

# Isolation of Plant Mitochondria for Genomic Applications

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- 1.) One-hundred grams of tissue is the minimum required amount of tissue for the procedure, it is best to use etiolated seedlings. If green plant tissue is used, it must be placed in the dark for 72 hours prior to isolation.
- 2.) Collect tissue and rinse in ice water for 10 minutes. Rinse tissue and blot dry, place plant material in a large beaker containing GB at 4 deg. C. At this point it may be necessary to cut large leaves into smaller pieces. Allow tissue to sit in grinding buffer at 4 deg. C in the dark for 15-30 minutes. Use 4 ml GB/ g fresh weight of tissue. Perform all subsequent steps in a cold-room or keep all tubes and solutions at 4 deg.C.
- 3.) Homogenize tissue in waring blender for 15 seconds on high, allow material to settle and repeat twice. Filter homogenate through 4 layers of cheesecloth and 2 layers of Miracloth into cold 250 mL centrifuge bottles.
- 4.) Centrifuge at 4,500 rpm in GSA rotor (3,000 xg) for 10 minutes. Centrifuge the supernatant at 10,000 rpm (16,000 xg) for 30 minutes.
- 5.) Resuspend pellet in GB using a soft paintbrush. Resuspend pellets in 150 ml of WB. Repeat step 4.
- 6.) Resuspend crude mitochondrial pellets in 20 ml of WB and combine samples into one or two GSA tubes.
- 7.) Centrifuge sample at 10,000 rpm (16,000 xg) for 30 minutes. Resuspend pellet in 25 ml of WB. With the addition of 25 mM MgCl<sub>2</sub>. (500 ul).
- 8.) Add 100 ug/ml DNase I (Sigma) mix well and place on ice for 30-60 minutes.
- 9.) Add 50 mM EDTA (2 ml), followed by the addition of 100 ml of WBE
- 10.) Centrifuge at 3,500 rpm in GSA rotor (2,000 xg) for 10 minutes. Centrifuge the supernatant at 10,000 rpm (16,000 xg) for 30 minutes.
- 11.) Resuspend pellet in 25 ml of WBE and place on ice
- 12.) Form Percoll gradients in Corex centrifuge tubes. Underlayer 18:23:40 % Percoll (dissolved in WBE) in the ratio of 8:10:8 ml.
- 13.) Add 6 ml of mitochondria suspension onto gradient and centrifuge in Beckman JA-13 at 9,000 rpm (12,000 xg) for 1 hour at 4 deg.C.
- 14.) Collect the mitochondria from the 23:40 interface with a 3 cc syringe. Dilute mitochondria with three volumes of WBE over a 15 minute period on ice.
- 15.) Collect mitochondria by centrifugation at 15,000 rpm in a SS34 rotor (20,000 xg) for 30 minutes at 4EC. Repeat wash, be careful not to lose mitochondrial pellet.
- 16.) Resuspend mitochondria in 5 ml of WB. Mitochondria can be stored at -20 deg. C with the addition of 1 volume 50% glycerol or used immediately for DNA or RNA extraction. For RNA extraction, follow Qiagen RNA isolation protocol.
- 17.) If mtDNA is desired Add 20 ml of WB and repeat step 15. Remove supernatant with aspirator.
- 18.) Resuspend pellet in 4.5 ml of LB (pre-warmed to 37 deg.C). Add Proteinase K (10mg/ml) and incubate at 37 deg.C for 1 hour with occasional mixing.

19.) Add 4.5 g CsCl to tube and dissolve. Add 200 ul of EtBr (5mg/ml stock) and place solution in Beckman heat-seal tubes. Seal, and centrifuge at 55,000 rpm for 20 hours at 20 deg.C.

20.) Remove mitochondrial DNA band, and partition EtBr with salt-saturated Isopropanol.

21.) Place DNA into 1.5 ml microfuge tubes and EtOH precipitate (use 90% to avoid CsCl precipitation). Wash with 70% and resuspend in 100 uL of TE. Quantify DNA and check quality with restriction analysis.

## Notes

a.) Procedure has been used with large seeded cucurbits, sunflower, and tobacco seedlings.

b.) Percoll centrifugation is only required when highly purified intact mitochondria are required, alternatively, DNase for a longer period, and wash mitochondria in the presence of WBE.

c.) If CsCl purity is not necessary two phenol extractions is sufficient.

## Solutions

(do no autoclave, store all solutions at 4 deg.C)

### **Grinding buffer (GB)**

Need 4 liters (Mix at least 2 hour)

0.35 M Sorbitol 255.08 g

50 mM Tris pH 7.6 200.00 ml (1.0M Stock)

5 mM EDTA 40.00 ml (0.5M Stock)

0.2% BSA 8.0 g

1.0% PVP 40.0 g

0.025% Spermine 1.0 g

0.025% Spermidine 1.0 g

0.125% B-ME 5.0 ml (Add day of extraction)

### **Wash Buffer (WB)**

Need 1 liter

0.35 M Sorbitol 63.77 g

50 mM Tris pH 7.6 50.00 ml

0.1% BSA 1.0 g

### **Wash Buffer + EDTA (WBE)**

Need 2 liters

0.35M Sorbitol 127.54 g

50 mM Tris pH 7.6 100.00 ml

20 mM EDTA 80.00 ml

### **Lysis Buffer (LB)**

Need 500 ml

1.0% Sarkosyl 50.00 ml (10% Stock)

50 mM Tris pH 8.0 25.00 ml

25 mM EDTA 50.00 ml

Proteinase K 10mg/ml (Add directly to lysis reaction)

### **Other solutions needed**

25:24:1 Phenol:Chloroform:Isoamyl Alcohol

TE (10mM Tris, 1mM EDTA (pH 8.0))

Percoll gradients

5X Gradient Buffer (1.5M Sucrose, 50mM MOPS, 10mM EDTA); Percoll (Sigma P-4937)

18% = 6 ml 5x, 5.5 ml Percoll, 18.5 ml ddH<sub>2</sub>O

23% = 6 ml 5x, 7 ml Percoll, 17 ml dd H<sub>2</sub>O

40% = 6 ml 5x, 12 ml Percoll, 12 ml ddH<sub>2</sub>O

### **Materials needed**

Waring blender

Large flasks and funnels

Cheesecloth/Miracloth (or 48uM nylon membrane)

Soft paintbrush

Waterbath

Corex tubes

Ultra-centrifuge tubes

GSA Rotor

SS-34 Rotor

Centrifuge tubes