Mitochondria Isolation Kit

Cat. No. MII-325K **Lot. No.** (See product label)

SPECIFICATION

Product Overview	The Mitochondria Isolation Kit provides a fast and easy method for the isolation of an enriched mitochondrial fraction from cells.
Applications	The kit may be used for isolating mitochondrial proteins for proteome studies.
Usage	1 Kit is sufficient for 50 applications (2-5 x 107 cells)
Storage	Store the kit at –20 centigrade. When stored, as supplied, the components of this kit are stable for at least 2 years. Store CelLytic M Cell Lysis Reagent at room temperature. CelLytic M Cell Lysis Reagent may appear cloudy after an extended period of storage. Product performance is unaffected and may be used, as is, without further filtration or clarification.
Kit Components	Extraction Buffer A, 5×25 ml Storage Buffer, 5×10 ml CelLytic M Cell Lysis Reagent 10 ml Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts 2.5 ml Cell Lysis Solution 0.6 ml Protein Extraction Reagent Type 4 1 btl
Materials Required but Not Supplied	· Table-top centrifuge · Cooled Eppendorf centrifuge · Dounce homogenizer: For small scale preparation - 2 ml glass tube and tight pestle

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	For Large scale preparation – 7 ml glass tube and tight pestle · Dulbecco's Phosphate Buffered Saline (PBS) · Ultrapure water · Tissue culture reagents: Trypsin/Trypsin EDTA Trypan blue · For purification on Percoll density gradient (optional) – Percoll · Tributylphosphine (TBP) Solution for two dimensional (2D) gel analysis · Alkylating reagent, iodoacetamide for 2D gel analysis
Preparation	Use ultrapure water for the preparation of reagents. 1× Extraction Buffer A (isotonic solution) - Defrost Extraction Buffer A, 5× at 37 centigrade. 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 centigrade. The Extraction Buffer A, 5× may be refrozen. Just prior to use, add the Protease Inhibitor Cocktail 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 and 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 ml of the Cell Lysis Solution to 2 ml of 1× Extraction Buffer A). Mix well by vortexing and 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× 5-fold with ultrapure water. Keep the 1× Storage Buffer at 2–8 centigrade before use. The Storage Buffer, 5× may be refrozen.

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	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 centigrade to entirely dissolve the solids. A 30 centigrade water bath will aid in the dissolution of the material. Do not allow the material to heat above 30 centigrade, since this product may begin to form cyanates that will be detrimental to the proteins. Freeze any unused solution in working aliquots at –20 centigrade for further use.
Separation Protocol	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 (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 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. Notes: All the isolation procedures should be performed at 2-8 centigrade with ice-cold solutions. The described procedures are for a small cell sample (2-5 ×10^7 cells). For a larger scale preparation calculate the amount of reagents required for the procedure accordingly. Preparation of mitochondria from cells A. Homogenization 1. Grow the cells to ~90% confluency. 2. For adherent cells, trypsinize the cells, add growth medium with 10% FCS, and centrifuge the cells for 5 minutes at 600 ×g. For cells in suspension, perform the centrifugation only. 3. Wash the cells - resuspend the cells in ice cold PBS, count the cells, and centrifuge them for 5 minutes at 600 ×g at 2-8 centigrade. Discard the supernatant. Repeat the wash step once again without counting the cells.

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4. 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.
5. Homogenize the cells on ice using a Dounce homogenizer, 10-30 strokes. Each cell type requires an optimization of the number of strokes. Note: Perform the homogenization gradually and follow it 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.
6. Centrifuge the homogenate at 600 ×g for 10 minutes at 2-8 centigrade.
7. Carefully transfer the supernatant liquid to a fresh tube. Centrifuge at 11,000 ×g for 10 minutes at 2-8 centigrade.
8. 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 ml 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 ml of 1×Storage Buffer.
· For profiling (2D gel) analysis it is recommended to use 200-400 ml of Protein Extraction Reagent Type 4 as a starting volume.
· For further fractionation add 150-200 ml of 1×Extraction Buffer A.

B. Detergent Lysis
1. Grow the cells to ~90% confluency.
2. For adherent cells - trypsinize the cells, add growth medium with 10% FCS, and centrifuge the cells for 5 minutes at 600 ×g. For cells in suspension perform only the centrifugation.

3. Wash the cells - resuspend the cells in ice cold PBS, count the cells, and centrifuge them for 5 minutes at 600 ×g at 2-8 centigrade. Discard the supernatant. 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 (not longer). 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 centigrade.
8. Carefully transfer the supernatant to a fresh tube. Centrifuge at 11,000 ×g for 10 minutes at 4 centigrade.
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 ml 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 ml of 1×Storage Buffer.
· For profiling (2D gel) analysis it is recommended to use 200-400 ml of Protein Extraction Reagent Type 4 as a starting volume.
· For further fractionation add 150-200 ml of 1×Extraction Buffer A.

C. Preparation of the sample for 2D gel electrophoresis
Reduce the protein extract prepared for profiling (in Protein Extraction Reagent Type 4) with 5 mM TBP (add 5 ml of 0.2 M TBP Solution to 200 ml of protein sample) for 30 minutes, and then alkylate it with 15 mM iodoacetamide (add 6 ml of prepared 0.5 M

iodoacetamide solution to 200 ml protein sample) for 30 minutes. The sample is now ready for loading onto IPG strips. 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 (Procedure A, step 8 or Procedure B, step 9) can be further fractionated by layering onto a Percoll density gradient.6 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 ×108 cells.

1. Suspend the mitochondrial pellet (Procedure A, step 8 or Procedure B, 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, and 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 centigrade.
4. Harvest the mitochondria that band at the lowest interface and dilute them 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 centigrade.
6. Remove the supernatant and suspend the mitochondria pellet in 1×Storage Buffer at a concentration of 1-5 mg-protein/ml.

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