVP
 - c. 5 ml of Grinding Buffer.
 - d. 200ul of the beta-mercaptoethanol
 - e. 200ul of the Tween (very thick, use the 1000ul micropipetter and cut the end off the tip with some scissors)
- G. Grind for 10-15 minutes until a smooth light-green paste is formed.
 - a. Scrape down the mortar and the pestle occasionally with a Popsicle stick.
 - 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.**
- H. Label a 50 ml tube and set it upright in a tube rack.
- I. Make a 'funnel' of the Mira cloth, poking it into the top of the 50ml tube.
- J. Scrape the plant material from the mortar and pestle into the Mira cloth funnel.
 - a. Rinse the mortar, pestle and spatula with 1 ml of Grinding Buffer and add to the funnel.

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- K. Squeeze the material through a Miracloth 'funnel' into the labeled 50 ml Falcon tube (label the cap as well)
- Discard the Miracloth in regular waste.
 - Note the volume of the solution in the tube: _____ml
 - Change your gloves!
- L. Add an equal volume of CTAB Buffer (should be ~4-6 ml)
- _____ml added
 - Cap carefully and mix thoroughly by inverting
- M. 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.
- Start time: _____
 - End time: _____
- N. Remove from the water bath
- Cool to room temperature (you can put in ice or run cool water over the tube to speed this up)
- O. Take to the chemical hood and add an equal volume of chloroform (should be around 7-10ml).
- _____ ml sample
 - _____ml Chloroform
- P. Cap and shake thoroughly, continue to shake for 10 minutes.
- Q. 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
- If you have less than 15ml, balance with another group. Add Chloroform to increase the mass of the lightest tube.
 - 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.
- R. Using a 10ml serological pipette, remove the (top) aqueous layer to a fresh labeled 15 ml tube.
- _____ Label
 - Dispose of the organic layer (bottom layer) by pouring it into the Organic Waste bottle in the chemical hood.
 - The original tube can go in regular trash once empty.
- S. 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.
- For example, if I have 8ml of solution, $8 \times 0.7 = 5.6$ ml, 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.
 - 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.

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- i. $0.7(\text{___ml your aqueous solution}) = \text{___ml isopropanol to add}$
 - 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: _____
 - T. **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
 - U. 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
 - V. 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.
 - W. 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 CHCl_3
 - c. Vortex for 10 second.
 - X. Spin in the microfuge for 30 minutes at 14,000 x g, making sure there is a balance tube.
 - Y. 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.
 - Z. 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 Vortexer (Low setting) for 60 minutes to overnight.

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- 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 Phenol-Chloroform reagent bottle in the chemical hood, 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. Banacing 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 Phenol-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 + 1/5th = 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 NaCal 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 1/5th volume of 3M NaOAc and 2.5 volumes of 95% ethanol.
 - ii. If the pellet does completely dissolve

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1. Precipitate with 1/5th 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.
- i. Storage location: _____

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