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ISOLATION OF MITOCHONDRIA FROM HEK293 CELLS

Lab equipment required:

1. Cell culture facility
2. Ultra-centrifuge with MLS 650 or equivalent rotor
3. Incubator

HEK cells were cultured in DMEM with 10% tetracycline-free FBS
The whole procedure was performed on ice or at 4°C.

1. Cells were detached by pipetting and pelleted by centrifugation for 10 min at 300 gmax, then washed twice with PBS and the final pellet was weighed to determine its volume (assuming a density of 1.25 g/ml).
2. Cells were then re-suspended in nine volumes of hypotonic buffer containing 20 mM HEPES (pH 8.0), 5 mM KCl, 1.5 mM MgCl2 with 1 mg/ml BSA, 2 mM DTT and protease inhibitors (Protease inhibitor cocktail 1 and 2; FabGennix Int. Inc.), incubated on ice for 10 min and homogenized with 10 strokes of a tight-fitting 15 ml glass Dounce homogenizer (cell disruption was monitored by microscopic examination).
3. Next, two-third of total volume of 2.5× MSH buffer [525 mM mannitol, 175 mM sucrose, 20 mM HEPES (pH 8.0), 5 mM EDTA with 1 mg/ml BSA, 2 mM DTT and protease inhibitor cocktail (FabGennix Inc)] was immediately added and the homogenate was centrifuged for 10 min at 1600gmax.
4. The pellet was discarded and the supernatant was centrifuged again.
5. The pellet containing the cell debris and nuclei was re-suspended in 0.5 ml of 1× MSH buffer [210 mM mannitol, 70 mM sucrose, 20 mM HEPES (pH 8.0), 2 mM EDTA with 1 mg/ml BSA, 2 mM DTT and protease inhibitors (FabGennix Inc)] and stored in −20°C.
6. The cytoplasmic fraction was centrifuged for 10 min at 8900gmax and a 0.5 ml sample the supernatant containing the cytosolic fraction was stored in −20°C.
7. The pellet containing the mitochondrial fraction was re-suspended in 1× MSH buffer supplemented with 10 mM MgCl2 to a final protein concentration of 2 mg/ml (assuming 10 mg of mitochondrial protein per 1 g of cell pellet) and DNase I (Sigma) was added to a final concentration of 0.2 mg/ml (DNase I treatment removes contamination by residual nuclear DNA and chromatin proteins). The sample was rotated for 30 min and the reaction was terminated by adding EDTA to a final concentration of 15 mM.
8. The sample was washed three times with 5 ml of MSH buffer without BSA by centrifugation for 10 min at 8 900 gmax. After washing, the pellet was suspended in 0.1–0.2 of volume of MSH buffer without BSA, loaded onto a sucrose gradient (see below) and centrifuged for 40 min at 117 000gmax.
9. The gradient was prepared by layering 1 M and 1.5 M sucrose solutions in fresh gradient buffer [10 mM HEPES (pH 7.8), 5 mM EDTA, 2 mM EDTA, 2 mM DTT] in polyallomer tubes (Beckman Coulter).

10. After the centrifugation, the interface between 1M and 1.5M sucrose solution was collected and four volumes of gradient buffer were slowly added with gentle vortexing. The sample was then centrifuged for 10 min at 8 900gmax, the supernatant discarded and the mitochondria re-suspended in MSH buffer without BSA

Preparation of Solutions:

a. Hypotonic buffer: 20mM HEPES, pH 8.0; 5mM KCl; 1.5mM MgC2; 2mM DTT with 1mg/ml BSA and 2ul/ml of protease inhibitor cocktail 1 and 2.
b. MSH buffer: 525 mM Mannitol; 175 mM Sucrose; 20mM HEPES; 5mM EDTA; with 1mg/ml BSA, 2mM DTT; 2ul/ml of protease inhibitor cocktail A/B
c. 1X MSH buffer containing 10mM MgCl2
d. DNAse
e. 15mM EDTA
f. MSH buffer without BSA
g. Gradient Buffer: 10mM HEPS, pH 7.8; 5mM EDTA; 2mM EDTA; 2mM DTT. Dissolve sucrose 1M and 1.5M for preparation of density gradient.

Note: