1. *CTAB DNA extraction from plant tissue:*

WHAT YOU NEED
Plant tissue - frozen or fresh
Mortar & pestle or 2 mL tubes and micro-pestles (all kept at –20°C for ~1 hour)
Microcentrifuge and dry block or water bath set at 65°C
Solvents—isopropanol, ethanol, Chloroform-isoamyl alcohol (24:1)
Buffers and solutions prepared beforehand (see page 24)
Stuart Tube Rotator (or you can mix by hand)

1. After sampling from the plant, immediately wash the fresh plant leaf with distilled water and blot dry with paper towel. Surface sterilise the leaf with 70% ethanol.
2. Grind 200–500 mg plant tissue in either a mortar or 2 mL tube until all the tissue is disrupted. With larger leaves, roll into a tube-shape and place into the 2 mL tube before macerating.
3. Add 400 µL of hot (65°C) CTAB extraction buffer to each tube.
4. Mix well by flicking the tube (do not vortex)[1] and incubate the samples for ~20 minutes at 65°C (ideally, shake the samples every 5 minutes or so during the incubation to keep the tissue saturated).
5. After incubation add 400 µL of chloroform-isoamyl alcohol (24:1) to each tube. Perform this using a fume hood or similar.
6. Shake the tubes using a rotary shaker at room temperature for 15 minutes.
7. Centrifuge the samples at ~12,000 x g for 5 minutes.

Notes:
1. Never vortex genomic DNA too vigorously as it shears it.

8. Transfer the upper, clear aqueous layer of each sample to a new tube (avoiding plant debris and chloroform layers) and add 400 µL of chloroform-isoamyl alcohol 24:1 (equal volume), then repeat steps 5 and 6.
9. Transfer the upper aqueous layer to a new tube (avoiding taking any of the chloroform layer) and add an equal volume of isopropanol (~400 µL).
10. Mix the samples (by gentle inversion) and incubate at room temperature for 15–30 minutes.
11. Centrifuge the samples at maximum speed in the micro centrifuge for 5 minutes.
12. Discard the supernatant. This can be done with care by aspirating off the supernatant with a 1 mL pipette.
13. Add 400 µL DNA washing solution (kept on ice) to each sample and agitate gently. Leave for 5 minutes before centrifuging for 3 minutes. Remove supernatant. If the pellet of the sample is not clean, then repeat this step again[2].
14. This is a good stopping point in the protocol if you find that time is limited. Add the DNA washing solution to each sample and leave overnight at 4°C.
15. Place the open tubes on a clean, absorbent surface inverted for 10 minutes to dry the pellet or until most of the reagent has evaporated from the tube.
16. Resuspend the pellet in 100 µL TE buffer and 6 µL RNAse A (10 mg/mL).
17. Incubate the samples at room temperature for 15-30 minutes.
18. Precipitate the DNA by adding 1 mL cold 100 % ethanol (kept on ice) and incubate at room temperature for 30 minutes.
19. Centrifuge the samples for 5 minutes and discard the supernatant. Allow the pellet to air dry for ~ 2 hours.
20. Re-suspend the pellet in 100 µL TE overnight at 4°C.

Note:
2. The pellet might not stick tightly to the tube after this step so care is advised.

2. Stock solutions of buffers and reagents:

1. 10 % hexadecyltrimethylammonium bromide (CTAB): Add 10 g CTAB to 80 mL H_2O. Heat to 65 °C to dissolve and adjust the volume to 100 mL with H_2O. Store at room temperature.
   *** Do NOT autoclave CTAB solution ***
2. 5 M sodium chloride (NaCl).
3. 1 M tris-hydrochloric acid (Tris-HCl) pH 8.0.
4. 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0[3].
5. DNA washing solution stored at 4°C: 70 % ethanol , 0.77 g/ L ammonium acetate.
   **** Do NOT autoclave ammonium acetate solution[4] **.***
   Filter solution using a 10 mL syringe through a 0.2 µm filter unit.
6. Dithiothreitol (DTT): Weigh out just before use and add to CTAB buffer.
7. RNase A (10 mg/mL).
8. Proteinase K (20 mg/mL).

3. CTAB working solution:

CTAB Extraction Buffer: 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0 and finally add DTT to 1% just before use.

Reagent	Final concentration	Stock concentration	To prepare 5 mL
CTAB	2 %	10 %	1 mL
NaCl	1.4 M	5 M	1.4 mL
EDTA pH 8	20 mM	0.5 M	200 µL
Tris-HCl pH 8	100 mM	1 M	500 µL
DTT	1 %	-	50 mg
dH_2O	-	-	1.85 mL

4. Stuart Tube Rotator (mixer):

1. Place tubes with lids firmly closed and facing inwards. Balance the tubes so the disc rotates evenly.
2. Switch the unit on using the green on/off switch. The unit will rotate at a fixed speed of 20 rpm.
3. Once mixing is complete switch the unit off using the green on / off switch and unload the tubes.

Stuart Tube Rotator
Position the tubes on the disc so they are balanced, for example place tubes opposite each other.

Note:
[3] EDTA will not go into solution below pH 8.0. Add solid NaOH sparingly and allow the solution to equilibrate between additions to avoid over-shooting the final pH.
[4] Ammonium acetate decomposes in hot water and should not be autoclaved.