Mitochondrial Isolation Protocol

This protocol is modified from the following two reference:

- Meyer et al. 2009 Plant Phys 151(2):603-19

Starting material: Protocol has successfully worked for *Arabidopsis*, *Silene*, *Solanum*, and *Agrostemma* in the isolation of leaf mitochondria. All aerial tissue can be used, younger plants produce more mitochondria

Comments:
- 350 ml of grinding buffer per 65 g of tissue
- Max 65 g of tissue per two gradients. Increase number of gradients if necessary. Maximum weight of tissue per gradient is species-specific.
- Keep mitochondria on ice and in a cold room as much as possible

1. Harvesting and grinding

- Collect the aerial parts of your plants, cut the stem at the basis of the rosette, and remove dead leaves and soil. Weigh your plants. Wash thoroughly in water and use a salad spinner for drying. Go in cold room.
- Add 4/5th of the extraction buffer, saving 1/5th to wash blender and collect remaining tissue in blender cup.
- For NutraBullet blender: One speed, blend for 2 seconds 2x times and 3 seconds 1x time. Shake blender after the first two short blending bursts
- Pre-wet the miracloth before pouring extraction into funnel.
- Filter through 4 layers of disposable cheese cloth and one layer of miracloth and collect flow-though. Squeeze cheese cloth <u>thoroughly</u>, very minimal squeezing of the mira cloth.

2. Differential centrifugation to remove nuclear and plastid components

Use 250 ml bottles for all actions 1-4 listed in step #2. Then transfer to 33 ml corex centrifuge tubes in step 5.

1. - Centrifuge 12 min at 500g, max break. Transfer the supernatant into a clean centrifuge tube.
2. - Centrifuge 12 min at 1500g, max break. Transfer the supernatant into a clean centrifuge tube.
3. - Centrifuge 12 min at 3000g, max break. Transfer the supernatant into a clean centrifuge tube.

Pellet mitochondria

4. - Centrifuge 12 min at 20000g, max break. Discard the supernatant. Resuspend the pellet in a small volume of wash buffer (using a ***pre-wet** paintbrush).

Add a very small amount of wash buffer or use remaining extraction buffer to resuspend pellet. Pellet should be gently but thoroughly resuspended with a paintbrush until no aggregates are visible. Failure to resuspend will cause aggregates to not migrate properly through a gradient. Place the end of a plastic bulb pipet against the bottom of the centrifuge tube and slowly aspirate the fluid, pulling the homogenate through the paintbrush fibers. This action breaks up the aggregates over the paintbrush fibres.

5. - Transfer homogenate to centrifuge tubes. Dilute homogenate well with about 25 mL of washing buffer. Passing the liquid back and forth between two centrifuge tubes can help further mix.

Remove residual nuclear and plastid components

6. - Centrifuge 12 min at 3000g. Save the supernatant, transfer to new centrifuge tubes.

Pellet mitochondria

7. - Centrifuge 12 min at 20000g. max break**.** Discard all supernatant.

8. - Use any remaining supernatant or use a very small amount of washing buffer (500 µL) to resuspend with a paintbrush. Repeat the aspiration of the supernatant over the paintbrush fibres and finally put inside the well of a Dounce homogenizer. Remove and collect residual homogenate from the paintbrush by flowing washing buffer over the fibres. Add any washing buffer used to clean paintbrushes to the Dounce homogenizer as well. Pass a small amount of washing buffer to the centrifuge tubes after collecting the majority of the pellect to recover any remaining mitochondria. Use **3** strokes of the Dounce homogenizer to eliminate aggregates.

3. Purification of mitochondria on Percoll gradient

- Load the homogenized suspension on Percoll step gradients. Be careful of not overloading your gradients (maximum loading was around 30 g per gradient for *Arabidopsis* and slightly more for *Silene*).

Isolate intact Mitochondria

- Centrifuge at 40000 g for 45 min. **Break off.**
- Mitochondria will form a white band in the bottom of the gradient on top of the 50% Percoll layer. Overloaded gradients will have a green 25% layer. Increase number of gradients to prevent overloaded gradients.
- Remove with a vacuum pump the green band in the top part of the gradient.
- Collect the bottom part of the gradient (don't take the bottom 2-3 mL) and dilute in wash buffer. Use 4 clean tubes per 2 collected gradients for this dilution step.
- Centrifuge at 20000 g for 10 min.
- Remove with a vacuum pump most of the supernatant. Keep ca. 3-4 mL per tube (Be careful, the pellet is loose) and resuspend the pellet by swirling the tube. Combine two tubes into 1 (4 tubes -> 2 tubes) for the next step.
- Add about 20 ml of new wash buffer. Gently mix wash buffer and mitochondria.
- Centrifuge at 10000 g for 10 min.
- Remove all supernatant with a vacuum pump. Cut the tip off of a p1000 pipette tip, resuspend pellets with a small amount of washing buffer with this tip. Combine both pellets by transferring into a 1.5 mL centrifuge tube, add washing buffer to almost fill the centrifuge tube.
- Centrifuge at 10000 g for 10 min on a tabletop centrifuge.
- Discard supernatant and flash freeze in liquid N_2.

BUFFERS :

Extraction Buffer		**350** ml	700ml
Mw: 342.30 g/mol	0.3 M **Sucrose**	35.9 g	71.88 g
Mw: 446.06 g/mol	5 mM **Tetrasodium pyrophosphate** (10 H_2O)	.781 g	1.56 g
Mw: /	2 mM **EDTA** (0.5M)	1.40 ml	2.80 ml
Mw: 136.09 g/mol	10 mM **KH_2PO_4**	.476 g	.953 g
Mw: 40,000	1% **PVP-40**	3.50 g	7 g
Added right before use:			
	1% **BSA**	3.50 g	7 g
176.13 g/mol	20 mM **Ascorbic acid**	1.23 g	2.47 g
121.16 g/mol	5 mM **Cysteine**	.212 g	.424 g
pH to 7.5 with KOH after addition of all reagents			

Wash Buffer		**1000** ml
Mw: 342.30 g/mol	0.3 M **Sucrose**	102.7 g
Mw: 209.26 g/mol	10 mM **MOPS**	2.1 g
Mw: 380.35 g/mol	1 mM **EGTA**	0.4 g
pH to 7.2 with KOH after addition of all reagents		

Mito gradient buffer 5x		100 ml
Mw: 342.30 g/mol	1.5 M **Sucrose**	51.4 g
Mw: 209.26 g/mol	50 mM **MOPS**	1 g
pH to 7.2 with KOH after addition of all reagents		

Other reagents and materials checklist:

Extraction	**RNA extraction**
Glass dounce homogenizer	Trizol
Plastic transfer pipets	Chloroform
Animal hair paintbrush	Isopropanol
Funnel	Glycogen
Mira cloth/cheesecloth	
P1000 pipets/tips/scissors	

Gradient Prep:

Solutions for gradients (2 gradients / 4 grad):

Note: Volumes of gradients had to be changed from IBMP because our centrifuge tubes are smaller. There is less 25% volume to pass through, so it's possible that this is doing less "filtering" – only possibility is to try and increasing gradient number per g tissue.

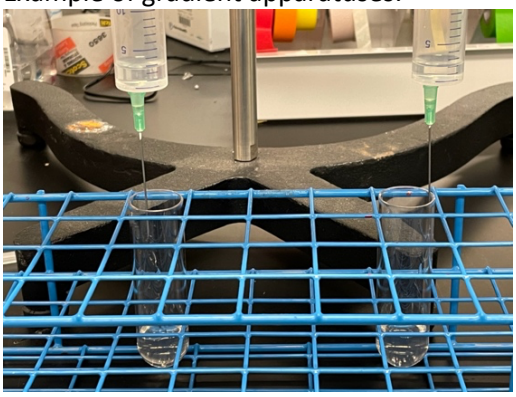
All measurements are in ml

Solution	Volume / grad	Gradient buffer 5X	Percoll 100%	Water
50%	4	2 / 4	5 / 10	3 / 6
25%	20	10 / 20	12.5 / 25	27.5 / 55
18%	4	2 / 4	1.8 / 3.6	6.2 / 12.4

Notes on gradient preparation:

+ Vortex each gradient mix and vortex briefly. These solutions will stick.
+ Set up needle apparatus for a slow drip of gradient. Ensure that the opening of the needle is flush with the centrifuge tube so that the solution is not dripping, but instead running down. Turbulence ruins gradient interfaces.
+ Watch the placement of the needle throughout the process, but the first 5 ml are most important for development of the gradient interface. Do not add all 20 ml of the 25% solution to the syringe at once, instead add 2-5 ml and let the interface form before adding the remaining solution.
+ The 18% solution is sometimes difficult to move down the needle. If the solution "sticks" use a pipette and generally aspirate some of the solution and push it down the exit of the syringe.

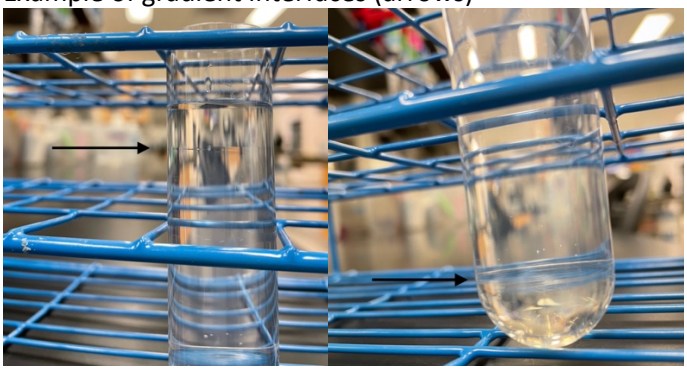
Example of gradient apparatuses:



Example of solution running down the side of the gradient tube wall.

Solution should not freely drip onto the solution below, but instead run down the side of the centrifuge tube. Use tape to apply pressure to the needle against the wall of the tube if necessary.

Example of gradient interfaces (arrows)



If gradient interfaces are not obvious when gently tilting the gradient (light should bend) do not use gradient and start over. Place gradients in cold room at least an hour before use (to reach temperature) but do not make overnight for morning prep.