Product Information

Mitochondria Isolation Kit

Sufficient for 50 applications (2-5 × 10^7 mL),
for isolation of enriched mitochondrial fraction from cells

MITOISO2

Product Description

Mitochondria are the site of most energy production in eukaryotic cells, with a double membrane structure, including an outer membrane and a folded inner membrane.[1] This kit enables the fast and easy isolation of an enriched mitochondrial fraction from cells. Most of the isolated mitochondria will contain intact inner and outer membranes. The outer membrane integrity may be measured by observing cytochrome c oxidase activity (Cat. No. CYTOCOX1, Cytochrome c Oxidase Assay Kit). The integrity of the inner membrane can be detected by measuring citrate synthase activity (Cat. No. CS0720, Citrate Synthase Assay Kit)).[1,3] The inner membrane integrity can also be assessed by testing the electrochemical proton gradient (ΔΨ) of the inner mitochondrial membrane by measuring the uptake of the fluorescent dye JC-1 into the mitochondria (with either Cat. Nos. CS0760 or T4069).[4]

MITOISO2 may be used for isolating mitochondrial proteins for proteome studies.[5] The kit includes two buffers for mitochondrial protein extraction:

- The first buffer is for protein extraction for functional studies.
- The second buffer is for profiling for proteome studies.

The kit includes also a storage buffer for use with intact mitochondria. The kit was tested on NIH 3T3, HeLa, HEK 293T, A431, HepG2, CHO, and Jurkat cell lines. Several theses[6,7] and dissertations[8-15] have cited use of product MITOISO2 in their protocols.

Components

The reagents are sufficient for 50 isolation procedures from 2-5 × 10^7 cells.

- Extraction Buffer A, 5×, 25 mL (Component E2778)
- Storage Buffer, 5×, 10 mL (Component S9689)
- CelLytic™ M, 10 mL (Component C2978)
- Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts, 2.5 mL (Component P8340)
- Cell Lysis Solution, 0.6 mL (Component C1242)
- Protein Extraction Reagent Type 4, 1 bottle (Component C0356)

Reagents and Equipment Required

(Not provided)

- Table-top centrifuge
- Cooled microcentrifuge
- Dounce homogenizer:
  - For small-scale preparation: 2 mL glass tube (Cat. No. T2690) and tight pestle (Cat. No. P1110)
  - For large-scale preparation: 7 mL glass tube (Cat. No. T0566) and tight pestle (Cat. No. P1235)
- Dulbecco's Phosphate Buffered Saline (D-PBS, Cat. No. D8537)
- Ultrapure water
- Tissue culture reagents:
  - Trypsin-EDTA (Cat. No. T4049 or equivalent)
  - Trypan Blue (Cat. No. T8154 or equivalent)
- Percoll® (optional), for purification on Percoll® density gradient (Cat. No. P1644)
- Tributylphosphine (TBP) Solution, for two-dimensional (2D) gel analysis (Cat. No. T7567)
- Iodoacetamide, alkylating reagent, for 2D gel analysis (Cat. No. A3221)

Storage/Stability

Store the kit at –20 °C. When stored, as supplied, the components of this kit are stable for at least 2 years.

Store CelLytic™ M Cell Lysis Reagent (Component C2978) at room temperature. CelLytic™ M Cell Lysis Reagent may appear cloudy after an extended period of storage. Product performance is unaffected and the CelLytic™ M may be used, as is, without further filtration or clarification.

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Use ultrapure water for the preparation of reagents.

1× Extraction Buffer A (isotonic solution)

- Defrost Extraction Buffer A, 5× (Component E2778) at 37 °C. A short heating (15 seconds in a microwave oven) may be needed to achieve a clear solution.
- Dilute an aliquot of the Extraction Buffer A, 5× five-fold with ultrapure water. The 5-fold diluted buffer may be stored at 2-8 °C.
- The Extraction Buffer A, 5× may be refrozen.
- Just prior to use, add the Protease Inhibitor Cocktail (Component P8340) to the 5-fold diluted buffer (1:100, [v/v]) to yield the 1× Extraction Buffer A.

Lysis Buffer

- Before use, thaw the Cell Lysis Solution (Component C1242). Mix to form a homogenous solution.
- Dilute the required amount of the Cell Lysis Solution 1:200 (v/v) in 1× Extraction Buffer A containing Protease Inhibitor Cocktail (add 10 µL of the Cell Lysis Solution to 2 mL of 1× Extraction Buffer A).
- Mix well by vortexing. Keep on ice until use.

  Note: For each specific cell line, it is recommended to optimize the dilution of the Cell Lysis Solution (in the range of 100 to 400-fold dilution) for the best yield of intact mitochondria.

1× Storage Buffer

- Dilute an aliquot of the Storage Buffer, 5× (Component S9689) 5-fold with ultrapure water.
- Keep the 1× Storage Buffer at 2-8 °C before use.
- The Storage Buffer, 5× may be refrozen.

Protein Extraction Reagent Type 4

- Add 15 mL of ultrapure water to the contents of the container.
  - This solution will become cold and needs to be warmed to 20-25 °C to dissolve the solids entirely.
  - A 30 °C water bath will aid in the dissolution of the material.
  - **Do not allow the material to heat above 30 °C, since this material may begin to form cyanates that will be detrimental to the proteins.**
- Freeze any unused solution in working aliquots at –20 °C for further use.

Procedure

Mitochondria can be easily prepared from mammalian cells by a simple method of homogenization or lysis with the use of a detergent, followed by low-speed (600 × g) and high-speed (11,000 × g) centrifugation. The final pellet represents a crude mitochondrial fraction that may be used for further experiments.

Another option is for a more purified "heavy" mitochondrial fraction that is less contaminated with lysosomes and peroxisomes. In this method, the low-speed and high-speed centrifugation steps are changed to 1,000 × g and 3,500 × g, respectively, so that the mitochondrial enriched fraction is obtained as the 3,500 × g pellet. The drawback of this method is a lower yield of mitochondria.

All the isolation procedures should be performed at 2-8 °C with ice-cold solutions. The described procedures are for a small cell sample ($2\text{-}5 \times 10^7$ cells). For a larger-scale preparation, calculate the amount of reagents required for the procedure accordingly.

Preparation of mitochondria from cells

Homogenization

1. Grow the cells to ~90% confluency.
2. For adherent cells:
3. Trypsinize the cells.
4. Add growth medium with 10% FCS (Fetal Calf Serum).
5. Centrifuge the cells for 5 minutes at 600 × g.

   Note: For cells in suspension, perform Step 2.3 only (the Centrifugation Step).

6. Wash the cells:
   6.1. Resuspend the cells in ice-cold PBS.
   6.2. Count the cells.
   6.3. Centrifuge the cells for 5 minutes at 600 × g at 2-8 °C.
   6.4. Discard the supernatant.
   6.5. Repeat the Wash Step once again without counting the cells.
7. Add 1-2.5 mL of the prepared 1× Extraction Buffer A per 2-5 × 10^7 cells. Incubate on ice for 10-15 minutes.
8. Homogenize the cells on ice using a Dounce homogenizer, with 10-30 strokes. Each cell type requires an optimization of the number of strokes.

   Notes:

   - Perform the homogenization gradually.
   - Follow the homogenization by staining an aliquot with Trypan Blue and counting the cells under a microscope.
   - It may be necessary to dilute the aliquot 20-fold, in order to count the cells.
   - If there are less than 50% damaged cells (blue cells), perform additional sequential homogenizations (5 additional strokes each time) until there is at least 50% damaged cells (blue cells).
   - Avoid over-homogenization of the cells. This can result in mitochondria breakage.
9. Centrifuge the homogenate at 600 × g for 10 minutes at 2-8 °C.
10. Carefully transfer the supernatant liquid to a fresh tube. Centrifuge at 11,000 × g for 10 minutes at 2-8 °C.
11. Carefully remove the supernatant, and suspend the pellet in a suitable buffer for your application:
    - For mitochondrial protein characterization or for performing functional assays, add 150-200 µL of CelLytic™ M Cell Lysis Reagent with Protease Inhibitor Cocktail (1:100 [v/v]).
    - For applications requiring intact mitochondria (measurement of JC-1 uptake, citrate synthase activity, or cytochrome c oxidase activity) add 150-250 µL of 1× Storage Buffer.
    - For profiling (2D gel) analysis, it is recommended to use 200-400 µL of Protein Extraction Reagent Type 4 as a starting volume.
    - For further fractionation, add 150–200 µL of 1× Extraction Buffer A.

Detergent Lysis

1. Grow the cells to ~90% confluency.
2. For adherent cells:
   2.1. Trypsinize the cells.
   2.2. Add growth medium with 10% FCS.
   2.3. Centrifuge the cells for 5 minutes at 600 × g.

   Note: For cells in suspension, perform Step 2.3 only (the Centrifugation Step).
3. Wash the cells:
   3.1. Resuspend the cells in ice cold PBS.
   3.2. Count the cells.
   3.3. Centrifuge them for 5 minutes at 600 × g at 2-8 °C.
   3.4. Discard the supernatant.
   3.5. Repeat the wash step once again without counting the cells.
4. Resuspend the cell pellet to a uniform suspension in 0.65-2 mL of Lysis Buffer per 2-5 × 10^7 cells.

   Note: For each specific cell line, it is recommended to optimize the volume of the Lysis Buffer added (in the range of 0.65-2 mL) for the best yield of intact mitochondria.
5. Incubate on ice for 5 minutes (**maximum**). It is recommended to suspend the sample at 1-minute intervals by pipetting up and down once.
6. Add 2 volumes of 1× Extraction Buffer.
7. Centrifuge the homogenate at 600 × g for 10 minutes at 4 °C.
8. Carefully transfer the supernatant to a fresh tube. Centrifuge at 11,000 × g for 10 minutes at 4 °C.
9. Carefully remove the supernatant, and suspend the pellet in a buffer suitable for your application:
   - For mitochondrial protein characterization or for performing functional assays, add 150-200 µL of CelLytic™ M Cell Lysis Reagent with Protease Inhibitor Cocktail (1:100 [v/v]).
   - For applications requiring intact mitochondria (measurement of JC-1 uptake, citrate synthase activity, or cytochrome c oxidase activity) add 150-250 µL of 1× Storage Buffer.
   - For profiling (2D gel) analysis, it is recommended to use 200-400 µL of Protein Extraction Reagent Type 4 as a starting volume.
   - For further fractionation, add 150-200 µL of 1× Extraction Buffer A.

Sample Preparation for 2D gel electrophoresis

1. Reduce the protein extract prepared for profiling (in Protein Extraction Reagent Type 4) with 5 mM TBP:

   1.1. Add 5 µL of 0.2 M TBP Solution to 200 µL of protein sample) for 30 minutes.

2. Then alkylate the sample with 15 mM iodoacetamide:

   2.1. Add 6 µL of prepared 0.5 M iodoacetamide solution to 200 µL protein sample) for 30 minutes.

3. The sample is now ready for loading onto IPG strips.

4. The sample may need to be further diluted with Protein Extraction Buffer Type 4 to obtain the desired 2D gel electrophoresis results.

Appendix

Further purification of the mitochondrial fraction on a Percoll density gradient

The mitochondrial pellet (from either the "Homogenization" procedure, Step 8, or the "Detergent Lysis" procedure, Step 9) can be further fractionated by layering onto a Percoll® (Cat. No. P1644) density gradient.[16]

Further purification using a Percoll® density gradient decreases the overall yield of mitochondria. Therefore, it is recommended to use a larger initial cell sample (5×10^8 cells) in order to obtain a significant quantity of mitochondria. This procedure is for an initial cell sample of 5×10^8 cells.

1. Suspend the mitochondrial pellet ("Homogenization" procedure, Step 8, or "Detergent Lysis" procedure, Step 9) in ~1 mL of 1× Storage Buffer containing 15% (v/v) Percoll®.

2. Use the mitochondrial suspension to form a Percoll® density gradient. The 5 mL gradient consists of:

   - A bottom layer of 2 mL of 40% Percoll® in 1× Storage Buffer
   - A middle layer of 2 ml of 23% Percoll® in 1× Storage Buffer
   - A top layer of 1 mL of the mitochondrial suspension in 1× Storage Buffer containing 15% Percoll®

3. Centrifuge the gradient in a swinging bucket rotor for 5 minutes at ~31,000 × g at 2-8 °C.

4. Harvest the mitochondria which band at the lowest interface. Dilute the mitochondria with 4 volumes of ice-cold 1× Storage Buffer.

5. Centrifuge the mitochondria in a fixed angle rotor for 10 minutes at ~17,000 × g at 2-8 °C.

6. Remove the supernatant. Suspend the mitochondria pellet in 1× Storage Buffer at a concentration of 1-5 mg-protein/mL.

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