Day 1: Plant Material Collection

Each team will collect leaf material for DNA extraction and flower blooms (later) for RNA extraction. Once the leaves have been harvested the plants are put into a growth chamber under prescribed light conditions and 'forced' to cause early blooming.

1. Label and weigh two baggies.
2. Each team will harvest leaves from one soybean variety and one pea variety. Since they have already been planted you will be given a picture of the seeds, so that you can track the color. You will observe the flower color for yourselves, later.
3. Per plant you must be able to obtain at least 3 leaves from at least 3 plants. If there are not enough leaves or plants we will have you replicate the sampling and processing of a plant assigned to another group (this is good practice anyway for a technical replicate). We are using the smaller leaves (above the first level of leaves nearest the soil) as they are easier to disrupt. Use a ruler to estimate the size of the leaves you will harvest.
4. Use a clean razor blade to cut off the leaves – put into a labeled Baggie on ice (on the label you should put the Team name/initials, Date and Plant ID number). Weigh the Baggies containing the plant material (and record), return to ice.
5. The Baggies will immediately be collected and placed in the -80°C freezer upstairs.

Note: If we were making RNA the material would be harvested into liquid nitrogen, but prior to cell disruption DNAase enzymes are sequestered away from the nucleus so the DNA will be intact.

Day 2: Plant DNA extraction

The logic to this protocol is that high salt in the CTAB buffer keeps the carbohydrate in solution in alcohols, while the DNA is precitpitated. The beta-mercaptoethanol helps prevent oxidation of small phenolic compounds and if you use polyvinyl pyrrolidone helps remove polyphenolic compounds.

Reagents (Extraction Buffer, beta-mercaptoethanol, 2xCTAB Buffer, 20% SDS, $CHCl_3$-isoamyl alcohol (24:1), Isopropanol, TE buffer, RNAase A @ 5ug/ul, 3M sodium acetate pH 5.2, Phenol-$CHCl_3$ (1:1), CHCl3, Ethanol (75%, 100%).

Preparation:

The water bath should be turned on and set at 60°C. Tubes of 2X CTAB buffer are in the water bath – collect when ready to use them. Collect reagent tubes of isopropanol, chloroform, ammonium acetate and beta-mercaptoethanol.

1. You will need about 1-5 gm of plant material to use the mortars and pestles that we have effectively. Make sure you collected sufficient material. Baggies are in a dry ice container.
2. You will need to wear protective gloves when handling the chilled mortars and pestles, and protective eye gear in case of splashes. Put the tissue into a chilled mortar. If available, add liquid nitrogen or powdered dry ice and grind to a fine powder for ~5 minutes. Add liquid nitrogen or

powdered dry ice as needed to keep the sample from liquefying (you want it to be a powder at all times), but be aware that it can send the powder is easily sent flying, so add carefully.

3. Deposit the powdered tissue in a 50 ml Falcon flask.
4. Add 10 ml per gram tissue of 60°C CTAB buffer. Add 0.2% Beta-mercatoethanol. Mix thoroughly but gently (any rough handling will shear the DNA). Rinse out the mortar with 1-2 ml of the buffer and add to the tube.
5. Add 1/10th volume of 20% SDS. Mix gently.
6. Return the tube to the 60°C water bath and incubate for 30 minutes, with periodic mixing.
7. Remove from water bathand let cool to room temperature for 10 minutes.
8. Add 8 ml Chloroform for every 10 ml of wash buffer. Mix the solution (invert ~30 times) to form an emulsion.
    a. Note: Change gloves immediately if you get chloroform on them. The chloroform will remove your tube labels if it drips, it is best to write on both the tube and the lid.
9. Spin in a centrifuge at 3000 rpm for 20 minutes to separate the layers (room temperature is fine). Don't forget to balance tubes!
10. Using a 10ml serological pipette and green pipette pump, transfer the top (aqueous) layer to a new tube.
    b. Note: Dispose of the chloroform in the designated waste bottle. Dispose of the tube in the biohazard waste.
11. Repeat the chloroform extraction, adding an equal volume to what you have remaining in the tube.
12. Repeat the centrifugation and remove the aqueous layer to a new tube.
    Don't forget to balance tubes!
    c. Dispose of the chloroform in the designated waste bottle. Dispose of the tube in the biohazard waste.
13. Determine the volume of the remaining sample. Add 1/5th volume of 5M NaCl and mix.
14. Add 2/3 volume of isopropanol. You may see DNA threads forming at the interface, which means you could spool out the DNA, but this is an not reliably seen and is not quantitative. Mix the solution carefully and thoroughly.
15. Let sit at room temperature overnight (or longer).
16. Spin in centrifuge at 3000 rpm for 6 minutes.
    d. Don't forget to balance tubes!
17. Pour off the isopropanol, wiping off the last drips of the solution from the tube lip with a Kimwipe.
    e. Note: if the pellet is 'loose' you will have to pipette off the solution instead. It can go down the sink . Remove as much liquid as possible from the pellet.
18. Add 700ul of 70% ethanol with 10mM ammonium acetate and invert the tube 5-10 times
19. Centrifuge at 14,000 rpm for 1 min in the microfuge.
20. Discard the solution (make sure you don't lose the pellet)
21. Let the pellet air dry for 10-20 minutes (you can loosely cover with a Kimwipe to prevent dust contamination).
22. Add 200ul of TE buffer, and dissolve the pellet (this may take 30 minutes or so, gentle agitation on the Genie Vortexer is OK).
23. Transfer the solution to an Eppendorf tube. Store at 4°C until the next period.

a. Note: f you want to compare purity before and after the next steps, measure the absorbance at 260nm and 280 nm with the Nanodrop 2000.
b. Note: if the solution is extremely 'slimy' this means that considerable carbohydrate remains in the sample.

Day 3: Removal of protein and RNA from the DNA sample. Check of concentration and purity of the sample.

Now you need to remove the RNA and remaining protein (probably the tightly bound histones and HMGs of chromatin) from the genomic DNA. You will check the concentration and purity, and length of the final product, prior to performing PCR. Collect tubes of RNAase A,Proteinase K, PCA, NaOAc, ethanol and TE.

1. Add 12ul of RNAase A (stock is at 1 mg/ml) and incubate at 37°C for 30 minutes.

2. Add 12 ul of Proteinase K (stock is at 10mg/ml) and incubate at 40°C for 30 minutes.

3. Add 300ul of Phenol-chloroform (1:1), shake to form and emulsion, centrifuge at 14,000 rpm for 10 minutes, remove the aqueous layer to a new tube. Dispose of the Phenol-chloroform in the closed Eppendorf tube in the Biohazard waste.

    a. Note: Phenol is dangerous on the skin – it causes contact burns. Handle carefully, wear protective gear and dispose of correctly. If any gets on eyes or skin use copious water to rinse it off.

4. Add 1/10th volume of 2M sodium acetate, then 2 volumes of absolute ethanol, mix thoroughly, put in the -20C freezer for one hour, or at room temperature overnight.

5. Microfuge at 14,000 rpm for 30 minutes. Make sure you can see the white pellet, pour off the ethanolic solution. Let pellet air dry (cap open) for 20 minutes. Add 200ul of TE buffer, close cap, resuspend with gentle shaking on a Vortex Genie for 30 minutes.

6. Measure the concentration of DNA on the Nanodrop 2000, and check the A260/A280 ratio (this gives information about the purity)

7. Estimate, using the Nanodrop 2000 values, how much of the solution contains 100ng of DNA. In successive lanes of a 1% agarose minigel (use 1X TBE buffer) load 100 and 200 ng of the DNA, and include high molecular weight size markers (depending on how badly sheared the DNA is, it could be anywhere from 10-30kbp in average length).

Extraction Buffer (used with freeze-dried tissue)

100mM Tris pH 8
0.35M sorbitol
5mM EDTA pH 8
1% beta-mercaptoethanol (add just before use)

2X CTAB Buffer (used with fresh-frozen tissue) Final:

100 ml 1 M Tris HCl pH 8.0	100mM Tris
280 ml 5 M NaCl	1.4M NaCl
40 ml of 0.5 M EDTA	20mM EDTA
20 g of CTAB (cetyltrimethyl ammonium bromide)	2% CTAB
Bring total volume to 1 L with ddH_2O.	0.2% BME is added just before use.

TE Buffer

10 ml 1 M Tris HCl pH 8.0
2 ml 0.5 M EDTA
Bring total volume to 1 L with ddH_2O.

1 M Tris HCl pH 8.0

121.1 g Tris
Dissolve in about 700 ml of H_2O.
Bring pH down to 8.0 by adding concentrated HCl (you'll need about 50 ml).
Bring total volume to 1 L with ddH_2O.

0.5 M EDTA

186.12 g EDTA
Add about 700 ml H_2O
16-18 g of NaOH pellets
Adjust pH to 8.0 by with a few more pellets, EDTA won't dissolve until the pH is near 8.0
Bring total volume to 1 L with ddH_2O.

5 M NaCl

292.2 g of NaCl
700 ml H_2O
Dissolve (don't add NaCl all at once, it will never go into solution) and bring to 1 L.

7.5 M Ammonium acetate

57.81 g ammonium acetate
~50 ml of H_2O
Bring to 100 ml total volume

Hydrated Ether used when PCR does not work – it removes a class of UV-absorbing inhibitors of PCR. However, it may inhibit some restriction enzymes and ligases, so it should not be used on an entire sample without testing first.

1. Shake ether with TE buffer or Molecular Biology Grade water.
2. Use an equal volume of this ether and your TE-dissolved sample, mix thoroughly and spin, remove the ether (the top layer). The compounds will initially be seen between the phases but will dissolve into the ether with mixing.
3. I would recommend re-precipitating the DNA with ethanol, this should help remove residual ether when you air-dry the sample
4. Re-test the absorbance of the sample in any case, as the ether is stated to remove absorbing compounds that can skew the estimation of DNA concentration.