Cytochrome *c* release from isolated mitochondria

Solution preparation

1. Mitochondria isolation buffer (MIB, 50 ml)

Mannitol (MW: 182.17, C6H14O6)	2.28 g (250 mM)
1 M Hepes	250 µl (5 mM)
0.5 M EGTA	50 µl (0.5 mM)
BSA	50.00 mg (1 mg/ml)

Note: add protease inhibitor tablet before use (1 tablet / 10 ml buffer) and 0.2 M PMSF (final concentration is 0.1 mM).

2. Mitochondria suspension buffer (MSB, 50 ml)

Mannitol	3.64 g (400 mM)
KH_2PO_4 (MW: 136.09)	68.05 mg (10 mM)
BSA	250.00 mg (5 mg/ml)
1 M Tris-HCl (pH 7.2)	2.5 ml (50 mM)

3. Reaction buffer (RB, 50 ml)

Mannitol	2.00 g (220 mM)
Sucrose (MW: 342.3)	1.16 g (68 mM)
1 M Hepes-KOH [pH 7.5]	1 ml (20 mM)
KCl (MW: 74.55)	37.28 mg (10 mM)
$MgCl_2.6H_2O$ (MW: 203.3)	15.26 mg (1.5 mM)
0.5 M sodium EDTA	100 µl (1 mM)
0.5 M sodium EGTA	100 µl (1 mM)

Note: add 1 M DTT stock solution (final concentration is 1 mM) and 0.2 M PMSF (final concentration is 0.1 mM).

Mitochondria Isolation

1. Pass 3T3 cell (3×10^6/plate, prepare 3 plates each time) to 150 mm tissue culture plates. When cells grow to 80% confluence (take about 48 hr) the cells are harvested by trypsinization and centrifugation at 600*g* for 10 min at 4°C and washed twice with ice-cold PBS (J Cell Biol, 1999 Mar 8, 144(5):891-901; Science, 1997 Feb 21, 275: 1129-1132; Cell, 1998 Aug 21, 94(4):481-90).

2. Resuspend the cell pellet in 1 ml ice-cold MIB by pipeting up and down using a 1 ml pipet tip. Complete cell disruption by using a 25-gauge needle and a syringe. Draw slowly into the syringe and eject with on stroke. Repeat 15 times.

3. Centrifuge the lysate at 1000 ×g for 10 min at 4 °C and carefully transfer the supernatant to a clean 1.5 ml tube using a 1 ml pipet tip.

4. Repeat step 2 and 3 two more times with 500 µl MIB and pool the supernatants.

5. The pooled supernatants are further centrifuged at 1000 ×g for 10 min at 4 ºC to pellet the unbroken cells and nuclei. The supernatants are centrifuged at 10,000×g for 10 min at 4 ºC to pellet the mitochondria.

6. Resuspend the pellet in 1 ml MIB. Centrifuge for 10 min at 6300×g at 4ºC. The mitochondria were then resuspended gently in 50 µl MSB.

7. Perform protein concentration assay to determine the mitochondria protein concentration.

Cyt c Release Assays

1. 3 µl (amount to 25 µg) mitochondria protein are incubated with stimulus in a final volume of 25 µl RB (1.5 ml tube) at 30°C for 30 min.

2. At the end of incubation, the reaction mixture is centrifuged at 12,000×g for 5 min at 4ºC to pellet the mitochondria. The mitochondria pellets are resuspended in 33 µl volume of 2 × SDS sample buffer. The samples are subjected to 15% SDS-PAGE to probe cytochrome c content.

3. 8 µl of 5×SDS sample buffer was added to the resulting supernatants and analyzed by 15% SDS-Page gels to probe cytochrome *c* release.