Preparation of Highly Purified Plant DNA

Collection of Plant Tissue

1. Cut off leaf tissue (with scissors). Avoiding collecting yellowed tissue or petioles, if possible.

2. Wrap tissue loosely in cheesecloth, tying with string, and attach cold-resistant label. Tape will crack and fall off at liquid nitrogen temperatures, a successful method is to punch a hole in a thick paper label, and pass the string through the hole.

3. Submerge in liquid nitrogen. From this point on, thawing must be avoided. Store in a styrofoam cooler with liquid nitrogen until transferred either to an ultracold freezer (-70°C), or place directly in freeze drier.

4. Freeze dry (usually about 3 days), taking care not to let tissue thaw. Transfer tissue from cooler or ultracold freezer directly to freeze drier chamber, don't let tissue touch the warm metal sides, and start freeze drier immediately. If tissue is handled correctly, dry tissue is whitish-green colored. If tissue has thawed in handling, it will be darker green on the thawed edges. If this occurs, throw the affected tissue away, it is ruined.

Tissue Extraction Protocol

1. Crush 2 grams (*or more*) of freeze-dried plant tissue with a small amount of Ottawa silica sand (*optional*) in an acid washed (see below for acid-washing procedure) and sterilized mortar and pestle. Grind as finely as possible.

2. Add crushed samples to 150 ml glass test tubes (acid-washed), with screw caps, *or to 250 ml centrifuge tubes,* with at least 50 ml of sterile extraction buffer. (50 mM Tris (pH 8.0), 0.7 M NaCl, 10 mM EDTA, 1% CTAB (hexadecyltrimethylammonium bromide), 0.1% β-mercaptoethanol)

To make:	100 ml	1 l	2 l	4 l
1 M Tris (pH 8.0)	5 ml	50 ml	100 ml	200 ml
NaCl	4.09 g	40.9 g	81.8 g	163.6 g
0.5 M EDTA	2 ml	20 ml	40 ml	80 ml
CTAB	1 g	10 g	20 g	40 g
β-mercaptoethanol	0.1 ml	1 ml	2 ml	4 ml

   Mix and sterilize the above ingredients except β-mercaptoethanol. Add β-mercaptoethanol after sterilization.

3. Mix tissue in buffer until thoroughly wet. Final product should not be highly viscous.

4. Incubate at 50°C for 45 minutes, mix gently (so as not to break DNA) about 10 every minutes.

5. Add equal volume of chloroform:isoamyl alcohol (24:1) and incubate for 45 minutes at 50°C. Mix gently about every 10 minutes.

6. Centrifuge in 50 ml tubes in SS34 rotors (use rubber sleeves) for 10 minutes at 8000 rpm, *or 250 ml tubes for 10 minutes at 6000 rpm.*

7. Using inverted 10 ml pipette (to provide large orifice), remove supernatant and place in a new acid-washed tube. Remove only the top phase.

8. Add an equal volume of isopropanol, or twice the volume of 95% ethanol. Invert gently until two phases are no longer evident. DNA will collect as white stringy mass. *Sample may be stored overnight at -20°C at this point.*

9. Close and hook the end of a pasteur pipette and gently wind DNA onto end. Let liquid drain off and put into acid-washed tube containing 76% ethanol, 10 mM ammonium acetate. Let stand for at least 20 minutes. DNA can be left on the pasteur pipette.

> 76% Ethanol, 10 mM Ammonium acetate:
> To make 475 ml, use 380 ml of 95% ethanol, 630 µl of 7.5 M NH_4OAc, and bring to 475 ml with H_2O.

10. Remove DNA by hooked pipette. Let liquid drain off (until almost dry) and dissolve in 4.0 to 4.5 ml of 0.10× SSC in the blue-capped disposable plastic test-tubes. It is important to dissolve the DNA completely.

11. For every ml of SSC + DNA, add 1.1 g of CsCl. Add 5 µl of ethidium bromide (5 mg/ml) for each gram of CsCl.

12. Use a pasteur pipette to draw up the mixture and place into Beckman ultra-centrifuge tubes.

13. Balance the tubes and run on Beckmann ultra-centrifuge at 50,000 rpm, 20°C for at least 6 hours. DNA will be visible with UV light.

14. Remove the DNA with a 18 gauge needle on a small syringe.

15. If there is another band close to the DNA band, the drawn out band will have to be run again. Transfer the DNA band to centrifuge tube and add 200 µl (5 mg/ml). Top off with solution of 1 g CsCl per 1 ml of TE (pH 8.0). Spin for 6 hours at 50,000 rpm at 20°C.

16. Place DNA into blue-capped tubes and add 2 to 3 volumes of isoamyl *or isopropyl* alcohol saturated with 20× SSC.

17. Slowly mix for >5 minutes. Ethidium bromide will enter the alcohol (top) layer. Discard alcohol and add more. Repeat this procedure until no more red (Ethidium bromide) moves into the alcohol layer.

18. To dialyze out the CsCl, remove DNA solution with pasteur pipette and place in dialysis tubes. Clamp both ends. Place dialysis tubes in a beaker and add 2000 ml of TE (pH 8.0). Place in cold room for at least 6 hours. Change buffer twice.

19. Pipette DNA in sterile microfuge tubes. Determine the concentration of DNA solution using UV spectrophotometer.