Isolation of mitochondria from Arabidopsis leaves (Keech et al., 2005)

Plant materials

Arabidopsis plants must be grown under short-day conditions as described in the literature and harvested before the completion of the light cycle.

Preparation of Percoll gradient

1. Centrifuge a 50% Percoll solution (6 ml Percoll solution/13 ml PC tube x 6) at 39 000 g for 40 min in a Hitachi RPS40T-410 rotor (18,000rpm) and keep the density gradient thus produced at 4˚C before use.

   After this centrifugation, the Percoll solution will form as a pellet at the bottom of the tube. Special care should be taken, at later steps, not to recover the pellet during Mit recovery.

Method B: to obtain purified mitochondria (Yield ~100-200 mg of total mitochondrial proteins)

Grind approximately 50 g of leaves in 4 portions. All equipment should be chilled at 4˚C and centrifuged at 4˚C.

1. Grind 12.5 g leaf tissue, using a mortar and pestle, in 15 ml of grinding buffer B with a small amount (0.5 g) of quartz (fine granular, Merck).

2. Add 4 x ~20 ml of grinding buffer B. Combine all portions of homogenates and filter through a nylon mesh (20 µm).

3. Centrifuge the extract (total ~200 ml) at 2,500 *g* for 5 min in a Hitachi RPR20-2 rotor (4,800 rpm, 4 ˚C) and centrifuge the resultant supernatant at 15,000 *g* for 15 min in an RPR20-2 rotor (11,500rpm, 4˚C).

4. Resuspend the pellet in ~750 µl of wash buffer B, using a small paintbrush, and very gently homogenize twice, using a 5 ml glass homogenizer.

5. Carefully layer the resuspended mitochondrial preparation on top of the percoll gradient (6 x 13PC tubes). The maximum load per gradient is the crude mitochondria from g fresh leaves per ml gradient. Centrifuge for 15 min at 15,000 g in a Hitachi RPS40T-410 rotor (11,000rpm).

6. The mitochondria will form a whitish band close to the bottom of the tube. This band is aspirated and resuspended in wash buffer B to obtain (at least) a 20-fold dilution. (Remove the major green band (broken thylakoids) by aspiration, and recover the mitochondria by pipetting).

7. Centrifuge at 15,000 *g* (4˚C) for 20 min in RPR20-2 rotor (11,500rpm). Mitochondria should form a soft pellet. Repeat this last centrifugation step if the pellet is not sufficiently firm.

8. Resuspend the pellet in 200–300 µl of wash buffer B. [Option for respiratory activity measurements: add four percent of the final volume of a stock solution of protease inhibitor cocktail (Roche Applied Science)].

Tips:

・Do not use a washed Miracloth.

・Grind plant material thoroughly.

・Do not use too large a volume of percoll gradient (6 ml/12 g initial leaf tissue is enough). Also, be sure to use swing bucket rotors for the Percoll steps.

Grinding buffer B (100 ml):

0.3 M sucrose (mw 342.30)		10.269 g
60 mM TES-KOH, pH 8.0	1.0 M stock	6 ml(1.376g)
2 mM EDTA-2Na· $2H_2O$ (mw. 372.24)		74.45 mg(0.4ml)
10 mM KH_2PO_4 (mw 136.09)		136.09 mg
25mM tetrasodium pyrophosphate (mw 446.06)		1.115 g
1 mM glycine (mw 75.07)		7.51 mg
1% (w/v) polyvinylpyrrolidone-40		1.0 g
1% (w/v) bovine serum albumin (BSA)		1.0 g
50 mM sodium ascorbate (mw 198.11)		990.55 mg

Plus 20 mM cysteine (mw 121.16, 242.32 mg) added just prior to grinding, and readjustment of the pH to 8.0 with 1 M KOH.

Wash buffer B (100 ml):

0.3 M sucrose (mw 342.30)	10.27 g
10 mM TES (mw 229.25)	229.25 mg
10 mM KH_2PO_4 (mw 136.09)	136.09 mg

Adjust pH 7.5 with 1 M KOH

Percoll solution (100 ml):

50% (v/v) Percoll	50 ml
0.3 M sucrose (mw 342.30)	10.27 g
10 mM TES (mw 229.25)	229.25 mg
1 mM EDTA-2Na· $2H_2O$ (mw 372.24)	37.22 mg
10 mM KH_2PO_4 (mw 136.09)	136.09 mg
1 mM glycine (mw 75.07)	7.51 mg

Adjust pH 7.5 with 1 M KOH

Reference:

Keech, O., Dizengremel, P. and Gardestrom P. (2005) Preparation of leaf mitochondria from *Arabidopsis thaliana*. Physiol Plant **124:** 403-409.

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