Isolation of Mitochondrial DNA

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Modified by Jason W. Lilly 4-1-98

1.) Select plant material which will be low in carbohydrates and chlorophyll. Best material is germinated seedlings which are dark grown for approximately one week. For adequate yields 500grams of fresh weight tissue will be required.

2.) Harvest above ground tissue and rinse in a light bleach solution (5%) and rinse with distilled water three times to remove all traces of bleach. This is needed to remove any fungal or bacterial contamination

3.) All solutions can only be 1 day old, and kept at 4C, DO NOT autoclave unless it is noted. All centrifugations are carried out at 4C unless noted

4.) See materials and solutions list at bottom for complete recipes

5.) Homogenize tissue in Buffer A (2mls/gram initial fresh weight... i.e. 1000mls/500grams) macerate all tissue until solution is highly viscous. Add an additional 500mls of solution and homogenize for an additional 2 minutes.

6.) Homogenate will need to be filtered prior to centrifugation. Two filtrations are required. First, using a 4L beaker, suspend 2 layers of sterilized cheesecloth (with enough extra to use to squeeze excess liquid). Pour homogenate SLOWLY over cheesecloth and allow to filter through. When liquid stops dripping from cheesecloth, while wearing gloves, squeeze out extra solution. Keeping filtrate and adding an additional 250-500mls of Buffer A and re-homogenize. Repeat the filtering process, but this time use 2 layers of MIRACLOTH, (note be careful when squeezing... MIRACLOTH has low tensile strength and you if you use too much POWER :) your filtrate will cover your lab bench... you have been warned!)

7.) Pour filtered solution into 6 250 mls GSA tubes. Mix while pouring to ensure even distribution of organelles.

8.) Centrifuge in GSA rotor for 12 minutes at 3,000rpm (1,000 X G)

9.) Clean up your mess from steps 4-7 (trust me it will be a big one)

10.) Pour the supernatant into clean GSA tubes (be careful, not to dis-lodge the pellet from the side of the tube), and spin at 11,000rpm (12,000 X G) for 25 minutes

11.) Decant liquid and try to remove all solution from tube, the resulting pellet contains mitochondria. CAREFULLY, resuspend the pellet, at this point I prefer to use horsehair paintbrushes which have been soaked in Buffer B, use a different brush for each sample. Resuspend pellet in 30-50 mls (depending on size of pellet) of Buffer B. Keeping

everything on ICE at all times. At this point you can begin to reduce the number of tubes you will use. I went from 6 tubes down to 2 tubes at this step, later on will be condense this down to a single oakridge (50ml) tube.

12.) Centrifuge again for 10 minutes at 3,000 rpm

13.) Carefully pour supernatant into clean GSA tubes, add $MgCl^{+2}$ to final conc of 15mM/L and add 3 vials of Sigma DNAse I (50ug/g initial fresh weight) You can dissolve the DNAse I in .15M NaCl if you wish and pipette it into the mitochondrial suspension mix gently.

14.) Let this sit at room temperature for 1 hour this should degrade all nuclear DNA's in the solution

15.) Underlay the suspension with 2 volumes of Buffer C (do this by carefully pipetting the buffer underneath the suspension... the suspension should begin to rise above (due to sucrose concentration differences) the underlayer/suspension interface should be visible

16.) Centrifuge for 25 minutes at 10,000rpm (10,000 X G)

17.) Decant supernatant and resuspend pellet with paintbrush in 50mls of Buffer C

18.) Centrifuge for 25 minutes at 10,000rpm (10,000 X G)

19.) Repeat steps 17 & 18

20.) Decant last wash. Resuspend pellet in 10mls of Buffer D (lysis buffer) Preheat lysis buffer to 37C, and carefully resuspend mitochondrial pellet. Try to place all mitochondrial lysis suspensions in a single Oakridge (with seal) tube. Allow lysis to occur for 1 hour at 37 degrees with occasional mixing.

21.) Add equal volume of chloroform:isoamyl (24:1) Invert gently 40 times, and spin in HS-4 rotor for 20 minutes at 6,000rpm

22.) Remove top (aqueous) phase, and place in clean oakridge tube and repeat step 21 at least once more... if solution is still cloudy do again.

23.) Add ice-cold 95% alcohol (fill tube completely) mix gently inverting 50 times, and place at -80C for 1 hour

24.) Precipitate DNA by centrifugation. Spin in HS-4 rotor for 30 minutes at 7,000 rpm

25.) Decant EtOH, allow tube to dry. Add a small amount of 75% EtOH + 10mM NH_4OAc, allow to sit for 5 minutes, and decant. Place tubes in front of Laminar flow hood for 20minutes to dry.

26.) Resuspend pellet in 4.5mls of .1XSSC. It helps to pre-heat SSC to 60C and allow resuspension to occur at 60C for 1 hour, this should dissolve all DNA. Some proteins still may be present, but don't worry about those.

27.) Add CsCl to a conc of 1.08g/ml of .1X SSC Dissolve completely

28.) Add EtBr. Add LOTS. You need a very deep red color (300ug/ml). I add 150ul of a 5ug/ml solution

29.) Place into Beckman HeatSeal tubes. (See CsCl DNA isolation protocol)

30.) Spin at 55,000rpm (20C) in Beckman Ultracentrifuge for at least 16 hours.

31.) Remove tubes and pull mtDNA bands. You should (this is where you know if it worked or not) see a very narrow band 1/4 from the top of the tube. And if you were really good an extremely small band just beneath it. Pull both bands as one. There should also be lots of illumination from the bottom of the tube... this is degraded nuclear DNA

32.) Partition against Isopropanol/20XSSC until all red color is removed

33.) Dialyze in TE buffer once for ½ hour, second for 6 hours, third for 4 hours.

34.) Spec DNA on USDA spec in room 203. Good mtDNA should yield about 200ul of a 60ng/ul concentration.

35.) To check quality of mtDNA. Run a .6% gel with the following lanes.
HindIII lambda marker, uncut genomic DNA (doesn't matter what species), uncut mtDNA, and Sal I digested mtDNA. Good quality mtDNA should be unsheared, and SalI digested should show many discrete bands.

Materials needed

Polytron homogenizer (from Spooner's lab)
Lots of acid washed GSA tubes, with lids, seals, and caps, chilled before use
Sterilized cheesecloth
Many large 4l beakers, very clean!!!. And chilled before use!
Miracloth
Large rubber bands
GSA rotor
Very soft (horsehair) paintbrushes
Waterbath
Many oakridge tubes (preferable with pink sealable caps)
10ml Pipettes
95% EtOH
15ml Polypropylene tubes
Beckman HeatSeal tubes
Needles and syringes
Dialyze tubing and clips

Buffers and Solutions

Buffer A (Grinding Buffer) Need 4L (for 1 species)
For 1 L add
- 171.15g Sucrose (.5M/L)
- 50mls 1M solution of TRIS pH 7.6 (50mM/L)
- 10mls .5M solution of EDTA pH 8.0 (5mM/L)
- 1 g of BSA

Buffer B (Resuspension Buffer) Need 2L
For 1L add
- 126.9g Sucrose (.3M/L)
- 50mls 1M solution of TRIS pH 7.6 (50mM/L)

Buffer C (Underlaying solution) Need 2L
For 1L Add
- 205.38g Sucrose (.6mol/L)
- 10mls 1M solution of TRIS pH 7.2 (10mM/L)
- 40mls .5M solution of EDTA pH 8.0 (20mM/L)

Buffer D (Lysis solution) Need .5L
For 1L Add
- 25g SDS
- .5g Proteinase K
- 50mls 1.0M solution of TRIS pH 8.0 (50mM/L)
- 20mls of .5M solution of EDTA pH 8.0 (10mM/L)

1M $MgCl^{+2}$
For 500mls add
- 101.65g $MgCl^{+2}$
- Bring up to 500mls with ddH_20
- Filter sterilize

Chloroform:Isoamayl
For 500mls add
- 480mls Chloroform
- 20mls Isoamayl Alcohol

76% EtOH + 10mM NH4OAc
For 1L Add
- 800mls of 95% EtOH
- 1.5 mls of 7.5M
- 198.5mls of H_20