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ab110171 – Mitochondria Isolation Kit for Cultured Cells (with Dounce Homogenizer)

Instructions for Use

For mitochondria isolation from mammalian cultured cells

This product is for research use only and is not intended for diagnostic use.

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1. Introduction

Abcam's benchtop mitochondria isolation kit allows for quick and efficient isolation of intact mitochondria from cultured cells using differential centrifugation. Sufficient reagents are provided in the kit for 20 isolations, each requiring approximately an hour.

Principles of mitochondria isolation: The key steps when isolating mitochondria from any tissue or cell are always the same: (i) rupturing of cells by mechanical and/or chemical means and (ii) differential centrifugation at low speed to remove debris and extremely large cellular organelles (SPIN 1) followed by centrifugation at a higher speed to isolate mitochondria which are collected (SPIN 2). This crude mitochondrial preparation is often enough for most applications. The procedures detailed in this manual have been designed to provide the highest possible yield of intact and enzymatically active mitochondria.

Suggested amounts of starting material for a small scale preparation from cultured cells are shown in Table 1 in addition to expected protein yields of mitochondria.

Sample	Starting Material	Expected Yield
MRC-5	4 – 150 mm plates	0.5 mg*
HepG2	4 – 150 mm plates	1.0 mg*
HDFN	2 – 150 mm plates	0.5 mg*

Table 1. Suggested starting amounts and expected yields of mitochondria.*Purity can be enhanced, while yield is lowered, by adjusting SPIN 2.

2. Assay Summary

Freeze and thaw cells to weaken membranes, suspend in Reagent A at 5.0 mg/ml, incubate on ice for 10 minutes.

DISRUPT CELLS: Homogenize with a Dounce Homogenizer.

SPIN 1: 1,000 g 10 minutes 4°C, collect & save supernatant #1. Repeat and save supernatant #2.

SPIN 2: 12,000 g 10 minutes 4°C, collect & save pellet.

Resuspend the pellet in Reagent C supplemented with PI, aliquot and freeze at -80°C.

Assay mitochondria: protein concentration, OXPHOS activities, Western Blot.

3. Kit Contents

- Reagent A: 50 ml
- Reagent B: 50 ml
- Reagent C: 10 ml
- Dounce Homogenizer (2 ml size) 1

4. Storage and Handling

Reagents A, B, and C should be stored at 4°C.

5. Additional Materials Required

Reagents:

- Double distilled water
- Protease inhibitor cocktail (PI)
- BCA Protein Assay

Equipment:

- Highspeed benchtop centrifuge
- 2.0 mL microtubes
- Weighing balance and other standard lab equipment

6. Protocol

The mitochondria preparation follows three simple steps: cell rupturing, centrifugation to remove large particles and centrifugation to isolate mitochondria. Below are guidelines for the preparation of mitochondria from cultured Human cells. Reagents and samples should be chilled when possible. Starting amount of 4 x 150 mm plates of confluent cells (approximately $4x10^7$ cells) is recommended. This amount allows for volumes that are compatible with a benchtop procedure, although this procedure may be downscaled.

1. Collect Cells: In the case of adherent cells they can be collected with a cell lifter and pelleted by centrifugation at 1,000 g. Each confluent plate typically yields 2 mg of whole cell protein. This should be checked by protein assay.

2. Freeze the cells and then thaw in order to weaken the cell membranes.

3. Resuspend the cells to a concentration of 5 mg of protein per ml in Reagent A, in a 2-ml microtube..

4. Incubate for 10 minutes on ice.

RUPTURING:

5. Transfer the cells into a pre-cooled 2.0 ml Dounce Homogenizer.

6. Homogenize the cells with 30 strokes using pestle B.

7. Transfer homogenate to a 2 ml centrifuge tube.

SPIN 1:

8. Centrifuge homogenate 1,000 g for 10 minutes at 4°C

9. Save supernatant #1.

10. Resuspend the pellet in Reagent B to the same volume as in the Step 3.

11. Repeat the Rupturing and Spin 1 steps.

12. Save as supernatant (SN) #2 and discard the pellet.

13. Combine SNs #1 & #2, mix thoroughly, and add to 2 ml centrifuge tube. If volume is over 2.0 ml, divide the SN in half and add to two centrifuge tubes.

SPIN 2:

14. Centrifuge combined supernatants at 12,000 g for 15 minutes at 4°C.

15. Discard the supernatant and collect the pellet.

16. Resuspend the pellet into 500 µl of Reagent C supplemented with Protease Inhibitors.

17. Freeze the aliquots at -80°C until use or until the mitochondrial quality assays described below are performed.

7. Mitochondrial Quality Analysis

There are several Abcam products that can be used to test mitochondrial quality. Figure 1 demonstrates a typical Western blot using 20 µg of isolated MRC5 mitochondria versus 20 µg whole cell MRC5 extract. Samples were probed with Abcam's ab110411, MitoProfile® Total OXPHOS Human WB Antibody Cocktail (MS601).

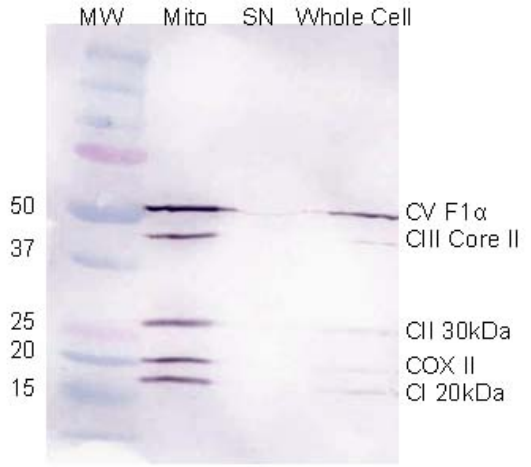

Figure 1. Isolated mitochondria show enriched signal when compared to the whole cell extract. In lane 1, MRC5 mitochondria isolated with Abcam's Benchtop Isolation Kit was loaded at 20 µg. In lanes 2 and 3, post-spin supernatant and whole cell extract were loaded at 20 µg.

Mitochondria integrity can also be tested by screening for cytochrome *c* (intermembrane space), Porin (outer membrane), Cyclophilin D (matrix), or Complex V (inner membrane) in the isolated mitochondria versus in the supernatant fraction using Abcam's antibodies ab110325 (MSA06), ab14734 (MSA03), ab110324 (MSA04), and ab110273 (MS502). These mAbs are components of ab110414, Abcam's MitoProfile® Membrane Integrity WB Antibody Cocktail (MS620). Figure 2 depicts MRC5 mitochondria and supernatant screened with these antibodies.

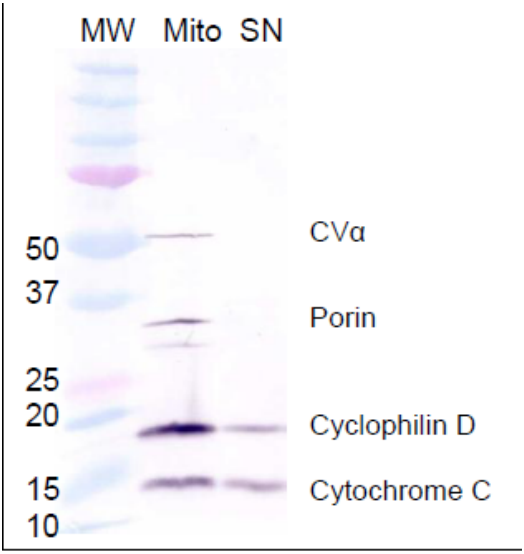

Figure 2. MRC5 mitochondria were isolated from 4x150mm plates. The supernatant fraction was saved after SPIN 2. 20 µg of MRC5 mitochondria and 20 µg of supernatant were loaded onto each lane and detected using ab110325/MSA06 (Cyt *c*), ab110324/MSA04 (Cyclophilin D), ab14734/MSA03 (Porin), and ab110273/MS502 (complex V alpha). Western blot analysis shows that minimal loss of cytochrome *c*, Cyclophilin D, Porin, and Complex V alpha occurs during mitochondria isolation.

8. Optimization Steps and General Tips

Problem	Probable Cause	Solution
Small mitochondrial pellet	Insufficient lysis occurred	Increase Dounce strokes
Large amount of Cytochrome *c* in the cytosol	Cells over-lysed/ Cells not fresh	Reduce Dounce strokes/Isolate from freshly pelleted cells
*Low mitochondria purity	Contaminants spun down with mitochondria	Decrease SPIN 2 to 6,000 g

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