Bioplatforms Australia Genomics for Australian Plants Initiative
Activity: Reference genomes
Species: *Thelymitra variegata* (Queen of Sheba orchid)
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CTAB protocol for extracting high molecular weight DNA

References:

- Doyle, J. J. & Doyle, J. L. (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11-15.
- Doyle, J. J. & Doyle, J. L. (1990): Isolation of plant DNA from fresh tissue. Focus 12: 13-15.
- Weising, K., Nybom, H., Wolff, K. & W. Meyer (1995): DNA fingerprinting in plants and fungi. CRC Press, Boca Raton.
- Furtado, A. (2014). DNA extraction from vegetative tissue for next-generation sequencing. In *Cereal genomics* (pp. 1-5). Humana Press, Totowa, NJ.

Preparation of chemicals and stock solutions:

CTAB buffer (Doyle and Doyle, 1987):
2% (w/v) CTAB (Corrosive powder)
1.4 M NaCL
0.1 M Tris-HCl, pH 8 (corrosive)
20 mM EDTA
0.2 % Mercaptoethanol (MET) (Toxic, handle with care under the fume hood)
MET should only to be added immediately before use of the buffer

- This CTAB extraction buffer cannot be stored for long and is also not autoclavable. Therefore, it is recommended to prepare an extraction stock solution (Table 1) which does not include CTAB and MET as well as a 10 % stock solution of CTAB (Table 2). Both can be stored at room temperature; the extraction buffer is autoclavable whereas the CTAB stock solution is not.
- The final CTAB extraction buffer is prepared on the day of extraction (Table 3).

Table 1. Extraction stock solution (excluding CTAB and MET)

	800 mL	**400 mL**	**200 mL**	**80 mL**
0.5 M EDTA	40 mL	20 mL	10 mL	4 mL
2 M Tris-HCl, pH 8	100 mL	50 mL	25 mL	5 mL
5 M NaCl	280 mL	140 mL	70 mL	28 mL
Molecular grade water, pH 7.5 – 8 Fill to final volume	Add to 800 mL	Add to 400 mL	Add to 200 mL	Add to 80 mL

Preparation:

All extraction stock solution components are mixed together and then autoclaved.

Table 2. 10% CTAB stock solution

	200 mL	100 mL	50 mL	20 mL
CTAB powder	20g	10g	5g	2g
Molecular grade water	Add to 200 mL	Add to 100 mL	Add to 50 mL	Add to 20 mL

Preparation:

- Autoclave a Schott glass bottle inclusive the magnetic stirrer, the spatulas and the water to sterilise everything.
- CTAB powder added in to the glass bottle (best to be weighted into the glass bottle)
- Add molecular grade water (but not yet fully, only to 80-90% of final volume.
- Stir, can be done with heating to 60-65°C to fully dissolve the powder
- When powder is dissolved fill with molecular grade water to final volume

Preparation of the CTAB extraction buffer:

- The extraction stock solution is mixed with the 10% CTAB stock solution 4 parts to 1 part.
- The Mercapto-Ethanol (MET) is mixed with the stock solution + 10% CTAB 1 part to 500 parts
- 10 mL of CTAB extraction buffer (stock solution + 10% CTAB + MET) is required for each sample of approximately 3g fresh plant tissue

Table 3. CTAB extraction buffer

Solution	Number of extractions	
	2 samples	4 samples
Extraction buffer stock solution	24 mL	44 mL
10 % CTAB stock solution	6 mL	11 mL
Mercapto-Ethanol (MET) **note this reagent is measured in microliters (µL)	60 **µL**	120 **µL**
Total	30.06 mL	55.12 mL

Chloroform/ Isoamylalcohol 24 + 1

Preparation:
24 parts Chloroform
1 part Isoamylalcohol
Fill in sterilised glass bottle. Work under the fume hood.

Reagent list:

Flammable chemicals:
Mercapto-Ethanol (MET)
Isopropanol
70 % Ethanol, purest grade
100% Ethanol, purest grade

General chemicals:
CTAB extraction buffer stock solution
10% CTAB stock solution
Chloroform/Isoamylalcohol 24+1
TE buffer, pH 8
RNase 10mg/µL
5M NaCl

Other equipment:
50 mL tubes

DAY 1: DNA isolation and purification:

Preparations:

- Dissolve the CTAB solution in a water bath at 60°C (while CTAB is dissolving, prepare everything else needed for the day, gather chemicals and equipment to bring to the extraction lab).
- Prepare the CTAB extraction buffer under the fume hood (!): Mix CTAB buffer stock solution, 10% CTAB stock solution and MET – see table above.

Grinding of material, CTAB/MET and RNA digest (Cell lysis step):

- Grind approximately 3 grams of fresh plant tissue to a fine powder in liquid nitrogen using a mortar and pestle. To avoid degrading DNA, do not grind further once a fine powder is achieved.
- Under the fume hood (!): add 10 mL of CTAB extraction buffer to the ground plant tissue.
- Using the pestle mix **gently** to create a homogenous mixture. Mix gently and not more than is required to avoid degrading DNA.
- Transfer mixture to a 50 mL tube
- Incubate for 60 min at 60°C. Sway the tube **very gently** about every 5 minutes.
- let samples cool down to room temperature before the next step.
- Add 5 µL of 10mg/µL RNaseA and incubate at room temperature for 10 minutes.

Wash with Chloroform/ Isoamylalcohol:

- Under the fume hood (!): Add 5 mL Chloroform/ **Isoamylalcohol** (24+1) into each tube.
- Mix by **gently** swaying tubes 50 times.
- Centrifuge 10 min at 3500xg, room temperature in a swing out bucket rotor
- Under the fume hood (!): Transfer upper phase (contains the DNA) carefully with a 1 mL pipette into new 50 mL tubes.

Responsible disposal of middle phase (solid plant tissue) and lower phase (organic reagents)

- Under the fume hood (!):
- Dispose of lower phase into a CTAB waste containers.
- Add water to rinse the plant tissue remaining in the tube.
- This step can be completed at a later stage – shake the tube containing plant tissue and water, centrifuge and dispose of liquid into CTAB waste container, evaporate remaining liquid off under the fume hood and dispose of tube containing dried tissue in the bin.

Precipitation of the DNA (further cleaning step):

- Estimate the volume of the upper phase containing DNA that was transferred into the new tube.
- Under the fume hood (!): Add 0.6 volumes of isopropanol and Sway the tube **very gently** to mix. DNA precipitates as fine threads. Incubate 1 hour at room temperature (it is not recommended to incubate at -20°C, because that increases the precipitation of polysaccharides).
- Centrifuge 10 min at 3500xg at 10°C in a swing out bucket rotor. The DNA will form a pellet.
- Pour the liquid carefully into the CTAB waste container, make sure the pellet does not move. In case it moves, try to remove liquid with pipette. As a safety measure you can pour the liquid into a new 50 mL tube to not lose a loose pellet.

Washing step with ethanol:

- Add 3 mL of 70% Ethanol to the pellet. Sway the tube to clean the sides. This step removes water soluble substances (e.g., salts, ethanol)
- Centrifuge 10 min at 3500xg at 10°C in a swing out bucket rotor.
- Carefully pour the Ethanol into the CTAB waste container, again you can pour into a new tube as a safety measure not to lose your DNA pellet. Gently invert the tube, ensuring the pellet is stuck to the side of the tube and place tube upside-down on paper sheets to drain any remaining liquid.

- Once the excess liquid is drained, invert the tube and leave without lid to dry the pellet at room temperature (ca. 30 min to 1 hour, until the tube does not smell of Ethanol anymore). Take care that the DNA does not fall completely dry.

Resolve DNA:

- Add 100 µL to 500 µL of TE buffer, pH 8 or molecular grade water to dissolve the DNA pellet overnight in the fridge at ca. 7°C. For highly concentrated DNA use less buffer. Transfer the dissolved DNA to a 2 mL Eppendorf tube.

DAY 2

**Day 2 of the protocol is optional and can be used when the QC of DNA extracts produced in Day 1 indicate sugar contamination. For WGS of *Thelymitra variegata,* NanoDrop readings of DNA extracted completed on Day 1 showed no indication of sugar contamination and Day 2 was not required.

Polysaccharide precipitation:

- For each 100 µL of DNA dissolved in buffer, add 5 µl 5M NaCl (final concentration will be 0.2 5M), mix well.
- Add 35% of the volume in tube of 100% Ethanol, purest grade (= 73 µl), vortex immediately to mix very quickly and thoroughly (or else the DNA will also precipitate), then incubate on ice for 10 min to precipitate polysaccharides.
- Centrifuge 15 min at 9,000 rpm, 10°C. Polysaccharides will form a pellet, DNA will stay in solution.
- Pour the liquid containing DNA into the new tubes.

DNA precipitation:

- Estimate the volume of the tube content and add the same volume of isopropanol (ca. 250 µl) to it.
- Sway solution **very gently** to mix. DNA will precipitate as white and fine threads.
- Incubate at room temperature for 10 mins.
- Centrifuge for 15 min, 13,000 rpm, 10°C.
- Decant liquid. Take care that pellet does not get lost!
- Add 500 µl 70% Ethanol, sway tube to clean tube walls.
- Centrifuge for 15 min, 13,000 rpm, 10°C.
- Decant liquid. Take care that pellet does not get lost!
- Dry the pellet at room temperature until the Ethanol has evaporated completely (ca. 1 h).

Redissolve DNA:

- Add 100 µl to 500 µl of TE buffer, pH 8 or molecular grade water to dissolve the DNA pellet overnight in the fridge at ca. 7°C. For highly concentrated DNA use less buffer.

Storage:

Store in the fridge at ca. 7°C, or in the freezer at –20°C to –80°C for longer term storage.